Screening for mutations in synaptotagmin XI in Parkinson's disease

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Summary. Parkinson's disease (PD) is characterized by selective degeneration of neurons in the substantia nigra and subsequent dysfunction of dopaminergic neurotransmission. Genes identified in familial forms of PD encode proteins that are linked to the ubiquitin-proteasome system indicating the pathogenic relevance of disturbed protein degradation in PD. Some of them, i.e. α-synuclein, parkin and synphilin-1, have been implicated in presynaptic neurotransmission based on their localization in synaptic vesicles. Synaptotagmin XI is linked to the pathogenesis of PD based on its identification as a substrate of the ubiquitin-E3-ligase parkin. Moreover synaptotagmin XI is involved in the maintainance of synaptic function and represents a component of Lewy bodies (LB) in brains of PD patients. Therefore, we performed a detailed mutation analysis of the synaptotagmin XI gene in a large sample of 393 familial and sporadic PD patients. We did not find any disease causing mutations arguing against a major role of mutations in the synaptotagmin XI gene in the pathogenesis of PD.

Introduction

The pathogenic causes of Parkinson's disease (PD) are widely unknown. Environmental and genetic causes are discussed as factors for the loss of dopaminergic neurons in the substantia nigra in affected individuals. In fact, there are an increasing number of genes in which mutations have been identified in autosomal recessive, autosomal dominant, and apparently sporadic patients. Altered gene products are thought to be involved in the ubiquitin-mediated protein degradation pathway (reviewed in Krüger et al., 2002). Two proteins, α-synuclein and parkin, play a key role in this pathway (Polymeropoulus et al., 1997; Kitada et al., 1998). Although the exact function of α-synuclein remains

to be identified, there is solid evidence based on cell culture and transgenic studies that it is involved in synaptic transmission. The second known gene in PD, parkin, encodes a synaptic protein, which is linked to the vesicular transport system of neuronal cells (Kubo et al., 2001). It is localized in the Golgi complex of neuronal cells and on secretory vesicles together with synaptotagmin I and synaptophysin (Kubo et al., 2001). Moreover parkin has been implicated in proteasomal protein degradation via its ubiquitin-E3-ligase function (Shimura et al., 2000) which results in ubiquitination and subsequent degradation of the glycosylated form of α-synuclein (Shimura et al., 2001), synphilin 1 (Chung et al., 2001), CDCrel (Zhang et al., 2000), Pael-receptor (Imai et al., 2001), CHIP (Imai et al., 2002), HSP70 (Tsai et al., 2003), p38 subunit of aminocyl tRNA synthetase (Corti et al., 2003), cyclin E (Staropoli et al., 2003), and α/β-tubulin heterodimers (Ren et al., 2003), and binds CASK/LIN-2 (Fallon et al., 2001). Based on the genetic characterization of parkin-interacting proteins, we recently identified a novel mutation in the synphilin-1 gene responsible for PD in two apparently sporadic PD patients (Marx et al., 2003). This illustrates that physiological substrates of the E3-ligase parkin are good candidates for mutation screenings in sporadic and familial PD patients.

Recently, some of us identified synaptotagmin XI as another substrate of parkin (Huynh et al., 2003). Synaptotagmin XI belongs to the family of synaptotagmins. Members of this family are vesicle proteins, probably playing a role in the docking and fusion of synaptic vesicles with the plasma membrane and hence in transmitter release. Moreover synaptotagmins might be involved in endocytosis, since all representatives studied so far show high affinity for AP2, a clathrin adaptor protein complex (Ullrich et al., 1994; Zhang et al., 1994; Li et al., 1995). Synaptotagmins are found in abundance in nerve cells and some endocrine cells. Their amino acid sequence comprises a single transmembrane region and two cytoplasmic C2 domains (C2A and C2B) in the C-terminal region, followed by a conserved C-terminus (A-latrotoxin receptor interaction domain).

