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ORIGINAL ARTICLE

Association of TLR4 gene polymorphisms with sporadic Parkinson's disease in a Han Chinese population

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Abstract Parkinson's disease (PD) is considered as a multifactorial disorder involving complex interactions between genetic and environmental factors, while previous studies point to a pivotal role of neuroinflammation in the pathophysiology of PD. As a member of pattern recognition receptors, TLR4 plays an important role in the immune response and inflammatory responses. Growing evidences suggest that mutation of TLR4 gene may be connected with the development of PD. The objective of this study was to evaluate whether genetic polymorphisms of the TLR4 gene are associated with PD susceptibility. We genotyped three single-nucleotide polymorphisms of the TLR4 gene (rs1927911, rs1927914 and rs10116253) by polymerase chain reaction and restriction fragment length polymorphism (PCR–RFLP) in unrelated 380 PD patients and 380 healthy-matched controls. Our study revealed that rs1927914 C allele carriers and C allele were probably related to a decreased risk of PD ($p = 0.032$ and $p = 0.028$, respectively) as well as male PD ($p = 0.034$) and early-onset PD (EOPD) ($p = 0.023$). In addition, there were significant differences in genotype and allele distribution in male PD patients and its healthy-matched control subgroup ($p = 0.035$ and $p = 0.012$, respectively). For rs1927911 and rs10116253 polymorphisms, genotype or allele frequencies did not differ between groups. Our data suggest that the TLR4 gene might contribute to the risk of developing PD in Han Chinese and rs1927914 polymorphism may be a protective factor for sporadic PD, male PD and EOPD.

Keywords Toll-like receptor 4 (TLR4) · Parkinson's disease (PD) · Single-nucleotide polymorphisms (SNPs)

Introduction

Parkinson's disease (PD) is one of the most common neurodegenerative disorder of the central nervous system(CNS), affecting over 1 % of the population over the age of 60 and about 5 % over 85 years old [1], and the latest data of the prevalence of PD that we can find is 1.7 % in the population over the age of 65 in China [2]. It is clinically characterized by progressive movement disorders including resting tremor, rigidity, bradykinesia and gait disturbance related to the loss of dopaminergic neurons in the substantia nigra (SN), and recently its non-motor deficiencies such as autonomic dysfunction, cognitive dysfunction, sleep disorders and mood disorders have received relatively attention [3–5]. While the disease mechanisms that ultimately cause PD are still not fully understood, substantial evidence has suggested that neuroinflammatory mechanisms might contribute to the cascade of events leading to neuronal degeneration [6–9].

Toll-like receptors (TLRs) are a member of pattern recognition receptors (PRRs) that recognize viruses, fungi, bacteria and protozoa known as pathogen-associated molecular patterns (PAMPs) playing an important role in the immune response and inflammatory responses [10, 11]. Toll-like receptor 4 (TLR4) which belongs to type I transmembrane glycoproteins is the first TLR explored in mammals and expressed on the surface of microglia in the CNS [12].

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After binding of ligands, such as lipopolysaccharide (LPS), lipoteichoic acid (LTA) and heat shock proteins HSP60, TLR4 will lead to the activation of microglia through the pathways of downstream signal transduction. Once activated, microglia will promote the production of pro-inflammatory and/or cytotoxic factors, including TNF-α, IL-1, IL-6, IL-8 and NO. It is also observed that the level of those inflammatory mediators, together with the activated microglia and astrocytes, are elevated in the brain of PD patients [13–15]. In addition, a recent study showed a significantly up-regulated TLR4 in a mouse MPTP model of Parkinson disease, which depended on the dopaminergic cell death to some extent [16]. It is therefore plausible to assume that TLR4 may be an important susceptibility gene to PD.

The TLR4 gene, comprising 4 exons, is located on chromosome 9q32-q33 [17], and a number of genetic variants in this gene have been identified. Previous data have shown the association between its polymorphisms and various multifactorial diseases, including Alzheimer's disease (AD), multiple sclerosis (MS) and cancer [17–20].

