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Polymorphisms of the α-synuclein promoter: expression analyses and association studies in Parkinson's disease

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Summary. Mutations of the α-synuclein gene have shown to be relevant in some rare families with autosomal dominant Parkinson's disease (PD). Furthermore, α-synuclein protein is a major component of the Lewy bodies also in sporadic PD patients. Increased levels of wildtype α-synuclein in the cell leads to increased intracellular hydrogen peroxide levels and causes death of dopaminergic neurons in rat primary culture. Subsequently, oxidative stress has been directly linked with α-synuclein aggregation in vitro. This raises the question whether increased α-synuclein expression might be linked to higher susceptibility to PD and whether α-synuclein promoter polymorphisms are associated with PD. Here, two polymorphisms (−116C>G and −668T>C) of the α-synuclein promoter defining four haplotypes have been characterized in 315 German PD patients. The influence of the four haplotypes on gene expression was studied by CAT reporter gene assays in neuronal SK-N-AS cells. The −668C/−116G haplotype revealed significant higher CAT expression than the −668T/−116G or the −668T/−116C haplotype, respectively. Although the −668C/−116G haplotype was more common in PD patients, this difference was not significant.

Keywords: Association, expression, Parkinson's disease, α-synuclein, promoter polymorphism.

Introduction

Two mutations in the α-synuclein gene have been described in some rare families with autosomal dominant Parkinson's disease (PD) (Polymeropoulos

et al., 1997; Krüger et al., 1998). Efforts to identify α-synuclein mutations in sporadic PD patients have failed (Krüger et al., 1998).

Mutant α-synuclein, a protein highly enriched in presynaptic terminals in unaffected individuals, is aggregated in PD patients in form of intraneuronal inclusions which have been described as Lewy bodies. Numerous studies implicate that mutant α-synuclein is incapable of vesicle binding (Jensen et al., 1998), and that mutant protein tends to form aggregates faster than native protein (Conway et al., 1998; Davidson et al., 1998; Giasson et al., 2001). However, native α-synuclein overexpressed in cell culture leads also to dopaminergic nerve terminal degeneration (Zhou et al., 2000) ascribed in part to oxidative stress rendering dopamine neurons more vulnerable to neurotoxic insults through a variety of agents (Kanda et al., 2000; Ko et al., 2000; Ostrerova-Golts et al. 2000; Tabrizi et al., 2000). It is in particular intriguing that α-synuclein forms toxic aggregates in the presence of iron which is considered to contribute to the formation of Lewy bodies via oxidative stress (reviewed in Youdim and Riederer, 2002).

Transgenic mice and flies overexpressing wild-type α-synuclein recapitulate some characteristics of PD including intracellular accumulation of α-synuclein in neuronal cells (Feany and Bender, 2000; Masliah et al., 2000). The demonstration that α-synuclein protein is a major component of the Lewy bodies also in sporadic patients (Spillantini et al., 1997) led us to ask whether different expression levels of α-synuclein might be associated with individual susceptibility to PD.

We have recently shown that a complex dinucleotide repeat (NACP-Rep1) in the 5′-region of the α-synuclein gene is associated with sporadic PD (Krüger et al., 1999). Sequence variations in this microsatellite repeat have been shown to influence the expression level in a cell culture luciferase reporter system (Chiba-Falek and Nussbaum, 2001). Here we searched for DNA polymorphisms in a 911 bp fragment upstream of exon 1 of the α-synuclein gene, studied the influence of two common polymorphisms on the expression level using CAT reporter gene analysis, and performed association studies in a large cohort of German PD patients.

Materials and methods

PD patients

A total of 315 German PD patients (mean age 66.01 years (SD ± 11.2 years); mean age at disease onset 55.41 years (SD ± 11.48 years; males 56.07 %, females 43.93 %) were evaluated by neurologists and were diagnosed as idiopathic PD based on the UK PD brain bank criteria. Sixty one patients had a family history of PD, the other patients are thought to be sporadic. None of the patients carried a mutation in the α-synuclein, parkin and ubiquitin C-terminal hydrolase L1 (UCHL1) genes, respectively (Krüger et al., 1998; Wintermeyer et al., 2000, and unpublished data). All subjects signed an informed consent.