C2 domains are modules found in a number of cellular proteins and are important in signal transduction and membrane transport. The two synaptotagmin C2 domains have been shown to have different functions: C2A is responsible for Ca^{2+}-dependent binding to phospholipid (Davletov, 1993; Sutton, 1995), while C2B binds phospholipid and inositol polyphosphate in a calcium-independent manner (Fukuda, 1994).

Synaptotagmin XI is highly expressed in brain cells, but is also detectable in other tissues. Together with synaptotagmin IV, being most closely related to synaptotagmin XI, it constitutes a separate subgroup of synaptotagmins. Characteristic feature of this subgroup is an amino acid substitution (aspartate → serine) within the Ca^{2+}-binding site of the C2A domain that abolishes Ca^{2+}-dependent phospholipid binding. Members of this subclass of synaptotagmins are therefore expected to have Ca^{2+}-independent functions that await further elucidation (von Poser, 1997).

Synaptotagmin XI is located on chromosome 1q21.2 and thus not in any of the as yet known candidate regions or susceptibility loci for Parkinson associated genes. The gene has an approximate length of 5 kb, contains four exons and encodes a protein of 430 amino acids.

Being substrate for parkin and involved in the synaptic vesicle function synaptotagmin XI is an interesting candidate for PD. We therefore searched for mutations in the *synaptotagmin XI* gene in a large group of PD patients.

Material and methods

PD patients

A total of 393 sporadic and familial PD patients (mean age at disease onset 56.0 years (S.D. ± 11.7 years); males 55.1%, females 44.9%) were screened for mutations in the *synaptotagmin XI* gene. All patients were evaluated by a neurologist experienced in the diagnosis of PD based on the UK Parkinson's disease brain bank criteria. All subjects gave informed consent. For all PD patients, mutations in the alpha-synuclein and UCHL1-gene were excluded, and for juvenile PD patients also mutations in the Parkin- and DJ1-gene, respectively.

Primers and PCR conditions

PCR and sequencing primers (Table 1) were designed using Synaptotagmin XI genomic sequence information (Ensembl Gene ID #ENSG00000132718) and the primer 3 program (http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi) (Rozen and Skaletsky, 2000). Exon 2 had to be divided into three overlapping fragments being suitable for denaturing high performance liquid chromatography (dHPLC) analysis (designated exon 2_1, 2_2 and 2_3 in the following). For exon 1, 2-1, 2-2, 2-3, 3, and 4, 393, 167, 359, 350, 359, and 345 DNA samples were analyzed by dHPLC as PCR and dHPLC did not reveal an evaluatable result in all cases.

Table 1. PCR primers used for amplification of the 4 exons of the synaptotagmin XI gene and optimized temperature and elution conditions for mutation detection

	Name	Sequence (5′ → 3′)	PCR product	Annealing temperatures (T_A)	dHPLC column temperature (T_C)	dHPLC gradient [in %B]
Exon 1	Ex1_F Ex1_R	gtctcaccattgcaaaaacg ccttgcaaccattctgacc	131 bp	53°C	59°C	50–60
Exon 2_1	Ex2_8416_F Ex2_8803_R	aagtccacccgccctgac gtccattttgatgggtaattggtc	388 bp	55°C	60.0°C	60–70
Exon 2_2	Ex2_8728_F Ex2_9120_R	cctgctaagccgagacaaag gggatgccatagaaggtgaa	393 bp	56°C	58.0°C and 60°C	61–71 and 59–69
Exon 2_3	Ex2_9023_F Ex2_9320_R	accatccttcctgacaaacg acctaccattgctccccaac	298 bp	58°C	62.0°C	56–66
Exon 3	Ex3_F Ex3_R	tctgccaggattctcacc gatggttcagacagtaagaaatagg	203 bp	53°C	59.5°C	54–64
Exon 4	Ex4_F Ex4_R	tgtcataggtctgtctccctttttt ccccggggattagaggag	387 bp	55°C	59.0°C	60–70

