Highlights

- Genotyping for four polymorphic sites in the VDR gene in patients with Parkinson's disease.
- Association was detected between FokI C polymorphism and Parkinson's disease.
- No association could be detected between ApaI, BsmI, TaqI polymorphisms and Parkinson's disease.

Association of vitamin D receptor gene polymorphisms and Parkinson's disease in Hungarians

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Running Title:

Vitamin D receptor polymorphisms in Parkinson's disease

Abstract

Vitamin D receptor (VDR) gene encodes a transcription factor that influences calcium homeostasis and immunoregulation, and may play a role in neurological disorders including Parkinson's disease (PD). The investigations of the association between VDR and PD in different populations revealed various results. In a present study 100 PD patients and 109 healthy controls from the Hungarian population were genotyped for four polymorphic sites (BsmI, ApaI, FokI and TaqI) in the VDR gene. The polymorphisms were determined by polymerase chain reaction and restriction fragment length polymorphism (PCR-RFLP). Our results demonstrate an association between the FokI C allele and PD; the frequency of the C allele was significantly higher in PD patients than in controls, suggesting that this polymorphism may have a role in the development of PD in these patients.

Keywords: FokI, Parkinson's disease, polymorphism, vitamin D receptor

1. Introduction

Parkinson's disease (PD) is one of the most common neurodegenerative disorders. The characteristic neuropathological features are the presence of Lewy bodies and the loss of dopaminergic cells in the substantia nigra pars compacta, but the precise pathomechanism is still not fully understood. The most common concepts are related to the genetic background and environmental effects [5]. Indeed, a number of genetic risk factors and gene-environment interactions have been implicated in the pathogenesis of PD [13, 14, 19-21, 24, 25, 29, 37]. Vitamin D, as an environmental factor, has been the subject of various studies on different neurological disorders, from which it has emerged that a vitamin D deficiency is associated with an increased risk of many diseases, including schizophrenia, autism, multiple sclerosis, Alzheimer's disease and PD [1, 3, 4, 7, 9, 22, 26-28, 30, 33, 35].

In humans, the majority of vitamin D is synthesized via the cleavage of a cholesterol metabolite in the epidermis by UVB, with further metabolism to the primary circulating form of vitamin D, 25-hydroxyvitamin D (25OHD), in the liver. This compound circulates in the blood in a form bound to vitamin D binding protein and in the kidneys 25OHD is metabolized by 1-α-hydroxylase to its active form, 1,25-dihydroxyvitamin D (1,25OHD). 1,25OHD binds to vitamin D receptors (VDRs) and influences calcium homeostasis, neurotrophic signalling, immunoregulation, cell growth and differentiation [8, 16, 17]. Both 1-α-hydroxylase and VDRs are expressed in numerous body tissues, including the brain [10].

The VDR gene encodes a nuclear transcription factor. The human gene is localized to 12q12 and various polymorphisms have been reported in it [38]. The level of vitamin D in PD has been analysed in number of studies. A Japanese population exhibited a higher incidence of hip fractures and lower serum levels of 25OHD in PD patients as compared with the healthy controls [34, 35], observations that were confirmed in a Caucasian population [9]. The

serum 25OHD level correlated negatively with the severity of PD, measured in terms of the Hoehn &Yahr stage and the Unified Parkinson's Disease Rating Scale (UPDRS) [34-36]. Whereas Sato et al. [34] reported a negative correlation between the 1,25OHD level and the UPDRS score, Suzuki et al. [36] could not confirm this finding.

However, only limited data are available regarding the association between VDR polymorphisms and PD. Suzuki et al. [36] found that FokI CC genotype was associated with milder forms of PD in a Japanese population. Furthermore, an association between the BsmI bb genotype and PD was demonstrated in a Korean population [18]. A recent genome-wide association study revealed the association of VDR polymorphisms with the risk of PD and the age at onset in a Caucasian population [6]. In a Chinese study it was described that FokI C allele associates with an increased risk of PD as well as early-onset PD [12].

The study we report here related to whether the known VDR gene polymorphisms ApaI, FokI, TaqI and BsmI are associated with PD in the Hungarian population, which belongs to the Caucasian race.

2. Material and methods

2.1 Subjects

100 idiopathic PD patients (mean age: 66.4±9.3 yr; 44 men and 56 women) were enrolled. The patients were evaluated by movement disorders specialists, who confirmed the diagnosis of idiopathic PD. Secondary forms of parkinsonism were excluded. The age at onset was determined from the medical records and the cases were categorized as early-onset (diagnosed ≤60 yr) or late-onset (diagnosed >60 yr) PD (Table 1). The Park2 and LRRK2 mutations were not present.

