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·Original Article·

Single-nucleotide polymorphisms and haplotypes of non-coding area in the *CP* gene are correlated with Parkinson's disease

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ABSTRACT

Our previous studies have demonstrated that ceruloplasmin (CP) dysmetabolism is correlated with Parkinson's disease (PD). However, the causes of decreased serum CP levels in PD patients remain to be clarified. This study aimed to explore the potential association between genetic variants of the *CP* gene and PD. Clinical features, serum CP levels, and the *CP* gene (both promoter and coding regions) were analyzed in 60 PD patients and 50 controls. A luciferase reporter system was used to investigate the function of promoter single-nucleotide polymorphisms (SNPs). High-density comparative genomic hybridization microarrays were also used to detect large-scale copy-number variations in *CP* and an additional 47 genes involved in PD and/or copper/iron metabolism. The frequencies of eight SNPs (one intronic SNP and seven promoter SNPs of the *CP* gene) and their haplotypes were significantly different between PD patients, especially those with lowered serum CP levels, and controls. However, the luciferase reporter system revealed no significant effect of the risk haplotype on promoter activity of the *CP* gene. Neither these SNPs nor their haplotypes were correlated with the Hoehn and Yahr staging of PD. The results of this study suggest that common genetic variants of *CP* are associated with PD and further investigation is needed to explore their functions in PD.

Keywords: Parkinson's disease; ceruloplasmin; single-nucleotide polymorphism; haplotype; copy-number variation

INTRODUCTION

Parkinson's disease (PD) is an age-related neurodegenerative disease characterized by the loss of dopaminergic neurons in the substantia nigra[1]. The presence of Lewy bodies in degenerating dopaminergic neurons seems to be the initial characterization of the pathology of PD. In the core of the Lewy body is the protein α-synuclein which binds iron. Iron seems to be a requisite for the deposition and accumulation of α-synuclein. Thus, iron might be deposited in a disorderly manner in extrapyramidal structures and result in the tissue damage in PD. However, little is known about the cause of iron deposition in PD.

Ceruloplasmin (CP) is a multi-copper enzyme with ferroxidase function and plays an important role in iron metabolism[2]. CP oxidizes ferrous iron into the ferric form and thus keeps the intracellular level of dangerous ferrous iron to a minimum[3, 4]. Hereditary aceruloplasminemia caused by mutation of the *CP* gene, resulting in the absence of circulating serum CP, presents with parkinsonism and retinal degeneration due to substantial iron accumulation in the basal ganglia and retina[5]. Patel and colleagues have also demonstrated that increased iron accumulation and free-radical injury occur in the central

nervous system of *Cp*$^{-/-}$ mice[6]. Thus, the association between CP and PD has been discussed since the ninth decade of the last century. It has been found that both the CP level and oxidative activity in serum and cerebrospinal fluid are significantly lower in PD patients than in age- and sex-matched healthy controls[7-13]. In addition, lower serum CP levels are correlated with a younger age of PD onset[14]. Furthermore, our previous studies and those of others have demonstrated that decreased serum CP level and oxidative activity specifically exacerbate nigral iron deposition in PD patients[15-17]. However, the causes of the decreased serum CP level in PD remain to be clarified.

As PD often has a hereditary basis and the genetic predisposition is seen as a major contributor to the underlying cause, we carried out this study to elucidate whether *CP* gene variations are generally found in PD patients and contribute to the decreased levels of CP.

MATERIALS AND METHODS

Patients and Controls

Sixty PD patients were recruited from the Department of Neurology, Zhongshan Hospital, Fudan University. PD was diagnosed by two independent movement-disorder specialists (CJ Zhong and LR Jin) according to the criteria of the United Kingdom Parkinson's Disease Society Brain Bank for idiopathic PD[19] and the modified version of Hoehn and Yahr[20]. Briefly, 33% of the PD patients showed unilateral motor impairments only, corresponding to Hoehn and Yahr stage I, 47% presented bilateral or midline involvement without balance impairment (Hoehn and Yahr stage II), and 20% showed bilateral impairments with mild to moderate disability and postural reflex dysfunction (Hoehn and Yahr stage III). The PD patients were divided into three subgroups: primarily rigidity and bradykinesia with minimal tremor (PD_{AR} subgroup), primarily tremor with minimal bradykinesia and rigidity (PD_T subgroup), and mixed classic motor symptoms with propinquity proportion (PD_M subgroup) according to the ratio of average UPDRS III Tremor score of each PD patient (sum of items 20 and 21 divided by 4) to his/her average UPDRS akinetic/rigid score (sum of items 22–27 and 31 divided by 15), as previously reported[16]. Patients showing signs of upper and/or lower motor neuron impairments, orthostatic hypotension within three years since PD onset, cognitive impairment assessed by the Mini-Mental State Examination (MMSE), and hepatic and/or renal dysfunction, were excluded from this study. Fifty control participants were recruited from the Xujiahui Community of Shanghai. No controls had a history of neurologic/psychiatric disorders and cognitive impairment as assessed by the MMSE (scores ≥28).

Serum CP levels were measured in both PD patients and controls using an immunonephelometry kit (N antiserum against human CP; Dade Behring, Marburg, Germany) according to the manufacturer's instructions. Accordingly, PD patients were further divided into two subgroups: those with lower serum CP (<0.20 g/L, PD_{LCP}) and those with normal serum CP (≥0.20 g/L, PD_{NCP}).

This study was approved by the Committee on Medical Ethics of Zhongshan Hospital, Fudan University, and all participants gave informed consent.