After amplification the best possible separation of homo- and heteroduplexes is achieved using the temperature and gradient parameters presented in this table. The acetonitril gradient (dHPLC gradient) is commonly indicated as the proportion of buffer B (25% (v/v) acetonitril) in the mobile phase. Exon 2_2 had to be analysed under two different temperature and elution conditions since two melting domains in the PCR product had arisen from the analysis of the melting profile (0–100 bp and 100–400 bp)

After optimizing PCR conditions by gradient PCR with a PTC-0200 DNA Engine (MJ Research, Inc.; Waltham, Mass.), PCR was performed on patient samples in a thermocycler (PTC-0200 DNA Engine, Perkin-Elmer 9600) and a final volume of 25 µl using 5 µl DNA, 1 µl of each primer (10 pmol/µl), 2.5 µl 10× PCR buffer (Genecraft), 0.5 µl 10 mM dNTPs and 1.5 U Taq polymerase (Genecraft) with the annealing temperatures given in Table 1. The following PCR conditions were applied: 2,5′ 94°C; [30″ 94°C; 30″ annealing temperature; 30″ 72°C] for 35 cycles; 5′ 72°C; 4°C ∝. Five µl of each PCR product were electrophoresed on 1% agarose gels to determine the presence and size of the amplified DNA. Only samples with positive PCR signals were pooled pair wise in the subsequent dHPLC analysis.

dHPLC

dHPLC (WAVE® DNA Fragment Analysis system [Transgenomic, Inc., San Jose, CA]) was used to screen for mutations in the *synaptotagmin XI* gene. Mutation detection relies on the different melting points of hetero- and homoduplexes. PCR products were pooled pairwise (10 µl each), subsequently denatured (94°C for 2 min), and slowly renatured (slow cooling to 10°C within 20 min) to allow the formation of heteroduplexes. Separation of homo- and heteroduplexes takes place in the cartridge of the wave system. DNA fragments bind to the stationary phase, a hydrophobic poly(styrene-divinylbenzene) bead matrix, via triethylammonium acetate (TEAA) as "bridging" molecule, single stranded fragments binding more tightly than double stranded fragments. The mobile phase consists of a buffer system composed of buffer A and B. Buffer A contains 0.1 M TEAA and 0.025% (v/v) acetonitrile (ACN) and buffer B contains 0.1 M TEAA and 25% (v/v) ACN. The hydrophobic interaction between stationary phase and TEAA is reduced with the increase of acetonitrile in the mobile phase. Under partially denaturing conditions (increased temperature) heteroduplexes are marginally more denatured than homoduplexes and elute therefore slightly before the homoduplexes. This difference is detected by the system's UV detector and can be seen in chromatograms, ideally as four distinct peaks (two homo- and two heteroduplexes). Complete resolution of the two homo- and heteroduplexes however is not obligatory. For more detailed information regarding the methodology please see (Huber et al., 1993; Kuklin et al., 1997–98).

The melting profiles of the PCR fragments under investigation were used to predict the temperature suitable for mutation scanning of the fragment. The ACN gradient (ratio of buffer A and B) was adjusted so that the retention times of homoduplexes lay between 1.6 and 1.9 minutes. Samples yielding conspicuous chromatograms were examined separately using the WAVE system and the samples to be considered were sequenced subsequently.

Gradient and temperature conditions suitable for mutation detection are presented in Table 1. These conditions have been confirmed for three different CEPH DNA samples.

Sequencing

After purifying PCR products using a PCR purification kit (Qiagen), both strands were sequenced using CEQ 2000 Dye Terminator Cycle Sequencing (Beckman Coulter). For each cycle sequencing reaction 200 ng of the amplicon and 12 pmol of primer (Table 1) were added to 3 µl of Quick Start Master Mix (Beckman Coulter) in a volume of 10 µl. The cycling profile was 96°C for 20 s, 50°C for 20 s and 60°C for 4 min for 30 cycles following a 90-s pre-denaturation at 94°C.