Control individuals

For the association study healthy German individuals of the MEMO-Study (Memory and Morbidity in Augsburg Elderly, Rothdach et al., 2000) served as controls (mean age 72+/−4.34 years; males 52%, females 48%). Cardinal signs of PD were defined as

UPDRS score ≥2 for tremor, rigidity or hypokinesia and resulted in exclusion from the controls.

PCR conditions

PCR was carried out in a thermocycler (Robocycler, Stratagene) under the following conditions: 50 ng DNA was amplified in a final volume of 10 μl in the presence of 10 mM Tris/HCl (pH 8.3), 50 mM KCl, 2 mM $MgCl_2$, 200 μM of each dNTP, 10 pmol of each PCR primer, 4 μl of Q-solution (Qiagen), and 1 Unit Taq Polymerase (Genecraft). Cycling conditions were as follows: 1 min at 95°C, 1 min at 59°C, and 1 min at 72°C for the 35 cycles, followed by a final elongation at 72°C for 5 min. We constructed the PCR primer pairs according to the published genomic sequence of the α-synuclein gene (GenBank U46896). 911 bp of the 5′ region upstream of the untranslated exon 1 were analyzed by SSCP analysis.

SSCP analysis and sequencing

The 5′-upstream region was analyzed by PCR amplification of two overlapping fragments using primer pairs 1718F (5′-TGGTGTGTAGCATCTCCCTTTC-3′) / 2270R (5′-ACCGAGGGCCTGAGGGTCGA-3′) and 2202F (5′-TGCCTGTCTCCTCCAGCA GC-3′) / 2719R (5′-CTTCCGCGTCGGCGCTCGG-3′). PCR fragments were electrophoresed on non-denaturating polyacrylamide gels using two different conditions (5% acrylamide (AA), 0.5× TBE, 5% glycerole and 6% AA, 1× TBE, 10% glycerole). SSCP gels were visualized using autoradiography. DNA samples exhibiting bandshifts on the SSCP gel were sequenced on a ABI 373 apparatus using BigDye Cycle Sequencing Kit (PE Biosystems).

Statistics

Data of each locus were evaluated for allele frequencies, genotype frequencies and Hardy-Weinberg equilibrium using the Genepop program designed by Michel Raymond and Francois Rousset (1995). The exact p-value of the Hardy-Weinberg equilibrium was calculated by the complete enumeration method (Louis and Dempster, 1987), as the number of alleles of the investigated loci was <5 (Rousset and Raymond, 1995). A level of $p < 0.1$ was considered as statistically significant for Hardy-Weinberg equilibrium test. Further statistical analysis [Chi square (χ^2), odds ratio (OR)] was performed as described previously (Svejgaard and Ryder, 1982). Estimated haplotypes of the α-synuclein promoter region were computed using the Arlequin program available from http://anthropologie.unige.ch/arlequin (Schneider et al., 2000). Maximum-likelihood haplotype frequencies are computed using an expectation-maximization (EM) algorithm (Excoffier and Slatkin, 1995).

Reporter gene constructs

PCR primer pairs (Syn1840F-HindIII: 5′-TCTAGAGCGAAAAGCTTTAGG ACCGCT-3′ and Syn2851R-NheI: 5′-AGGCAAACCCGCTAGCCTGTCGTCGA-3′) were constructed according to the published genomic sequence of the α-synuclein gene (accession U46896) and changed at one base to create a restriction site suitable for cloning into the vector pCAT-basic (Promega). PCR was carried out in a thermocycler (Robocycler, Stratagene) under the following conditions: 100 ng DNA were amplified in a final volume of 20 μl in the presence of 20 mM Tris-Cl (pH 8.75), 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 0.1% Triton® X-100, 0.1 mg/ml BSA, 2 mM $MgSO_4$, 0.2 μM of each dNTP, 8 pmol of each PCR primer and 2.5 Unit Pfu turbo Polymerase (Stratagene). Standard cycling conditions were performed with optimized annealing temperatures: 1 min at 95°C; 1 min at 58°C, and 2 min at 72°C for the 35 cycles, followed by a final elongation at 72°C

for 10 min. PCR was carried out with DNA of Parkinson's disease patients homozygote for each haplotype combination. Subsequently, PCR products were digested with Hind III and Nhe I and subcloned upstream of a promoterless CAT gene into the vector pCAT-basic (Promega). The sequence of every construct was confirmed by complete sequencing.