CP Gene Sequencing

Blood samples were collected into EDTA-containing tubes. A QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany) was used to extract genomic DNA from the white blood cell fraction. The sequences of all exons, intron–exon boundaries, and the DNase cluster region of the promoter in the *CP* gene were determined by aligning GenBank with genomic sequence records (NM 000096.1). The primers are shown in Table 1. *CP* amplicons were obtained by polymerase chain reaction (PCR) amplification and purified by cutting out the DNA band from agarose gel electrophoresis. DNA sequencing was performed on an ABI3730XL automated sequencer, using version 3.1 of the Big Dye fluorescent method according to the manufacturer's instructions (Applied Biosystems, Foster City, CA). Sequence data were analyzed using VECTOR NTI Advance 11 software (Invitrogen Corp., Carlsbad, CA).

Plasmid Construction

To construct the *CP* promoter reporter plasmid, we designed primers that could amplify the 878-bp DNA fragment containing rs66508328(GG/AA), rs67870152(CC/TT), rs16861642(GG/AA), rs73020328(TT/GG), rs11708215(GG/AA), rs73166855(AA/GG), and rs66953613(CC/TT) (Primer F: 5'-CTTGCCTGAGACCATTTTACATCC-3'; Primer R: 5'-CAACAGCACAGACTGGGGTTAG-3'). The wild-type and mutant DNA fragments were amplified from the genomic

Table 1. Primers for the *CP* gene and conditions of PCR

PCR location	Oligonucleotide of primers	Size of PCR product (bp)	Annealing temperature (°C)
Exon 1	F: 5'ACACGTTCTCTGCCCTCCTGGAA3'		
	R: 5'CGGAGATGCAGTTACGACCATGGGA3'	716	60
Exon 2	F: 5'GGAGGCATCCCTACAACAGGCA3'		
	R: 5'TGTCTTATCCAGGAGAGAAGTCCAC3'	482	62
Exon 3	F: 5'TCAGACTCCCATACCATGACCCGA3'		
	R: 5'TCACCGTGGAGTGCCCTTTTGG3'	832	63
Exon 4	F: 5'TGGTCAGTGGACATCCAGACACAGG3'		
	R: 5'ACCAGTTGGGGAACAAGTTTGGTGT3'	616	62
Exon 5	F: 5'GGCAAGAATACCAGCATGTGTGCCA3'		
	R: 5'AACAGGGTGCTTTCCAGTGCAACA3'	706	63
Exon 6	F: 5'CCAAGTGAAACCCCACAGAAACTGG3'		
	R: 5'TGCTGCTGAATCGTACAGTGCCA3'	758	63
Exon 7	F: 5' TGAGTGGACTGGAACTGTCTGCT 3'		
	R: 5' GCCCATGGGAAGAGTAAACCAGCC 3'	364	58
Exon 8	F: 5'TGACACACCTCCAGCCAACAGA3'		
	R: 5'GCGGTTTCCTTGGGAGTTCCTGC3'	592	61
Exon 9	F: 5'CCAGGAGGAGGTTTAGAAG3'		
	R: 5'GAACATTGATTGGCTATTTG3'	633	55
Exon 10	F: 5'TGTGCACATGGAAGTCTTCTGCT3'		
	R: 5'ATGAGCCTGTCATTTTTGAGCCA3'	652	60
Exon 11	F: 5'GGTCCTGGAAAGTCTGTGA3'		
	R: 5'ATCTTGAGGAGCCTATGGA3'	547	55
Exon 12	F: 5' AAAGGATGGATGGAGCAGG 3'		
	R: 5' AGCGGAAATGAATAAGGACAA 3'	532	57
Exon 13	F: 5'AGTGACTAGCTGGAGGAAAT3'		
	R: 5'AAATGAAACCCATAGACATG3'	492	59
Exon 14	F: 5'GGACTTTCAGGCCAAACCTCCCC3'		
	R: 5'ACAGACACCTCCTTGCATCCCCT3'	495	61
Exon 15	F: 5'GCTTTGTGGTATGGCAAGTGGGTT3'		
	R: 5'TCAGTGGCTACCTGTGACCCACAA3'	633	62
Exon 16	F: 5'AGCATCACCCACATGACCTACCT3'		
	R: 5'TGCTTTTCTAGGCACTTTGCACCA3'	696	60
Exon 17	F: 5'TAATCCAAAACTAAGATTAAGGC3'		
	R: 5'AATCCACGGATATGAAGCA3'	490	56
Exon 18	F: 5'GACAAACAGGCAAACCAGA3'		
	R: 5'ATCCCTCACCATTTAGCAG3'	631	55
Exon 19	1 F: 5'TCTTGTAGGGAAGAAAGTAATC3'		
	1 R: 5'GACATCTGCCACAGGTCTA3'	858	55
	2 F: 5'TGAGTTTTGTGAACCCCTGAA3'		
	2 R: 5'GGCTGCTTACCTTACCGTGTA3'	847	57
Promoter	1 F 5' AGGGAGAAGGCACTGGGAGCTAATA3'		
	1 R 5' AGGATTCAGCTAAATACTTATGCCAT3'	1260	60
	2 F 5'AGGCATAATCCCAAGTCGTTTCA3'		
	2 R 5' ATCGCATGCTTTCTTCTATACCAATC3'	420	65
	3 5' ACATGATATCTAGCATGCCAAAT3'	330	65
	4 5' AAGTTATTAGCCCCTGTTAGGCT3'	580	65

DNA of controls and patients carrying mutant single-nucleotide polymorphisms (SNPs), respectively. The DNA was constructed to the PGL3-Basic vector (Promega, San Luis Obispo, CA). All plasmids were verified by Sanger sequencing.

Cell Culture and Luciferase Assay

U251 and HepG2 cells were cultured in DMEM with 10% FBS. Then the cells were digested with trypsin and seeded in 24-well plates at 1×10^5/well with 500 μL culture medium. After 24 h in culture, 500 ng of *CP* promoter reporter plasmids were co-transfected with 10 ng pRL-TK plasmid as a normalizing control using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. After 24 h in culture, the cells were lysed, and 20 μL of supernatant was used to assay the luciferase activity using the Dual-Luciferase Reporter Assay System (Promega). The relative reporter activity was normalized by firefly activity to *Renilla* activity. Each assay was performed at three independent experiments with four replications.