To the best of our knowledge so far, a detailed study of the TLR4 polymorphisms in PD patients is lacking. In this study, we analyzed three genetic variants, rs1927911, rs1927914, rs10116253, which may alter the expression or function of TLR4 [17, 18, 21, 22], to investigate whether they are related to the risk of PD in Han Chinese population.

Materials and methods

Study subjects

380 patients (144 females and 236 males, mean age 62.5 ± 10.8) who were diagnosed with sporadic PD were recruited from the Department of Neurology of the Affiliated Hospital of Qingdao University Medical College, between 2009 and 2013. For single-nucleotide polymorphism (SNP) analysis, a total of 380 healthy controls (151 females and 229 males, mean age 70.4 ± 8.2) without any known history of neurodegenerative disorders were randomly selected from Health Examination Center of the hospital and frequency matched with the cases on age and sex. The diagnosis of PD was established by the United Kingdom Parkinson's Disease Society Brain Bank Clinical Diagnostic Criteria [23], and Hoehn and Yahr scale was used to analyze the clinical staging of disease [24]. The patients with family history of Parkinsonism or secondary forms of parkinsonism or neurological or psychiatric conditions were excluded. All the above subjects were genetically unrelated northern Han Chinese in origin. The study was approved by the Institute Ethical Committee, in compliance with the Declaration of Helsinki. All participants provided written informed consent for the study.

Table 1 Characteristics of the PD patients and health control (HC) subjects

Variable	PD group ($n = 380$)	HC group ($n = 380$)
Gender (male/female)	236/144	229/151
Age range	30–88	46–89
MMSE score[b]	27 (22–30)	29 (24–30)
Duration of PD (years)[a]	4.4 ± 3.6	–
UPDRS III score[b]	23 (5–36)	–
Hoehn and Yahr stage[b]	2 (1–4)	–

[a] Values are expressed as mean ± SD

[b] Values are expressed as median (range)

To examine the TLR4 gene polymorphisms, all participants were subdivided into two groups: patients with early-onset PD (EOPD, age at onset ≤50 years, 100 patients) and late-onset PD patients (LOPD, age at onset >50 years, 280 patients) and each control subgroup, patients with male and female PD patients and each healthy-matched controls subgroup. We provide an overview on the demographic and clinical characteristics of all the subjects in Table 1.

SNP selection and genotyping

A Genomic DNA extraction kit (Roche, USA) was used to extract genomic DNA from peripheral blood samples of patients and healthy individuals, and then genomic DNA was stored at −80 °C until further use. The rs1927911, rs1927914 and rs10116253 polymorphisms for TLR4 were genotyped in all subjects by polymerase chain reaction and restriction fragment length polymorphism (PCR–RFLP) method with primers designed as previously reported [21] or designed by Genetool. Table 2 shows the primer sequences, the length of PCR and restriction fragments. PCR reaction was carried out in a total volume of 10 μL containing 37.5 ng genomic DNA, 0.25 U Taq DNA polymerase, 2 pmol of each primer, 200 μM of dNTP (2.5 Mm) and 1 μl 10× PCR buffer.

The PCR conditions used for the rs1927911 and rs1927914 polymorphism were: initial denaturation at 94 °C for 5 min followed by 35 cycles of denaturation at 94 °C for 20 s, annealing at 60 °C for 20 s, and extension at 72 °C for 20 s, with a final extension at 72 °C for 5 min. For the rs10116253 polymorphism, the PCR conditions were as follows: after denaturation at 94 °C for 5 min, 35 cycles were performed for 30 s at 94 °C, 60 °C annealing for 30 s, and 72 °C for 1 min, and a final elongation at 72 °C for 5 min. The PCR products were digested by restriction enzymes *Bsa*JI (for rs1927911), *Sph*I (for rs1927914) and *Bsm*AI (for rs10116253) (New England BioLabs NEB, Beijing), respectively, and the reaction