Computational analysis

Differences in the CAT concentration was determined by simple variance analysis using ANOVA and subsequently by post hoc comparison using LSD tests (SPSS computer programm package). The analysis of potential transcription factor binding sites was performed with the MatInspector V2.2 software using the TRANSFAC 4.0 matrices (Quandt et al., 1995).

Cell culture

Human neuroblastoma cells (SK-N-AS) cells were cultured in Dulbecco's modified Eagles medium (DMEM) containing 10% fetal bovine serum, 2 mM glutamine, 100 U/ml penicillin and 100 mg/ml streptomycin.

Transfections and reporter gene expression studies

1×10^6 SK-N-AS cells were plated onto each well of a 6-well dish the day prior to transfection. 1 μg of each CAT-reporter construct was mixed and cotransfected with 0.1 μg pCMV-β (Clontech) using FuGENE™6 transfection reagent (Roche) according to the manufacturer's instructions. Each construct was transfected in quadruple on four wells of cultured cells with four individually prepared transfection reactions. For confirmation of the results each quadruplicate experiment was repeated on a separate day.

Cells were harvested after 48–60 h and lysed by adding 1 ml of lysis buffer (Roche). The cellular debris was removed by centrifugation and the supernatant was used to measure β-galactosidase activity as described (Sambrook et al., 1989).

50 μl of the remaining cell extract was used to measure CAT concentrations using the Roche CAT-ELISA kit. To normalize for variations of transfection efficiencies, the CAT activity was divided by the β-galactosidase activity. Every construct was tested twice (in total 8 transfections) in four independent transfection experiments. The results from four transfections were averaged. To exclude concentration effects as cause for different CAT-expression, two independent DNA preparations of each construct were used for separate transfections.

Results

We recently reported an association of a complex dinucleotide repeat in the promoter region of the α-synuclein gene with PD (Krüger et al., 1999). Therefore we searched for additional polymorphisms in the promoter region by PCR amplification of 911 bp upstream of exon 1 and subsequent SSCP analysis. Two common polymorphisms (−116G>C, and −668T>C) were identified upstream of the transcription start site with frequencies of 0.488 for the C polymorphism at position −116 according to the published transcription initiation site and of 0.276 for the T at position −668, respectively (Table 1). Similar allele frequencies for both polymorphisms were found in a group of more than 300 PD patients (Table 1). Genotype frequencies of both loci did also not reveal significant differences between the groups of PD patients and controls.

Table 1. Allelic and genotypic distribution of the promoter polymorphisms of the α-synuclein gene in PD patients and controls, and in early and late onset PD, respectively. For the latter analysis we exclusively included patients for which the age at onset was well documented by the physician

		Allele frequencies		Genotype frequencies		
−668T>C	n	T	C	T,T	T,C	C,C
PD	303	0.295	0.705	0.10	0.392	0.508
EOPD	82	0.299	0.701	0.085	0.427	0.488
LOPD	188	0.303	0.697	0.112	0.383	0.505
Controls	361	0.276	0.724	0.072	0.407	0.521
−116C>G	n	G	C	G,G	G,C	C,C
PD	280	0.523	0.477	0.261	0.525	0.214
EOPD	74	0.493	0.507	0.270	0.446	0.284
LOPD	176	0.511	0.489	0.227	0.568	0.205
Controls	345	0.512	0.488	0.275	0.472	0.252

We then investigated whether the −116G>C and −668T>C polymorphisms might be more frequent in the group of early onset PD patients compared to late onset PD (Table 1). In this analysis we only included patients for which the age at onset was well documented by the physician. However, for none of the polymorphisms significant differences have been found between both groups.

Finally, we combined both polymorphisms and generated estimated haplotypes. This was easily achieved by different running patterns of the polymorphisms. However, for some of the patients other SSCP band shifts due to additional DNA variations have been observed. These patients were therefore excluded from the statistical analysis. However, haplotype analysis did not reveal significant differences between PD patients and controls (Table 2).