Comparative Genomic Hybridization Microarray Analysis

We designed a high-density oligonucleotide-based comparative genomic hybridization (CGH) microarray in the Agilent 8×60K format. Briefly, we first selected 48 candidate genes associated with PD or iron/copper metabolism. Using the Agilent eArray online system, we selected 52828 oligonucleotide probes for the 48 candidates and their 150-kb flanking regions on both sides (Table S1). The genomic DNAs extracted from PD patients and sex-matched standard DNAs were fragmented *via* AluI and RsaI enzyme digestion. DNA was labeled using an Agilent SureTag DNA Labeling Kit (Agilent Technologies, Santa Clara, CA). Different fluorescent dyes were used for DNA labeling of patient DNA (Cy5-dUTP) and standard DNA (Cy3-dUTP). The labeled product from each patient was mixed with a standard product before being hybridized onto the Agilent CGH microarray (Agilent Technologies, Santa Clara, CA) for 24 h at 65°C. DNA processing, microarray handling, and scanning were conducted following the Agilent oligonucleotide CGH protocol (version 6.0). The microarray scanning profiles were processed by Agilent Feature Extraction 10.7.3.1. The extracted data were analyzed and plotted by Agilent Workbench 7.0. ADM-2 was selected as the statistical algorithm with a threshold of 6.0 and the Fuzzy Zero turned on.

Statistical Analysis

Data are presented as mean ± SEM. The unpaired *t*-test, Mann-Whitney test, or Kruskal-Wallis test (for continuous variables), or the χ^2 and Fisher exact tests (for categorical variables) were used as the main methods of statistical analysis. Genotype frequency data were also tested for Hardy-Weinberg equilibrium. Linkage disequilibrium (LD) of the *CP* gene variants was analyzed using SHEsis software (http://analysis.bio-x.cn/myAnalysis.php)[21]. Spearman correlation analysis was used to evaluate correlations. The level of significance was assumed to be 5%, and all tests were two-sided. All the statistical analyses were performed with SPSS software (version 18.0).

RESULTS

Characteristics of Study Participants

The average age was 61.52 ± 1.25 years in PD patients and 72.82 ± 0.79 years in controls. The gender ratio was 52% male and 48% female in PD patients, and 40% male and 60% female in controls. There was a statistically significant difference in the serum CP levels between PD patients (0.206 ± 0.007 g/L) and normal controls (0.224 ± 0.006 g/L) (P = 0.043). The PD patients included 28 PD_{LCP} patients (age: 60.57 ± 1.83; gender: male 71%, female 29%; serum CP level 0.165 ± 0.003 g/L) and 32 PD_{NCP} patients (age: 62.34 ± 1.73; gender: male 38%, female 62%; serum CP level 0.248 ± 0.008 g/L). The age-difference between PD_{LCP} and PD_{NCP} patients was not statistically significant (P = 0.837). There as a dramatic difference in the gender distribution in PD_{LCP} cases compared with PD_{NCP} cases (P = 0.010). Male PD patients were more prone to present low serum CP than females.

CP Gene Variants in PD Patients

The distributions and genotypes of the *CP* gene SNPs in PD patients and controls are shown in Table 2. Genotype data were first tested for Hardy-Weinberg equilibrium (HWE) before further genetic analysis. There was no significant deviation from the HWE in either the PD or control group (data not shown). Ten SNPs were found in the intronic and promoter regions of *CP*: two located at introns (rs3736282

Table 2. Distributions and genotypes of SNPs in PD cases and controls.

SNP	Location	Genotype	CTRL	PD			PD/CTRL		PD_{LCP}/CTRL	
			n=50	Total *n*=60	PD_{NCP} *n*=32	PD_{LCP} *n*=28	*P*	OR (95%CI)	*P*	OR (95%CI)
rs3736282	Intron 1	CC+CT	26+22 (96%)	29+20 (81.7%)	16+11 (84.4%)	13+9 (78.6%)	0.03	5.39 (1.13-25.61)	0.02	6.55 (1.22-35.06)
		TT	2 (4%)	11 (18.3%)	5 (15.6%)	6 (21.4%)				
rs17847023	Intron 2	GG+AG	22+25 (94%)	23+26 (81.7%)	13+15 (87.5%)	10+11 (75%)	0.08	3.52 (0.92-13.41)	0.03	5.22 (1.23-22.21)
		AA	3 (6%)	11 (18.3%)	4 (12.5%)	7 (25%)				
rs17838831	Promoter	AA+AG	21+26 (94%)	27+21 (80%)	15+11 (81.3%)	12+10 (78.6%)	0.05	3.92 (1.04-14.78)	0.06	4.27 (0.98-18.69)
		GG	3 (6%)	12 (20%)	6 (18.7%)	6 (21.4%)				
rs66508328	Promoter	GG+AG	26+23 (98%)	30+21 (85%)	17+11 (87.5%)	13+10 (82.1%)	0.02	8.65 (1.06-70.85)	0.02	10.65 (1.18-96.52)
		AA	1 (2%)	9 (15%)	4 (12.5%)	5 (17.9%)				
rs67870152	Promoter	CC+CT	25+24 (98%)	30+21 (85%)	17+11 (87.5%)	13+10 (82.1%)	0.02	8.65 (1.06-70.85)	0.02	10.65 (1.18-96.52)
		TT	1 (2%)	9 (15%)	4 (12.5%)	5 (17.9%)				
rs16861642	Promoter	GG+AG	25+24 (98%)	30+21 (85%)	17+11 (87.5%)	13+10 (82.1%)	0.02	8.65 (1.06-70.85)	0.02	10.65 (1.18-96.52)
		AA	1 (2%)	9 (15%)	4 (12.5%)	5 (17.9%)				
rs73020328	Promoter	TT+TG	27+22 (98%)	32+19 (85%)	18+10 (87.5%)	14+9 (82.1%)	0.02	8.65 (1.06-70.85)	0.02	10.65 (1.18-96.52)
		GG	1 (2%)	9 (15%)	4 (12.5%)	5 (17.9%)				
rs11708215	Promoter	GG+AG	26+23 (98%)	32+19 (85%)	18+10 (87.5%)	14+9 (82.1%)	0.02	8.65 (1.06-70.85)	0.02	10.65 (1.18-96.52)
		AA	1 (2%)	9 (15%)	4 (12.5%)	5 (17.9%)				
rs73166855	Promoter	AA+AG	22+26 (96%)	27+21 (80%)	15+11 (81.3%)	12+10 (78.6%)	0.02	3.69 (0.75-18.27)	0.02	6.55 (1.22-35.06)
		GG	2 (4%)	12 (20%)	6 (18.7%)	6 (21.4%)				
rs66953613	Promoter	CC+CT	26+23 (98%)	32+19 (85%)	18+10 (87.5%)	14+9 (82.1%)	0.02	8.65 (1.06-70.85)	0.02	10.65 (1.18-96.52)
		TT	1 (2%)	9 (15%)	4 (12.5%)	5 (17.9%)				