Table 2 Primary information on genotyping assays for three SNPs of TLR4 gene

SNP	Primer sequence	Product size (bp)	Restriction enzyme	Restriction fragments of genotype (bp)
rs1927911	F: TCACTTTGCTCAAGGGTCAA	203	*Bsa*JI	203 = TT
	R: AAACCTGCATGCTCTGCAC			203, 178, 25 = TC
				178, 25 = CC
rs1927914	F: ACAAAATGGTCCCTCACAGC	157	*Sph*I	157 = TT
	R: TGGAAAGTAGCAAGTGCAATG			157, 90, 67 = TC
				90, 67 = CC
rs10116253	F: GGGTGTAAAAGCCAGGTAGAGGAGGT	601	*Bsm*AI	601 = CC
	R: TGGAAAGTAGCAAGTGCAATGTAAGT			601, 470, 131 = TC
				470, 131 = TT

system of rs1927911 was incubated at 60 °C overnight, the PCR products of re1927914 were incubated at 37 °C overnight, while the system of rs10116253 was incubated at 55 °C for 30 min. Then the digestion products of rs1927911 and rs1927914 were separated by electrophoresis on a 2.5 %agarose gels containing ethidium bromide and visualized under UV light. And 1 %agarose electrophoresis was used to analyze the results of rs10116253. Genotypes and the size of fragments are shown in Table 2. To validate the results, the genotyping experiments were repeated and 10 % of PCR-amplified DNA samples were examined by DNA sequencing. Results between PCR and DNA sequencing analysis were 100 % concordant.

Statistical analyses

Genotype and allele frequencies were estimated by direct counting. All the statistical analyses were performed with the SPSS Statistics 19.0 software. Hardy–Weinberg equilibrium (HWE) between expected and observed genotype distributions was assessed by Chi-square (χ^2) test. Demographic and clinical data of the study population between two groups were compared using the χ^2 test or Student's t test. The χ^2 test was also used to analyze the genotype and allele distributions. To assess the relative risk conferred by a particular allele and genotype, odds ratio (OR) and 95 % confidence intervals (CI) were calculated. $p < 0.05$ was considered statistically significant.

Results

For the three SNPs, the HWE test was performed among PD cases and control subjects to examine possible genotyping error and selection bias. Statistical analysis indicated that no bias occurred ($p > 0.05$ for all the analyses, Table 3). The allele and genotype distributions for TLR4 gene rs1927911, rs1927914 and rs10116253 polymorphisms in PD groups and controls are shown in Table 4.

For rs1927914 polymorphism, there was no significant difference in genotype distribution between PD and control in the total sample ($p = 0.069$), but in patient group, the frequency of genotype with C (CC+TC) was significantly lower than the corresponding value for the control group (OR = 0.718, 95 % CI = 0.531–0.972, $p = 0.032$ for CC+TC vs. TT; OR = 0.742, 95 % CI = 0.494–1.114, $p = 0.149$ for CC vs. TC+TT). And the PD patients showed a lower C allele frequency than the healthy-matched control (OR = 0.795, 95 % CI = 0.647–0.976, $p = 0.028$). In addition, there was significant difference in genotype distribution between male PD and its healthy-matched control subgroup ($p = 0.035$) and CC+TC genotype is a reduced risk compared with the TT genotype (OR = 0.659, 95 % CI = 0.448–0.970, $p = 0.034$). It was also found that the frequency of rs1927914 C allele was significantly reduced in male PD relative to healthy-matched control subgroup (OR = 0.714, 95 % CI = 0.549–0.929, $p = 0.012$). Moreover, CC+TC genotype is a reduced risk compared with the TT genotype between EOPD patients and healthy controls (OR = 0.592, 95 % CI = 0.376–0.932, $p = 0.023$). However, there were no significant differences in the genotype and allele frequencies between male and female PD patients, female PD patients and healthy-matched controls, LOPD patients and controls, as well as EOPD and LOPD (Table 5).

For rs1927911 polymorphisms, there was no significant difference in genotype and allele distribution between PD and control ($p = 0.557$ and $p = 0.272$, respectively). Similarly, rs10116253 had no significant differences in the genotype ($p = 0.399$) and allele ($p = 0.174$) frequencies between PD patients and the controls. And no significant difference was observed in the genotype distributions and allele frequencies between any age and gender subgroup for both rs1927911 and rs10116253 (Tables 6, 7).