The control group comprised 109 healthy individuals (mean age: 64.0±8.2 yr; 54 men and 55 women) who had no history of neurological or psychiatric disorders (Table 1). The patients and healthy controls, all of Hungarian origin, were selected from the Department of Neurology and Department of Medical Microbiology and Immunobiology at the University of Szeged. The study was approved by the Ethics Committee of the Faculty of Medicine, University of Szeged. All study participants gave their written informed consent, in accordance with the Declaration of Helsinki.

Table 1.
Characteristics of PD patients and healthy controls

	PD group (%)	Control group (%)
No.	100	109
Age (mean±SEM)	66.4±9.3	64.0±8.2
Age at onset ≤ 60	52 (52)	
Age at onset > 60	48 (48)	
Gender		
Male	44 (44)	54 (49.5)
Female	56 (56)	55 (50.5)

2.2 DNA isolation

Genomic DNA was isolated from the peripheral blood by a standard desalting method [23], and stored at –20°C until further use.

The VDR polymorphisms were determined by polymerase chain reaction (PCR) techniques in a thermal cycler (Applied Biosystems 2720 Thermal Cycler, Applied Biosystems, Foster City, CA, USA) and restriction fragment-length polymorphism (RFLP).

2.3 Genotyping

For amplification of the FokI C/T polymorphism (rs10735810) the following primers were used [15]: forward primer 5'-AGC TGG CCC TGG CAC TGA CTC TGC TCT-3' and reverse primer 5'-ATG GAA ACA CCT TGC TTC TTC TCC CTC-3'. The PCR amplification was carried out with the following cycling parameters: 95°C for 5 min, and then 30 cycles of 95°C for 30 s, 60°C for 30 s, and 72°C for 30 s, and finally 72°C for 7 min. The PCR products were digested with the FokI restriction enzyme (Fermentas, Vilnius, Lithuania) at 55°C for 3 h. The digested products were separated by agarose gel electrophoresis. The genotypes were defined as CC (265 bp), TT (169 and 96 bp) or CT (265, 169 and 96 bp).

The BsmI A/G polymorphic site of intron 8 (rs1544410) was amplified with previously described primers [18]: forward primer 5'-CAA CCA AGA CTA CAA GTA CCG CGT CAG TGA-3' and reverse primer 5'-AAC CAG CGG GAA GAG GTC AAG GG-3'. The PCR conditions were as follows: 95°C for 10 min, 95°C for 30 s, 59°C for 30 s and 72°C for 50 s for 35 cycles, and finally 72°C for 10 min. The PCR products were digested with the restriction enzyme Mva12691 (Fermentas, Vilnius, Lithuania) at 37°C overnight. Fragments were separated by electrophoresis in 2% stained agarose gels and visualized in UV light. The genotypes were defined as AA (825 bp), GG (650 and 175 bp) or AG (825, 650 and 175 bp).

PCR amplification of the polymorphic TaqI T/C site (rs731236) was performed with the following primers [31]: forward 5'-CAG AGC ATG GAC AGG GAG CAA-3' and reverse 5'-CAC TTC GAG CAC AAG GGG CGT TAG C-3'. The PCR conditions were identical to those for the BsmI polymorphism. The PCR products were digested with the TaqI restriction enzyme (Fermentas, Vilnius, Lithuania) at 65°C for 3 h and fragments were analysed by electrophoresis in 2% agarose gel. The absence of the TaqI restriction site on both alleles (TT) led to the 501 bp fragment, whereas the presence of the restriction site on both alleles (CC) yielded bands of 295 and 206 bp. The presence of the 501, 295 and 206 bp fragments reflected the TC heterozygotes.

The ApaI G/T polymorphic site (rs7976091) was amplified with previously described primers [32]: forward primer 5'-CAA CCA AGA CTA CAA GTA CCG CGT CAG TGA-3' and reverse primer 5'-CAC TTC GAG CAC AAG GGG CGT TAG C-3' The PCR conditions were: 95°C for 10 min, followed by 35 cycles of 95°C for 30 s, 59°C for 30 s and 72°C for 2 min, and finally 72°C for 7 min. The PCR products were incubated with the ApaI restriction enzyme (Fermentas, Vilnius, Lithuania) at 37°C overnight. The genotypes were defined as TT (absence of restriction site, one band at 2000 bp), TG (heterozygote, three bands at 2000, 1700 and 300 bp) and GG (presence of the restriction site, two bands at 1700 and 300 bp).