Statistical analysis was performed using the Fisher exact test.

C>T and rs17847023 G>A) and the other eight at the promoter (rs17838831 A>G, rs66508328 G>A, rs67870152 C>T, rs16861642 G>A, rs73020328 T>G, rs11708215 G>A, rs73166855 A>G, and rs66953613 C>T). Moreover, the frequencies of eight SNPs (rs3736282 C>T, rs66508328 G>A, rs67870152 C>T, rs16861642 G>A, rs73020328 T>G, rs11708215 G>A, rs73166855 A>G and rs66953613 C>T) were significantly higher in the PD group than in the control group. Compared with controls, PD patients, especially PD_{LCP} patients, carried higher frequencies of homozygous mutant alleles of rs3736282 TT (*P* = 0.03 for PD, *P* = 0.02 for PD_{LCP}), rs66508328 AA (*P* = 0.02, *P* = 0.02), rs67870152 TT (*P* = 0.02, *P* = 0.02), rs16861642 AA (*P* = 0.02, *P* = 0.02), rs73020328 GG (*P* = 0.02, *P* = 0.02), rs11708215 AA (*P* = 0.02, *P* = 0.02), rs73166855 GG (*P* = 0.02, *P* = 0.02), and rs66953613 TT (*P* = 0.02, *P* = 0.02).

To examine the combined effect of these eight SNPs in the *CP* gene, we performed haplotype analysis (Table 3), which revealed high LD between the eight SNPs (Fig.1). The results from haplotype analysis were generally consistent with those from single SNPs; the haplotype defined by the mutant alleles of these eight SNPs (TATAGAGT) was associated with risk for PD, especially PD_{LCP}. For example, the PD patients (15%), especially PD_{LCP} (17.8%), had higher frequencies of TATAGAGT/TATAGAGT than controls (2%) (*P* = 0.02, *P* = 0.02). The distributions of the other *CP* SNP haplotypes did not differ significantly between patients and controls (Table 3).

No Association of *CP* Gene Variants with Hoehn and Yahr Stage and Motor Phenotype

We also analyzed whether the frequencies of SNPs in the

Table 3. Haplotype analysis of the block of rs3736282, rs66508328, rs67870152, rs16861642, rs73020328, rs11708215, rs73166855, and rs66953613 in PD patients and controls

Haplotype block	rs3736282	rs66508328	rs67870152	rs16861642	rs73020328	rs11708215	rs73166855	rs66953613
Haplotype	CTRL	PD			PD/CTRL		PD_{LCP}/CTRL	
	n=50	Total *n*=60	PD_{NCP} *n*=32	PD_{LCP} *n*=28	*P*	OR(95%CI)	*P*	OR (95%CI)
CGCGTGAC/CGCGTGAC 00000000/00000000	22 (44%)	25 (41.7%)	14 (43.8%)	11 (39.3%)	0.84	0.91 (0.43-1.94)	0.81	0.82 (0.32-2.11)
CGCGTGAC/TATAGAGT 00000000/11111111	20 (40%)	16 (26.7%)	9 (28.1%)	7 (25%)	0.16	0.55 (0.24-1.22)	1.00	0.95 (0.32-2.88)
TATAGAGT/TATAGAGT 11111111/11111111	1 (2%)	9 (15%)	4 (12.5%)	5 (17.9%)	0.02	8.65 (1.06-70.85)	0.02	10.65 (1.18-96.52)
TGCGTGAC/TATAGAGT 10000000/11111111	2 (4%)	2 (3.3%)	1 (3.1%)	1 (3.6%)	1.00	0.83 (0.11-6.10)	1.00	0.89 (0.08-10.27)
CGCGTGAC/CGCGTGGC 00000000/00000010	3 (6%)	2 (3.3%)	1 (3.1%)	1 (3.6%)	1.00	0.65 (0.10-4.09)	1.00	0.58 (0.06-5.86)
CGCGTGAC/CATATGAC 00000000/01110000	0 (0%)	1 (1.7%)	0 (0%)	1 (3.6%)	/	/	/	/
CGCGTGAC/TGCGTGAC 00000000/10000000	0 (0%)	2 (3.3%)	1 (3.1%)	1 (3.6%)	/	/	/	/
CGCGTGAC/TATATAAT 00000000/11110101	1 (2%)	0 (0%)	0 (0%)	0 (0%)	/	/	/	/
CGCGTGGC/CGCGTGGC 00000010/00000010	0 (0%)	1 (1.7%)	1 (3.1%)	0 (0%)	/	/	/	/
CGCGTGGC/TATAGAGT 00000010/11111111	0 (0%)	1 (1.7%)	0 (0%)	1 (3.6%)	/	/	/	/
CGCGTGGC/TATATGGC 00000010/11110010	0 (0%)	1 (1.7%)	1 (3.1%)	0 (0%)	/	/	/	/
TGCGTGAC/TGCGTGAC 10000000/10000000	1 (2%)	0 (0%)	0 (0%)	0 (0%)	/	/	/	/

0=major genotype, 1=minor genotype. Statistical analysis was performed using the Fisher exact test.