Independent from the association studies we were interested whether the identified polymorphisms might be relevant for the expression of α-synuclein. Analysis of the polymorphisms using the computer program MatInsp (Quandt et al., 1995) revealed differences in putative transcription factor binding sites. The −116G>C polymorphism creates new putative binding sites for transcription factors GKLF, IK2, and MZF1, respectively. The −668T>C polymorphism creates also a binding site for MZF1 which is

Table 2. Haplotype distribution of the promoter polymorphisms of the α-synuclein gene in PD patients and controls

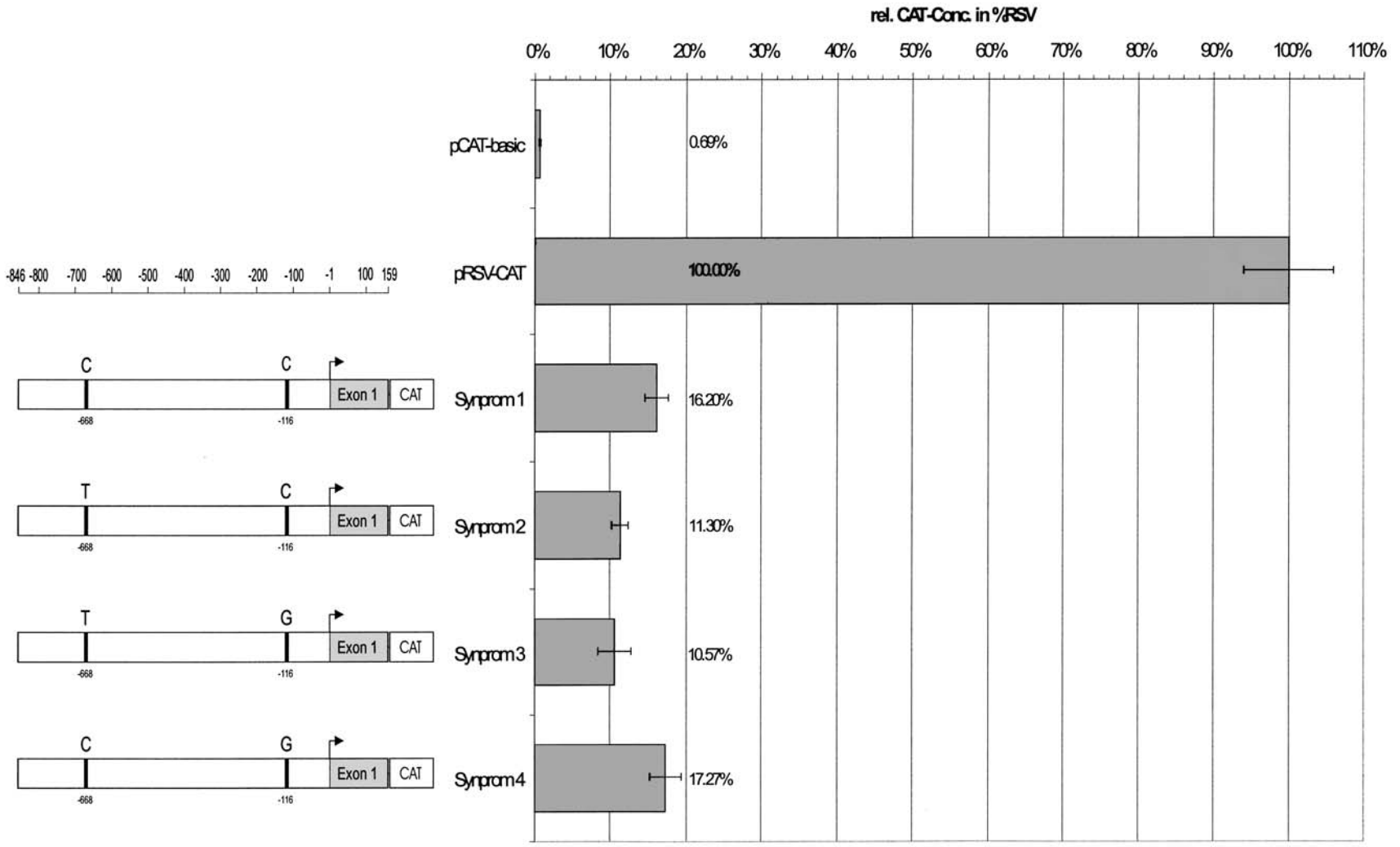
		Haplotype frequencies (−668/−116)			
	n	T/G	T/C	C/G	C/C
PD	277	0.00872	0.29994	0.51294	0.17839
Controls	336	0.00756	0.27220	0.50434	0.21589