2.4 Statistical analysis

All statistical analyses were performed with the SPSS Statistics 17.0 software (SPSS Inc., Chicago, IL, USA). The difference in genotype frequencies was analysed by using the Fisher's exact test or the χ^2 test. The associations between the genotypes and the PD were estimated via the odds ratio (OR), with a 95% confidence interval (CI) (95% CI). A p value of less than 0.05 was considered statistically significant. The observed FokI, BsmI, ApaI and

TaqI genotype frequencies were in accordance with the Hardy–Weinberg equilibrium in both the patients and the controls.

3. Results

3.1 *VDR FokI polymorphism*

The distributions of FokI restriction site genotypes in the PD patients and the controls are shown in Table 2. There was a significant difference in genotypes between the PD patients and the healthy controls ($\chi^2 = 6.7$; $p = 0.035$). The frequency of genotype with C (CC+CT) was significantly higher among the patients with PD relative to the controls: OR = 2.677 and 95% CI = 1.214-5.91, $p = 0.015$ for CC+CT vs. TT. Moreover, the C allele showed a significant association with PD group (OR = 1.615, 95% CI = 1.087-2.399, $p = 0.017$). There was no difference between the FokI polymorphism and gender in PD group, and no significant association was found between this polymorphism and the age at onset (Table 3).

Table 2.
VDR FokI genotypes and allele frequencies in the PD patients and the controls

	Genotype				Allele frequency		
	CC (%)	CT (%)	TT (%)	p	C (%)	T (%)	p
PD patients	42 (42)	48 (48)	10 (10)	0.035	132 (66)	68 (34)	0.017
Control	35 (32.1)	49 (45)	25(22.9)		119 (54.6)	99 (45.4)	

Table 3.
Relationship between age at onset and gender in the PD patients as a function of the VDR FokI genotyping

	CC (%)	CT (%)	TT (%)	p
Age at onset				
≤60 yr	22 (42.3)	24 (46.2)	6 (11.5)	0.841
>60 yr	20 (41.7)	24 (50)	4 (8.3)	
Gender				
Male	18 (40.9)	22 (50)	4 (9.1)	0.958
Female	24 (42.9)	26 (46.4)	6 (10.7)	

3.2 *VDR BsmI polymorphism*

There was no significant difference in the BsmI genotypic distribution (OR = 0.890, 95% CI = 0.478-1.654, p = 0.753 for GG vs. AA+AG) and allele frequency (OR = 0.977, 95% CI = 0.665-1.434, p = 0.905) between the PD patients and the healthy controls. The BsmI genotypic distribution, the allele frequency, the male to female ratio and the age at onset of the PD patients are presented in Table 4.

Table 4.
VDR BsmI genotypes and allele distributions in patients with PD and controls

	Genotype				Allele frequency		
	AA (%)	AG (%)	GG (%)	p	A (%)	G (%)	p
PD patients	24 (24)	49 (49)	27 (27)	0.902	97 (48.5)	103 (51.5)	0.905
Controls	25 (22.9)	57 (52.3)	27 (24.8)		107 (49)	111 (51)	
Age at onset							
≤60 yr	9 (17.3)	27 (51.9)	16 (30.8)	0.261			
>60 yr	15 (31.3)	22 (45.8)	11 (22.9)				
Gender							
Male	7 (15.9)	22 (50)	15 (34.1)	0.161			
Female	17 (30.4)	27 (48.2)	12 (21.4)				

3.3 *VDR TaqI polymorphism*

The frequencies of the TaqI genotype in the PD group and the controls were similar (OR = 0.840, 95% CI = 0.399-1.767, p = 0.646 for TT+TC vs. CC) and we did not find differences in allele distribution (OR = 0.802, 95% CI = 0.540-1.190, p = 0.273). There was no significant difference in the male to female ratio and the age at onset in the various TaqI polymorphism subgroups (Table 5).