CP gene and/or haplotypes are correlated with disease severity and motor phenotypes. The results showed that the frequencies of the SNPs and haplotype did not differ significantly among the PD subgroups assayed by Hoehn and Yahr staging and by motor phenotype (Tables 4 and 5).

Promoter Activity of the *CP* Gene

Since seven of the eight significantly changed SNPs were located in the promoter region of the *CP* gene, we investigated the function of the promoter SNPs/haplotypes of the *CP* gene using the luciferase reporter system in U251 and HepG2 cells. We found no significant difference in *CP* promoter activity between the WT haplotype (GCGTGAC/GCGTGAC) and the mutant haplotype (ATAGAGT/ATAGAGT) in both U251 and HepG2 cells (Fig. 2). Although the *in vitro* luciferase reporter assay

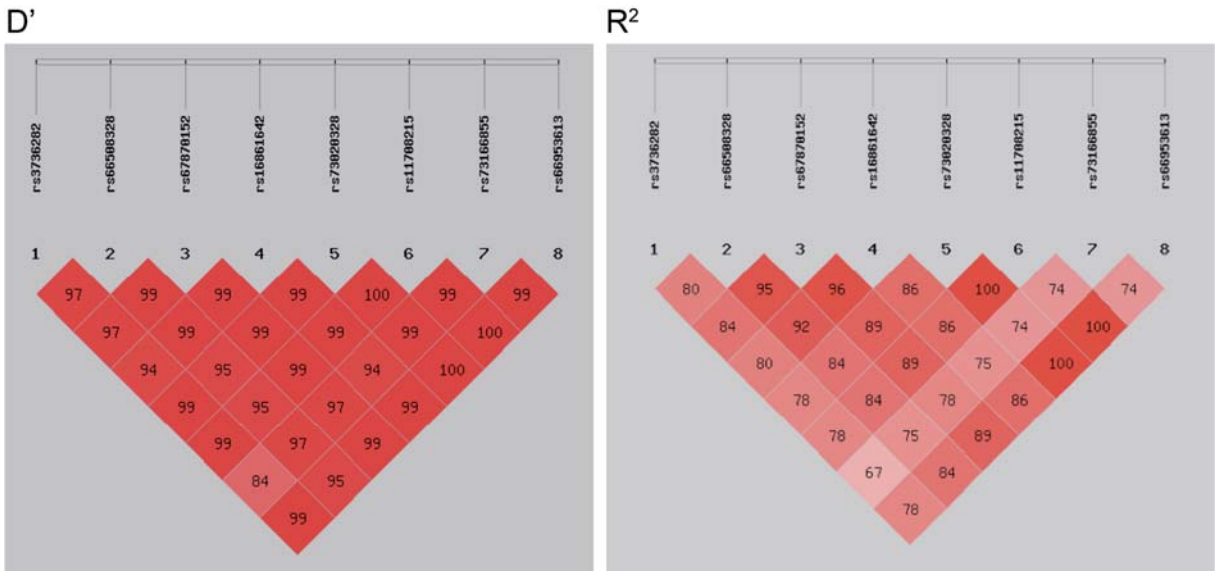

Fig. 1. Linkage disequilibrium (LD) test of *CP* gene polymorphisms (D', coefficient of LD; R^2, correlation coefficient of LD). The strength of the LD between SNPs is indicated by the color scheme. The number in each square indicates the magnitude of LD between respective pairs of SNPs. Dark red color indicating high D' and R^2; light red color indicating low D' and R^2. The blocks of dark red represent SNPs that are all in high LD with each other and thus are all inherited together.