Cycle sequencing reactions were stopped with 5 µl of a mix containing one portion glycogen and two portions of each 100 mM EDTA (pH 8.0) and 3 M NaAc (pH 5.2) and precipitated with 60 µl 100% ethanol. After a 30-min spin (4°C, 16100 rpm) the pellet was washed twice with 70% ethanol, vacuum dried for 12.5 min and resuspended in 40 µl CEQ sample loading solution (Beckman Coulter). Sequences were obtained using a capillary sequencer (CEQ 8000, Beckman Coulter). Conditions included a capillary temperature of 50°C, 120″ denaturation at 90°C, 15″ injection at 2.0 kV and 85-min separation at 4.2 kV. Sequencing data were analysed with the CEQ 8000 analysis software.

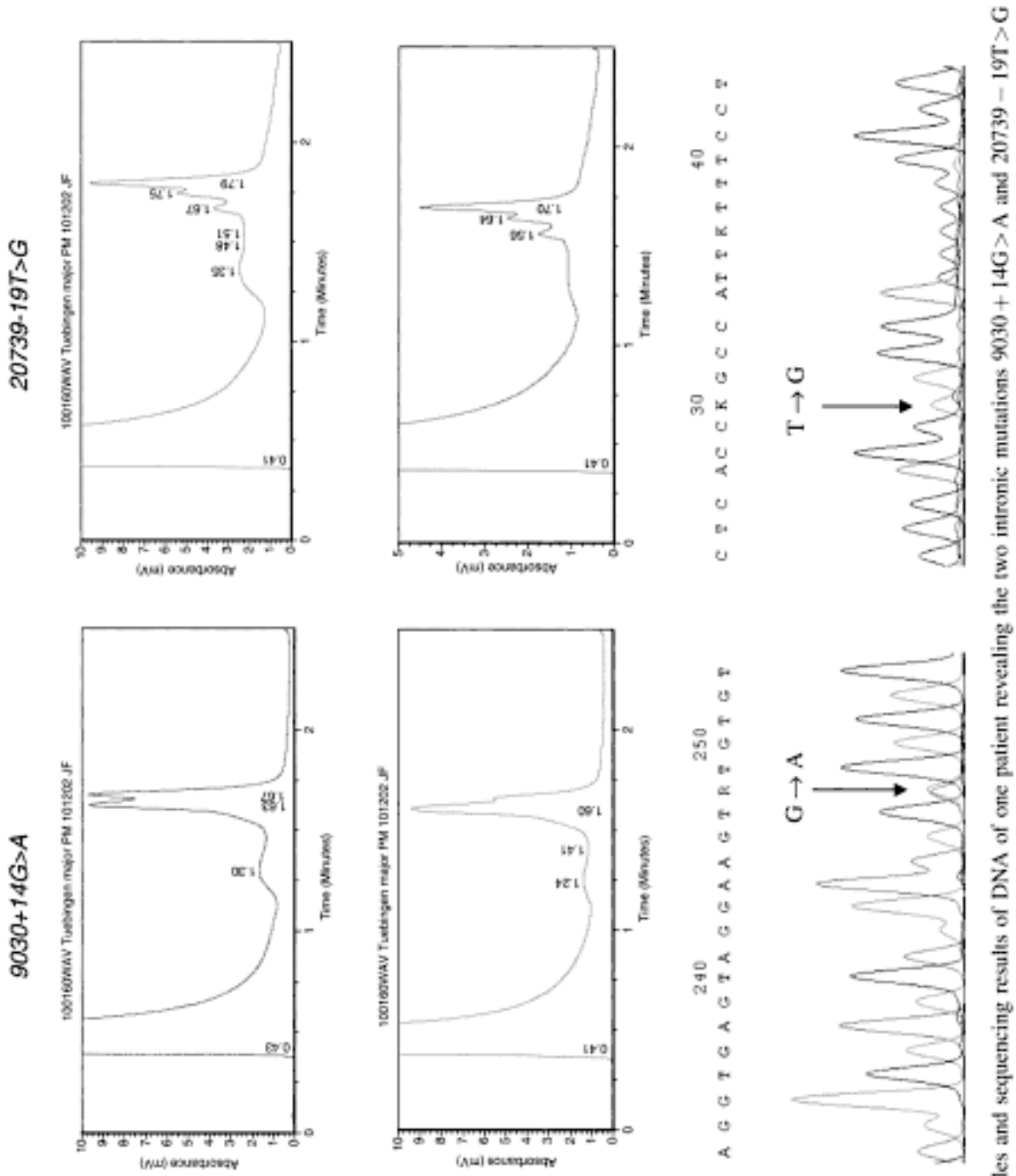

Fig. 1. dHPLC profiles and sequencing results of DNA of one patient revealing the two intronic mutations 9030 + 14G > A and 20739 – 19T > G

Results

dHPLC was performed to search for mutations in the *synaptotagmin XI* gene in up to 393 DNA samples of PD patients. Peak shapes typical for heteroduplexes appeared twice, namely in one PCR product of exon 2_3 and exon 3, respectively. No other obviously aberrant heteroduplex pattern was observed. Interestingly, both conspicuous patterns could be assigned to the same patient. Sequencing revealed two intronic mutations adjacent to exon 2_3 (9030 + 14G > A) and exon 3 (20739 − 19T > G), respectively (Fig. 1).

Other conspicuous peak shapes, such as "pre-peaks" and "shoulders" did not prove to be new sequence variants. However, sequencing of six representatives for exon 2_2 due to pre-peaks revealed an already described single nucleotide polymorphism (SNP) at genomic position 8715 (8715T > C) or cDNA position 546 (546T > C), respectively. This SNP does not change the amino acid composition and was observed in all six representatives independently of the pattern of pre-peaks.

Discussion

This is the first study to search for mutations in the *synaptotagmin XI* gene in PD. Synaptotagmin XI has been recently shown to interact with and serve as a substrate of Parkin, an E3 ubiquitin ligase (Shimura et al., 2000) which is commonly mutated in early onset autosomal recessive PD (Kitada et al., 1998). Therefore, and as the vesicle