Table 3 Hardy–Weinberg equilibrium of TLR4 gene polymorphisms

SNP	Group	*N*	Genotype			Hardy–Weinberg equilibrium	
						χ^2	*p* value
rs1927911	PD	380	61	173	146	0.655	0.418
	Control	380	69	178	133	0.487	0.485
rs1927914	PD	380	48	190	142	1.614	0.204
	Control	380	62	204	114	3.370	0.066
rs10116253	PD	380	57	179	144	0.013	0.910
	Control	380	68	183	129	0.049	0.825

Table 4 Genotype and allele frequencies of TLR4 gene in patients and controls

rs1927911	Genotype					Allele		
	N	TT (%)	TC (%)	CC (%)	*p*	T (%)	C (%)	*p*
PD	380	61 (16.1)	173 (45.5)	146 (38.4)	0.557	295 (38.8)	465 (61.2)	0.272
Control	380	69 (18.2)	178 (46.8)	133 (35.0)		316 (41.6)	444 (58.4)	
rs1927914	Genotype					Allele		
	N	CC (%)	TC (%)	TT (%)	*p*	C (%)	T (%)	*p*
PD	380	48 (12.6)	190 (50.0)	142 (37.4)	0.069	286 (37.6)	474 (62.4)	0.028
Control	380	62 (16.3)	204 (53.7)	114 (30.0)		328 (43.2)	432 (56.8)	
rs1927914	Genotype					Allele		
	N	CC + TC (%)	TC (%)	TT (%)	*p*	C (%)	T (%)	*p*
PD	380	238 (62.6)	0	142 (37.4)	0.032	0	0	0
Control	380	266 (70.0)	0	114 (30.0)		0	0	
rs10116253	Genotype					Allele		
	N	CC (%)	TC (%)	TT (%)	*p*	C (%)	T (%)	*p*
PD	380	57 (15.0)	179 (47.1)	144 (37.9)	0.399	293 (38.6)	467 (61.4)	0.174
Control	380	68 (17.9)	183 (48.2)	129 (33.9)		319 (42.0)	441 (58.0)	

Discussion

There is a consensus that inflammatory process has a fundamental role in the pathogenesis of progressive PD, and anti-inflammatory therapies have already been developed in animal model systems of PD [6–9, 25]. One of the most important functions of TLR4 in the brain is involvement in the microglia-mediated inflammatory response. Once activated, TLR4 initiates signal transduction cascades that involve the nuclear NF-κB pathway and the toll/IL-1 receptor-containing adaptor-inducing IFN-β (TRIF) pathway, inducing a multitude of pro-inflammatory [14, 26]. Moreover, specific NF-κB inhibitors have been demonstrated to halt the progression of neurodegeneration induced by the neurotoxin MPTP in murine models of PD, or by activation of CNS inflammation by the intracranial injection of LPS [25]. It is well documented that the abnormal aggregation of α-synuclein that was detected in the neuron of PD patients may participate in the pathogenesis of the disease. Recent data also indicate that α-synuclein could directly activate local microglia, inciting the production of pro-inflammatory molecules and augmentation of the expression of TLR4. And then TLR4 activation initiates downstream molecular pathway [16, 27–29]. There is evidence that TLR4 is associated with AD [18] which may own common pathogenetic mechanisms with PD. These findings suggest that TLR4 represents a reasonable functional candidate gene for PD.