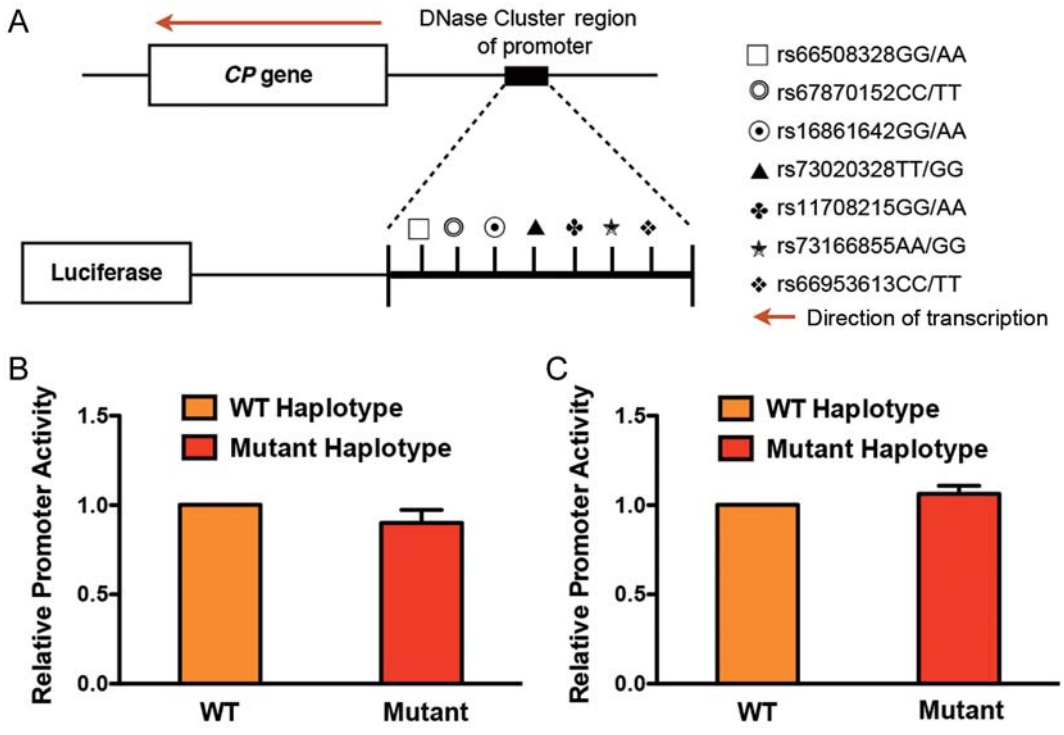

Fig. 2. Luciferase reporter assays in U251 (B) and HepG2 (C) cell lines. A. Schematic of the luciferase reporter construct with the seven SNPs represented by distinct symbols from the DNase cluster region of the *CP* gene promoter. No significant changes in *CP* promoter activity were found between the wild-type haplotype (GCGTGAC) and the mutant haplotype (ATAGAGT). Statistical analysis was performed using the unpaired *t*-test. Luciferase activity was normalized to the construct consisting of the wild-type haplotype (GCGTGAC).

Table 4. Correlation between SNPs/haplotype and PD disease severity

Subjects	Hoehn and Yahr stage I (*n*=20)	Hoehn and Yahr stage II (*n*=28)	Hoehn and Yahr stage III (*n*=12)	*P*
Age (years)	57.40±1.94	64.68±1.67	61.00±3.23	0.04
Gender	M: 11(55%)	M: 12(43%)	M: 9(75%)	
	F: 9(45%)	F: 16(57%)	F: 3(25%)	0.50
Serum CP (g/L)	0.20±0.01	0.22±0.01	0.23±0.03	0.66
Ratio of low serum CP (< 0.2g/L)	PD_{NCP}: 10(50%)	PD_{NCP}: 17(61%)	PD_{NCP}: 5(42%)	0.51
PD_{LCP}: 10(50%)	PD_{LCP}: 11(39%)	PD_{LCP}: 7(58%)		
rs3736282	CC+CT:18(90%)	CC+CT: 23(82.1%)	CC+CT: 8(66.7%)	0.26
	TT: 2(10%)	TT: 5(17.9%)	TT: 4(33.3%)	
rs17847023	GG+AG:18(60%)	GG+AG: 23(82.1%)	GG+AG: 8(66.7%)	0.26
	AA: 2(10%)	AA: 5(17.9%)	AA: 4(33.3%)	
rs17838831	AA+AG:18(90%)	AA+AG: 23(82.1%)	AA+AG: 7(58.3%)	0.09
	GG: 2(10%)	GG: 5(17.9%)	GG: 5(41.7%)	
rs66508328	GG+AG: 18(90%)	GG+AG: 25(89.3%)	GG+AG: 8(66.7%)	0.26
	AA: 2(10%)	AA: 3(10.7%)	AA: 4(33.3%)	
rs67870152	CC+CT: 18(90%)	CC+CT: 25(89.3%)	CC+CT: 8(66.7%)	0.26
	TT: 2(10%)	TT: 3(10.7%)	TT: 4(33.3%)	
rs16861642	GG+AG: 18(90%)	GG+AG: 25(89.3%)	GG+AG: 8(66.7%)	0.26
	AA: 2(10%)	AA: 3(10.7%)	AA: 4(33.3%)	
rs73020328	TT+TG:18(90%)	TT+TG: 25(89.3%)	TT+TG: 8(66.7%)	0.26
	GG: 2(10%)	GG: 3(10.7%)	GG: 4(33.3%)	
rs11708215	GG+GA: 18(90%)	GG+GA: 25(89.3%)	GG+GA: 8(66.7%)	0.26
	AA: 2(10%)	AA: 3(10.7%)	AA: 4(33.3%)	
rs73166855	AA+AG: 18(90%)	AA+AG: 23(82.1%)	AA+AG: 7(58.3%)	0.09
	GG: 2(10%)	GG: 5(11.9%)	GG: 5(41.7%)	
rs66953613	CC+CT: 18(90%)	CC+CT: 25(89.3%)	CC+CT: 8(66.7%)	0.26
	TT: 2(10%)	TT: 3(10.7%)	TT: 4(33.3%)	
TATAGAGT/TATAGAGT	2(10%)	3(10.7%)	4(33.3%)	0.14
11111111/11111111				

0=major genotype; 1=minor genotype. Statistical analysis for age, gender and the serum CP concentration was performed using the Kruskal-Wallis tests; and for the others was performed using X^2 test.

did not reveal the function of the *CP* promoter haplotype (ATAGAGT), its potential involvement in gene expression cannot be readily excluded.

No Copy Number Variations of the *CP* gene in PD Patients

Copy number variations (CNVs), including deletions and duplications, were investigated in 24 PD patients (12 PD_{LCP} and 12 PD_{NCP}) using high-density oligonucleotide CGH microarrays. Among the 48 candidate genes, we only found compound heterozygous deletions of the *PARK2* gene in one PD_{LCP} patient (Fig. 3E). No CNVs were identified in the other 47 candidate genes, including *CP*, *ATP7A*, *ATP7B*, and *ATOX1*, which are associated with iron/copper metabolism (Fig. 3A–D).