Table 5.
VDR TaqI genotypes and allele frequencies in patients with PD and controls

	Genotype				Allele frequency		
	TT (%)	TC (%)	CC (%)	p	T (%)	C (%)	p
PD patients	35 (35)	48 (48)	17 (17)	0.485	118 (59)	82 (41)	0.273
Controls	47 (43.1)	46 (42.2)	16 (14.7)		140 (64.2)	78 (35.8)	
Age at onset							
≤60 yr	22 (42.3)	23 (44.2)	7 (13.5)	0.265			
>60 yr	13 (27.1)	25 (52.1)	10 (20.8)				
Gender							
Male	18 (40.9)	20 (45.5)	6 (13.6)	0.528			
Female	17 (30.4)	28 (50)	11 (19.6)				

3.4 *VDR ApaI polymorphism*

The ApaI genotype frequencies (OR = 1.352, 95% CI = 0.654-2.796, p = 0.466 for GG vs. TT+TG) and the allele distribution (OR = 1.177, 95% CI = 0.793-1.748, p = 0.417) were similar in the healthy controls and the patients with PD (Table 6). There was no statistically significant association between the ApaI polymorphism and the age at onset in PD patients, and no significant difference was found between this polymorphism and gender in the PD group.

Table 6.
VDR ApaI genotypes and allele frequencies in the PD patients and the controls

	Genotype				Allele frequency		
	TT (%)	TG (%)	GG (%)	p	T (%)	G (%)	p
PD patients	42 (42)	43 (43)	15 (15)	0.691	127 (63.5)	73 (36.5)	0.417
Controls	42 (38.5)	46 (42.2)	21 (19.3)		130 (59.6)	88 (40.4)	
Age at onset							
≤60 yr	18 (34.6)	23 (44.2)	11 (21.2)	0.130			
>60 yr	24 (50)	20 (41.7)	4 (8.3)				
Gender							
Male	13 (29.5)	22 (50)	9 (20.5)	0.07			
Female	29 (51.8)	21 (37.5)	6 (10.7)				

4. Discussion

This work involved a study of VDR polymorphism in PD patients among Hungarians. Earlier reports revealed that 25OHD levels were decreased in PD patients in Japanese and Caucasian populations [9, 34, 35] and demonstrated a higher incidence of osteoporosis in PD patients of both genders, with a decreased bone mass index and a reduced bone mineral density [11, 34]. Overall, it has been clearly shown that the vitamin D metabolism is affected in PD patients.

Differences have also been demonstrated in the VDR polymorphisms in PD in Japanese, Korean, Chinese and Caucasian populations [6, 12, 18, 36].

Our results have indicated a significant difference in the FokI genotype distribution between PD and controls in Hungarian population; the frequency of the C allele was significantly higher in PD patients than in the healthy control group, suggesting that the C allele may have a role in the development of PD.

Previously, a Japanese and a Chinese study detected difference in this polymorphism between healthy subjects and PD patients. In Japan, FokI CC genotype was associated with milder forms of PD [36]. Han et al. [12] suggested that FokI C allele might be a risk factor for sporadic PD development.

FokI polymorphism is located in exon 2 at the 5′ coding region of the gene. This polymorphism results in different translation initiation sites: if the VDR gene contains C allele, the protein will be three amino acids shorter. Difference in length may result in altered VDR function [2, 38].

BsmI, ApaI and TaqI polymorphisms are located in the 3'-end region of the VDR gene, which do not result in changes in the amino acid sequence of the VDR [38]. We did not identify significant associations with these VDR polymorphisms. A Korean study detected difference in the genotype frequency of BsmI polymorphism between healthy subjects and PD patients; the bb genotype in that study was more common in Korean PD patients than in

controls [18]. No difference in BsmI polymorphism was identified in Japanese patients [36], where the FokI CC genotype displayed a strong association with the milder forms of PD.

Our data demonstrated no significant associations between VDR ApaI polymorphism and PD, whereas the ApaI polymorphism was associated with the early-onset form of PD in American Caucasians. Interestingly, there was no association between FokI polymorphism and PD patients among American Caucasians, reflecting differences in ethnicity among Caucasians [6].

These diverse data suggest that the Caucasian population is not homogeneous in this respect. We did not detect an association between the age at onset, the male-female ratio and the VDR polymorphisms in the PD group. The differences between the results of the various authors must be interpreted with regard to the facts that the study populations and sample sizes differed, with the additional possibility of certain ethnic variations.

As far as we are aware, this is the first report on the potential correlation between a VDR polymorphism and PD from a European country. We conclude overall that the C allele of the VDR FokI polymorphism may be associated with PD in a Caucasian population.

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6. Conflict of interest

The authors declare there is no conflict of interest.

7. Author contributions

Conceived and designed the experiments: RT, PK. Performed the experiments: RT, NT.

Collected the samples: NT, FS, ZSZ, PK. Analyzed the data: LSZ, RT. Wrote the paper: RT,

LSZ, IP, PK. Study supervision or coordination: LV, PK.

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