Most of the studies about TLR4 were focused on rs4896790 and rs4896791 polymorphisms, which have been demonstrated to affect the risk of various multifactorial diseases, especially in age-related diseases [12, 17, 26]. However, these two polymorphisms are rare in the Chinese population. Consequently, we investigated the potential relevance of TLR4 gene rs1927911, rs1927914 and rs10116253 polymorphisms with the risk of PD in a

Table 5 Distribution of rs1927914 polymorphisms observed in every PD patient and each healthy-matched control subgroup

Rs1927914	Genotype					Allele		
	N	CC (%)	TC (%)	TT (%)	*p*	C (%)	T (%)	*p*
Male PD	236	28 (11.9)	117 (49.6)	91 (38.5)	0.760	173 (36.7)	299 (63.3)	0.476
Female PD	144	20 (13.9)	73 (50.7)	51 (35.4)		113 (39.2)	175 (60.8)	
Female PD	144	20 (13.9)	73 (50.7)	51 (35.4)	0.627	113 (39.2)	175 (60.8)	0.711
Female health	151	19 (12.6)	85 (56.3)	47 (31.1)		123 (40.7)	179 (59.3)	
Male PD	236	28 (11.9)	117 (49.6)	91 (38.5)	0.035	173 (36.7)	299 (63.3)	0.012
Male health	229	43 (18.8)	119 (52.0)	67 (29.2)		205 (44.8)	253 (55.2)	
EOPD	100	15 (15.0)	43 (43.0)	42 (42.0)	0.069	73 (36.5)	127 (63.5)	0.089
Health	380	62 (16.3)	204 (53.7)	114 (30.0)		328 (43.2)	432 (56.8)	
LOPD	280	33 (11.8)	147 (52.5)	100 (35.7)	0.138	213 (38.0)	347 (62.0)	0.061
Health	380	62 (16.3)	204 (53.7)	114 (30.0)		328 (43.2)	432 (56.8)	
EOPD	100	15 (15.0)	43 (43.0)	42 (42.0)	0.258	73 (36.5)	127 (63.5)	0.700
LOPD	280	33 (11.8)	147 (52.5)	100 (35.7)		213 (38.0)	347 (62.0)	

Table 6 Distribution of rs1927911 polymorphisms observed in every PD patient and each healthy-matched control subgroup

Rs1927911	Genotype					Allele		
	N	TT (%)	TC (%)	CC (%)	*p*	T (%)	C (%)	*p*
Male PD	236	33 (14.0)	110 (46.6)	93 (39.4)	0.372	176 (37.3)	296 (62.7)	0.269
Female PD	144	28 (19.4)	63 (43.8)	53 (36.8)		119 (41.3)	169 (58.7)	
Female PD	144	28 (19.4)	63 (43.8)	53 (36.8)	0.891	119 (41.3)	169 (58.7)	0.949
Female health	151	27 (17.9)	70 (46.4)	54 (35.7)		124 (41.1)	178 (58.9)	
Male PD	236	33 (14.0)	110 (46.6)	93 (39.4)	0.344	176 (37.3)	296 (62.7)	0.149
Male health	229	42 (18.3)	108 (47.2)	79 (34.5)		192 (42.0)	266 (58.0)	
EOPD	100	17 (17.0)	38 (38.0)	45 (45.0)	0.168	72 (36.0)	128 (64.0)	0.153
Health	380	69 (18.2)	178 (46.8)	133 (35.0)		316 (41.6)	444 (58.4)	
LOPD	280	44 (15.7)	135 (48.2)	101 (36.1)	0.712	223 (40.0)	337 (60.0)	0.521
Health	380	69 (18.2)	178 (46.8)	133 (35.0)		316 (41.6)	444 (58.4)	
EOPD	100	17 (17.0)	38 (38.0)	45 (45.0)	0.193	72 (36.0)	128 (64.0)	0.341
LOPD	280	44 (15.7)	135 (48.2)	101 (36.1)		223 (40.0)	337 (60.0)	

Han Chinese population. Rs1927914 and rs10116253 SNP are located in 5′-UTR, while rs1912911 SNP is located in Intron 1. Based on the data presented in Table 4, genotyping 380 cases of healthy population shows that rs1927911 C/T, rs1927914 T/C and rs10116253 T/C all exist in Han Chinese population, with the frequencies 41.6, 43.2 and 42.0 %, respectively, were nearly in agreement with the minor allele frequency(MAF)reported in the database(36.4, 36.7 and 36.7 %, respectively). Our advanced analysis demonstrated that only rs1927914 polymorphisms may affect the development of PD. In our study, rs1927914 CC+TC genotype in PD patients is significantly lower than controls. There was also significant difference in allele distribution between PD and control in the total sample. The results demonstrated that rs1927914 polymorphism may decrease the risk of development of PD, and rs1927914C allele may be a protective factor against sporadic PD in a Han Chinese population. In contrary, our data indicated that no significant association was observed between TLR4 rs1927911 and rs10116253 polymorphisms and the risk of PD implying that these polymorphisms may not contribute to the susceptibility to PD.