Table 5. Correlation between SNPs/haplotype and motor phenotype in PD patients

Subjects	PD_T subgroup (*n*=17)	PD_{AR} subgroup (*n*=28)	PD_M subgroup (*n*=15)	*P*
Age (years)	60.41±7.86	59.79±9.57	66.00±10.93	0.11
Gender	M: 8(47%)	M: 13(46%)	M: 11(73%)	0.20
	F: 9(53%)	F: 15(54%)	F: 4(27%)	
Serum CP (g/L)	0.23±0.08	0.21±0.06	0.20±0.05	0.27
Ratio of low serum CP (<0.2 g/L)	PD_{NCP}: 12(71%)	PD_{NCP}: 14(50%)	PD_{NCP}: 6(40%)	0.20
	PD_{LCP}: 5(29%)	PD_{LCP}: 14(50%)	PD_{LCP}: 9(60%)	
rs3736282	CC+CT: 14(82.4%)	CC+CT: 25(89.3%)	CC+CT: 12(80%)	0.67
	TT: 3(17.6%)	TT: 3(10.7%)	TT: 3(20%)	
rs17847023	GG+AG: 13(76.5%)	GG+AG: 24(85.7%)	GG+AG: 11(73.3%)	0.60
	AA: 4(13.5%)	AA: 4(14.3%)	AA: 4(26.7%)	
rs17838831	AA+AG: 11(64.7%)	AA+AG: 23(82.1%)	AA+AG: 11(73.3%)	0.42
	GG: 6(35.3%)	GG: 5(17.9%)	GG: 4(26.7%)	
rs66508328	GG+AG: 14(82.4%)	GG+AG: 25(89.3%)	GG+AG: 12(80%)	0.78
	AA: 3(17.6%)	AA: 3(10.7%)	AA: 3(20%)	
rs67870152	CC+CT: 14(82.4%)	CC+CT: 25(89.3%)	CC+CT: 12(80%)	0.78
	TT: 3(17.6%)	TT: 3(10.7%)	TT: 3(20%)	
rs16861642	GG+AG: 14(82.4%)	GG+AG:25(89.3%)	GG+AG: 12(80%)	0.78
	AA: 3(17.6%)	AA:3(10.7%)	AA: 3(20%)	
rs73020328	TT+TG: 14(82.4%)	TT+TG: 25(89.3%)	TT+TG: 11(73.3%)	0.41
	GG: 3(17.6%)	GG: 3(10.7%)	GG: 4(26.7%)	
rs11708215	GG+GA: 14(82.4%)	GG+GA: 25(89.3%)	GG+GA: 11(73.3%)	0.41
	AA: 3(17.6%)	AA: 3(10.7%)	AA: 4(26.7%)	
rs73166855	AA+AG: 14(82.4%)	AA+AG: 23(82.1%)	AA+AG: 11(73.3%)	0.97
	GG: 3(17.6%)	GG: 5(17.9%)	GG: 4(26.7%)	
rs66953613	CC+CT: 14(82.4%)	CC+CT: 25(89.3%)	CC+CT: 11(73.3%)	0.41
	TT: 3(17.6%)	TT: 3(10.7%)	TT: 4(26.7%)	
TATAGAGT/TATAGAGT	3(17.6%)	4(14.3%)	2(13.3%)	0.98
11111111/11111111				

0=major genotype; 1=minor genotype. Statistical analysis for age, gender and the serum CP concentration was performed using the Kruskal-Wallis tests; others were performed using X^2 test.

DISCUSSION

CP gene mutation of a 5-bp insertion at amino-acid 410 in exon 7 resulting in a frame-shift mutation and a truncated open reading frame was reported by Harris *et al.* in 1995[5]. The patient presented with parkinsonism and had a total absence of circulating serum CP with retinal and basal ganglia iron deposition caused by the genetic defect of the *CP* gene. Another nonsense mutation of this gene (Trp858ter) was reported by Miyajima in a patient who presented with cerebellar ataxia and hypoceruloplasminemia, as well as iron deposition in the basal ganglia[22]. The presence of the mutation in conjunction with the clinical and pathologic findings

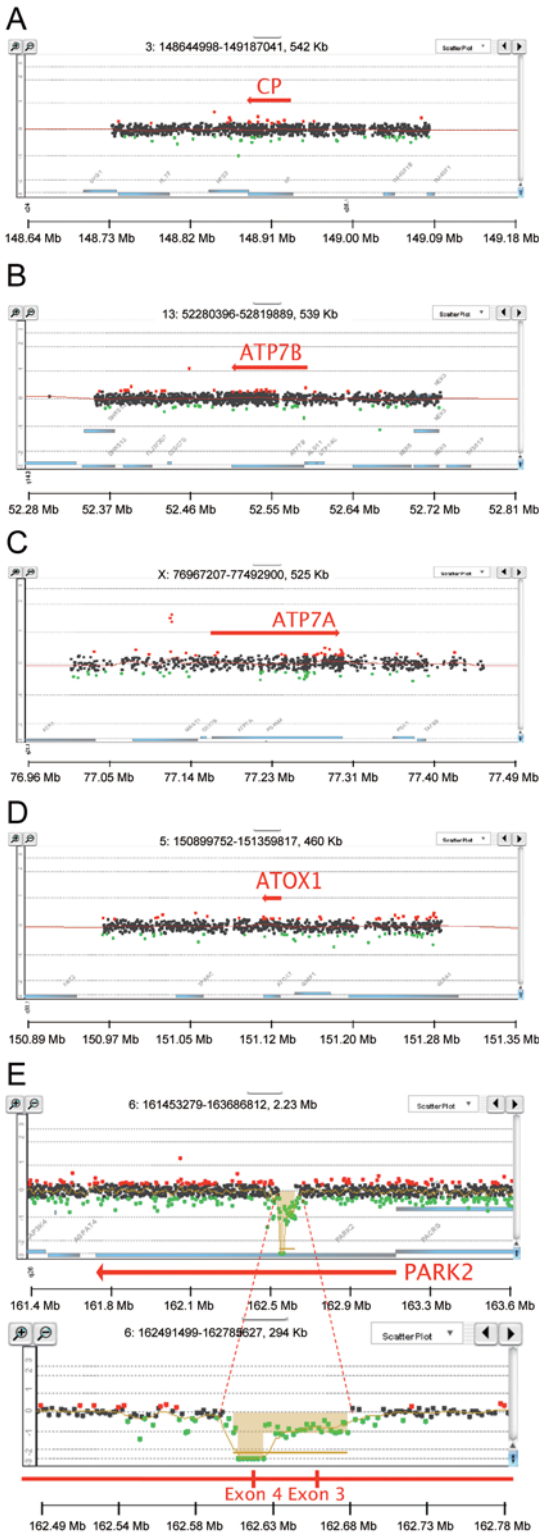

Fig. 3. CNV analysis. The CGH microarray results are shown for the *CP*, *ATP7A*, *ATP7B*, *ATOX1* and *PARK2* genes and their flanking regions.