transport or release seems to be altered in PD, synaptotagmin XI was considered an excellent candidate for PD. Recently, for another synaptic vesicle associated protein that is ubiquitinated by parkin, synphilin-1, a novel mutation responsible for an apparently sporadic form of PD was identified (Marx et al., 2003). Synaptotagmin XI is highly expressed in brain (von Poser et al., 1997) and has been shown to be a component of the Lewy bodies (Huynh et al., 2003), a pathological hallmark of PD. Disturbed synaptotagmin XI function or an altered conformation could therefore be causative for the disease process for instance via increased release of dopamine into the cytoplasm resulting in increased oxidative damage (reviewed in Betarbet et al., 2002). This process might be coupled by the regulation of Ca^{2+} influx by syntaxin, SNAP-25, and synaptotagmin (reviewed in Atlas, 2001). Disturbed synaptic function seems to be important in the pathogenesis of PD, since several proteins known to be mutated in familial forms are closely related to synaptic integrity and function, i.e. α-synuclein, parkin and synphilin-1. Loss of α-synuclein function, for instance, leads to impaired response to prolonged, low-frequency stimulation, indicating disturbed vesicle recruitment from reserve pools (Cabin et al., 2002). In this context known synaptotagmin-XI-mediated down-regulation of presynaptic neurotransmission might be involved in dopaminergic dysfunction in PD (Wang et al., 2001).

However, in our study including a large sample of up to 393 PD patients, we did not identify any disease causing mutations. In contrast, synaptotagmin XI turned out to be highly conserved since no amino acid variations were found. This might reflect that peptide sequence variations of synaptotagmin XI dramatically alter its function and may not be compatible with physiological

embryonic development. However, further investigations of the 5′UTR and promoter regions including functional analyses of detected sequence variations should be performed in further studies. Moreover, the generation of knock out animals is essential to prove this hypothesis.

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