Since genetic variations vary among the populations of age at onset and gender, we assessed the differences between PD cases and control subjects at the age at onset and gender subgroup level. As to the subgroup analyses, rs1927914 shows significant difference between male and healthy-matched control subgroup. Based on our findings, we can infer that the pathogenesis of male and female PD might be different and it probably is the cause of sex hormones and rs1927914C allele may be a protective factor against male PD. The male who possess rs1927914 missense mutation may have less risk for PD. Moreover, for rs1927914 polymorphism, our study shows that CC+TC genotype in EOPD is significantly lower than healthy controls. The conclusion of rs1927914 polymorphisms is

Table 7 Distribution of rs10116253 polymorphisms observed in every PD patient and each healthy-matched control subgroup

Rs10116253	Genotype					Allele		
	N	CC (%)	TC (%)	TT (%)	*p*	C (%)	T (%)	*p*
Male PD	236	32 (13.5)	113 (47.9)	91 (38.6)	0.602	177 (37.5)	295 (62.5)	0.445
Female PD	144	25 (17.4)	66 (45.8)	53 (36.8)		116 (40.3)	172 (59.7)	
Female PD	144	25 (17.4)	66 (45.8)	53 (36.8)	0.674	116 (40.3)	172 (59.7)	0.783
Female health	151	24 (15.9)	77 (51.0)	50 (33.1)		125 (41.4)	177 (58.6)	
Male PD	236	32 (13.5)	113 (47.9)	91 (38.6)	0.239	177 (37.5)	295 (62.5)	0.130
Male health	229	44 (19.2)	106 (46.3)	79 (34.5)		194 (42.4)	264 (57.6)	
EOPD	100	17 (17.0)	41 (41.0)	42 (42.0)	0.309	75 (37.5)	125 (62.5)	0.252
Health	380	68 (17.9)	183 (48.2)	129 (33.9)		319 (42.0)	441 (58.0)	
LOPD	280	40 (14.3)	138 (49.3)	102 (36.4)	0.447	218 (38.9)	342 (61.1)	0.266
Health	380	68 (17.9)	183 (48.2)	129 (33.9)		319 (42.0)	441 (58.0)	
EOPD	100	17 (17.0)	41 (41.0)	42 (42.0)	0.361	75 (37.5)	125 (62.5)	0.722
LOPD	280	40 (14.3)	138 (49.3)	102 (36.4)		218 (38.9)	342 (61.1)	

protective in all patients and the analyzing of patients by sex, male are protected while female are not may be the result of statistical power, 144 female PD vs 236 male PD. The male to female ratio was similar in patient group and healthy controls so that we can consider that the influence on the result is small. It indicated that genetic factors probably affect the development of PD that age at onset $\leq$50 years. However, for rs1927911 and rs10116253, there was no significant difference between any subgroups.

The major limitation of study was the relatively small sample sizes for both the case and control groups. With increasing sample size, the significances are supposed to approach the true population values. More studies are needed to confirm, refine the present findings in other populations of different ethnic and countries.

In summary, this study provides evidence that rs1927914 polymorphisms may decrease susceptibility and severity of sporadic PD, male PD and EOPD and the C allele of rs1927914 may be a protective factor for PD. TLR4 might be used as a novel therapeutic target and a prognostic marker in the future. Functional assays are also needed to elucidate the molecular mechanisms that the TLR4 sequence variants affect the function of the TLR4 gene.

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Conflict of interest The authors declare that they have no conflict of interest.

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