demonstrated an essential role of CP in iron metabolism in the central nervous system. The fact that Cp-knockout mice develop parkinsonism and are rescued by iron chelation further demonstrates the role of CP in the pathogenesis of PD[12].

Previous studies from our group and others also demonstrated that decreased serum CP levels are associated with movement disorders, including PD, by disturbing brain iron metabolism[14, 15, 17, 23]. However, less is known about the cause of low serum CP levels in PD patients. In this study, we found that the *CP* gene in controls and PD patients had a number of SNPs located at the introns and promoter region, but no exonic SNPs were found. The frequencies of eight SNPs of the *CP* gene (rs3736282 C>T, rs66508328 G>A, rs67870152 C>T, rs16861642 G>A, rs73020328 T>G, rs11708215 G>A, rs73166855 A>G and rs66953613 C>T) and their haplotype (TATAGAGT/TATAGAGT) defined by the mutant alleles of these SNPs in PD patients, especially PD_{LCP} patients, were significantly higher than those in controls, implying a possible genetic risk for PD.

Hochstrasser and colleagues screened the entire coding region of the *CP* gene in 176 German patients with idiopathic PD and 180 ethnically-matched heathy individuals, and found six missense variants in the coding region: I63T, P477L, D544E, T551I, R793H, and T841R. The frequency of D544E differed significantly between PD patients and controls[24]. Another study by Castiglioni *et al.* of 103 Italian PD patients revealed 24 nucleotide substitutions, of which 11 were in the coding region, one in the 3′ UTR, and 12 in the introns. The D544E substitution, which was previously found to be associated with PD, was not significantly different from that reported in dbSNP of Pubmed and similar to the control population of 180 individuals reported by Hochstrasser. None of the other 23 *CP* gene variants seemed to be associated with PD in this study[25]. In another study involving 21 Mexican PD patients and 13 healthy volunteers, Martınez-Hernandez *et al.* found no D544E mutation of the *CP* gene in the PD patients[17]. Thus, the *CP* gene variants in PD patients are inconsistent in different reports. Compared with these studies, we did not find any exonic variants of the *CP* gene like those in Martınez-Hernandez's report, but two intronic SNPs (rs3736282 and rs17847023) consistent with Castiglioni's report[25], as well as eight promoter SNPs. These differences may be explained by the different ethnicity and

location of the participants. Another important factor is the problem of our small sample size.

Genetic reporter systems are widely used to study eukaryotic gene expression and cellular physiology. Thus, we further explored the promoter activity of the *CP* gene using a dual luciferase reporter system transfected with the wild-type haplotype and mutant haplotype DNA fragments in U251 and HepG2 cells which have abundant *CP* gene expression, in order to verify the function of *CP* gene variants, especially the promoter haplotype. No significant ELECTRONIC difference was found in the promoter activity between the wild-type and mutant haplotype in both U251 and HepG2 cells. However, this luciferase reporter system was affected by the specificity of cells, thus it might not precisely reflect the fact of gene regulation *in vivo*. Thus, further investigation is needed to explore the association of *CP* gene variants and CP levels in PD.

Human genomic rearrangement can cause CNVs[18]. Rare CNVs are important genetic causes of human diseases, especially neurological disorders including PD[26-32]. Therefore, defining the genetic content and genomic location by high-resolution CNV breakpoint analysis is vital to elucidating the etiology of CNV-associated disorders. The *PARK2* gene is a molecular diagnostic test for parkinsonism. Kay *et al.* reported that a total of 0.95% of controls and 0.86% of patients carry a heterozygous CNV mutation of the *PARK2* gene[30]. Thus, there is no compelling evidence for an association of heterozygous *PARK2* gene CNV mutation with PD. The compound heterozygous deletions of the *PARK2* gene found in one PD_{LCP} patient in our study did not indicate a relationship with the low CP level in PD. Although no CNVs of the *CP* gene were identified in this study and others, their potential involvement in low serum CP and/or PD cannot be readily excluded. Further studies in more human populations will be informative for revealing more genetic risk factors of PD.

In conclusion, this study demonstrated CP genetic variants associated with PD, especially in PD_{LCP} patients. To our knowledge, this is the first study to reveal such variants and their relation with serum CP levels in a Chinese population. More in-depth studies in larger populations from this and other ethnic groups are needed. In addition, further investigations of epigenetic changes, like aberrant methylation of cytosine residues in genomic DNA, are needed to explore their contribution to PD and the decreased CP expression in PD patients. Furthermore, microRNAs that act as post-transcriptional regulators of gene expression have been recognized as contributors to pathological states in PD[33-35]. For instance, miR-133b is involved in PD through regulating the expression of paired-like homeodomain transcription factor 3 (Pitx3)[36]. Further investigation of the expression of microRNAs that might regulate CP gene expression in PD patients is needed.

ELECTRONIC SUPPLEMENTARY MATERIAL

Supplementary material is available in the online version of this article at http://dx.doi.org/10.1007/s12264-014-1512-6.

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