located on the (−) strand. We therefore cloned each of the four haplotypes into a CAT reporter gene expression vector and determined the CAT concentrations relative to the expression of a control plasmid containing the 3′-LTR of the rous sarcoma virus (Fig. 1). This assay is in particular sensitive to study slight differences in weak promoters as the unspecific background is rather low (Porsch et al., 1993). Significant differences in the CAT concentrations were found in particular between constructs 3 (−668T; −116G) and 4 (−668C; −116G) ($p = 0.021$), but also between constructs 2 and 4 ($p = 0.036$) and constructs 1 and 3 ($p = 0.045$), respectively (Fig. 1). Comparing the CAT concentrations between construct 1 and 2 did not reveal significant differences ($p = 0.076$).

Discussion

The aim of this study was to investigate whether promoter polymorphisms of the α-synuclein gene might be associated with sporadic PD. We used two approaches, first we searched for DNA polymorphisms in the promoter region and estimated their frequencies in up to 315 PD patients and up to 370 control individuals, both of German ancestry. Second we investigated whether the two identified common polymorphisms potentially influence the level of expression of α-synuclein. Our hypothesis was, that a certain haplotype is more frequent in PD patients and that this haplotype might confer a higher expression level than haplotypes which are less common in PD. We found two common polymorphisms, a −116C>G and a −668T>C substitution, in the investigated promoter region of the α-synuclein gene. However, none of them was significantly associated with PD in the investigated individuals. Similar results for the C-116G polymorphism were recently described in smaller patient groups (Farrer et al., 2001; Pastor et al., 2001). Despite these results there is mounting evidence that the expression of α-synuclein is linked to sporadic PD. First there is biological evidence obtained by overexpression of wild-type α-synuclein in cell culture and in transgenic animals leading to α-synuclein accumulation (Kahle et al., 2000) and aggregation (Feany et al., 2000). Wild-type α-synuclein has also found to be aggregated in form of Lewy bodies in the brains of sporadic PD patients (Spillantini et al., 1997). Secondly, a complex dinucleotide repeat in the promoter region of the α-synuclein gene is associated with PD (Krüger et al., 1999; Tan et al., 2000; Farrer et al., 2001) and significantly influences the expression of α-synuclein as revealed by luciferase reporter gene analysis (Touchman et al., 2001). The same group was also able to show that the repeat composition contributes to a significant variation of the α-synuclein expression level (Chiba-Falek and Nussbaum, 2001). In our reporter gene assay which did not contain the dinucleotide repeat we also found differences in the expression level investigating four constructs with different combinations of the C-116G and the

Fig. 1. Influence of the four analyzed polymorphisms in the promoter region of the α-synuclein gene on the relative expression level measured by CAT concentration and compared to an RVS construct


rel. CAT-Conc. in %RSV
0%
10%
20%
30%
40%
50%
60%
70%
80%
90%
100%
110%
pCAT-basic
0.69%
pRSV-CAT
100.00%
Symprom 1
16.20%
Symprom 2
11.30%
Symprom 3
10.57%
Symprom 4
17.27%
-846 -800 -700 -600 -500 -400 -300 -200 -100 -1 100 159
C
C
Exon 1
CAT
-668
-116
T
C
Exon 1
CAT
-668
-116
T
G
Exon 1
CAT
-668
-116
C
G
Exon 1
CAT
-668
-116

T-668C polymorphisms, respectively. Indeed, the Synprom 4 construct (−668C; −116G) which revealed significant higher expression than Synprom 3 (−668T; −116G) and Synprom 2 (−668T; −116C) did represent a haplotype more common in our group of PD patients although this difference was not significant. It will now be important to extend these analysis to the entire α-synuclein promoter region including different alleles of the complex dinucleotide repeat. These studies also have to take other polymorphisms in this region into account, in particular the −770C>A polymorphism which is part of a risk haplotype for PD (Farrer et al., 2001).

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