Lack of Association between *DMT1* Polymorphism and Iron Overload in Chinese Patients with Parkinson's Disease

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Abstract

Objective: Iron overload in the substantia nigra (SN) has been suggested playing a role in causing Parkinson's disease (PD), but the underlying mechanism leading to iron accumulation is not clear. Divalent metal transporter 1 (*DMT1*), an endogenous transporter for ferrous iron, has been suggested being involved in iron metabolism in both animal model and PD patients. However, previous studies failed to reveal *DMT1* as strong risk factor for PD patients. One reason might be that abnormal iron accumulation is not a universal pathogenesis for PD patients. This study aims to explore whether *DMT1* is a risk factor for PD patients with or without iron overload.

Methods: Transcranial sonography (TCS) was used to classify PD patients into two subgroups, PD with SN hyperechogenicity (SN+) and PD without SN hyperechogenicity (SN-), to study the possible association between *DMT1* gene variants and iron overload in PD patients. One mutation (1303C/A) and two single nucleotide polymorphisms (SNPs) (1254T/C and IVS4 + 44C/A) of *DMT1* gene were tested in 67 PD SN+ patients and 53 PD SN- patients of Southern Han Chinese population by method of polymerase chain reaction-restriction fragment length polymorphism (PCR–RFLP).

Results: Distribution of Genotypes and allele frequencies of all these three sites didn't show significant difference between PD SN+ and PD SN- patients. Haplotype analysis of 1254T/C and IVS4 + 44C/A didn't reveal potential risk factor for iron overload.

Conclusion: Our results suggested that *DMT1* gene variants (1303C/A, 1254T/C and IVS4 + 44C/A) are not correlated with iron accumulation in PD patients.

Keywords: Parkinson's Disease (PD); Iron overload; Divalent Metal Transporter 1 (*DMT1*); Single Nucleotide Polymorphism (SNP)

Introduction

Parkinson' disease (PD) is the most common movement disorder, characterized by degeneration of dopaminergic neurons in the substantia nigra (SN) of midbrain and the formation of intracytoplasmic inclusions called Lewy bodies (LBs) in the remaining dopaminergic neurons [1]. The pathogenesis of idiopathic PD has not been fully understood so far, but evidence suggests that abnormal aggregation of iron in SN perhaps have an important role in the etiology of idiopathic PD [2].

Brain iron abnormalities in SN were first observed in idiopathic PD patients about 90 years ago [3]. Subsequent studies also demonstrated an increase of total iron in SN [4-6], where dopaminergic neurons undergo selective degeneration. In 1995, Becker firstly described SN hyperechogenicity as a typical characteristic of idiopathic PD [7]. Hyperechogenicity was defined when the intensity of the ultrasound signal of the detected structure was abnormally increased compared with a reference structure, usually the surrounding white matter [8]. Related researches showed a sensitive and stable hyperechogenicity in idiopathic PD patients [9,10]. Animal studies and post mortem analyses of human brain tissue revealed that this hyperechogenicity is associated with increased iron levels of the substantia nigra [11,12].

To date, the mechanism of unusual aggregation of iron in SN is still an unsolved problem. The iron metabolism or homeostasis related genes such as, transferrin, transferrin receptor, iron responsive element binding protein 2 (IREB2), divalent metal transporter 1 (*DMT1*) and parkin [13] might change the expression or function of proteins involved in iron homeostasis, which might lead to iron abnormal accumulation in the brain. *DMT1*, also known as natural resistance-associated macrophage protein 2 (NRAMP2), divalent cation transporter 1 (DCT1) or solute carrier family 11 member 2 (SLC11A2), is essential for dietary iron absorption and iron translocation from the endosome. Up-regulation of *DMT1* in the SN of both MPTP-induced PD models and PD patients indicated that *DMT1* might involve in the process of iron accumulation in SN [14,15] Previous studies failed to reveal

DMT1 gene variants as strong risk factors for iron-related disease, such as PD, RLS and ALS [16-18]. But, haplotype analysis of 1254T/C and IVS4 + 44C/A showed that CC haplotype of *DMT1* gene was a possible risk factor for PD in Han Chinese population [16]. Two *DMT1* intronic SNPs (rs224589 and rs407135) showed positive association with RLS in patients with a history of anemia compared to RLS patients without anemia [17]. C allele of rs407135 in *DMT1* was associated with shorter disease duration in sporadic ALS patients with onset in the legs [18]. Thus, these results suggested *DMT1* gene variants were still candidates to explore the mechanism of abnormal iron accumulation in PD. However, abnormal iron accumulation is not a universal mechanism for all PD patients. Since hyperechogenicity is associated with increased iron levels, thus abnormal iron accumulation was more likely involved in those PD patients with SN hyperechogenicity than those PD patients without SN hyperechogenicity. In order to explore the possible association between *DMT1* gene and abnormal iron accumulation, in our study, TCS was used to classify PD patients into two subgroups PD with SN hyperechogenicity (SN+) and PD without SN hyperechogenicity (SN-). One mutation (1303C/A) and two single nucleotide polymorphisms (SNPs) (1254T/C and IVS4 + 44C/A) of *DMT1* was tested in these two groups.

Materials and Methods

Subjects

A total of 120 idiopathic PD patients (90 male and 30 female) were recruited from the Movement Disorder Clinic at the Department of Neurology, Ruijin Hospital affiliated to Shanghai Jiao Tong University School of Medicine. Patients were divided into PD SN+ group (male 50, female 17, mean age: 65.3 ± 10.5 years) and PD SN-group (male 40, female 13, mean age 64.8 ± 10.9 years) according to TCS results. Two groups were matched for gender and age (**Table 1**), and none of them had a positive family history of PD. Patients with Parkinson's-plus syndrome or secondary Parkinsonism was excluded. The clinical diagnosis of idiopathic PD was given by two independent movement disorder specialists according to the diagnosis criteria [19]. The study was approved by the Ethics Committee of Rujin Hospital.

Transcranial sonography

A color-coded phase array ultra-sound system (MyLab90, ESAOTE, Italy) with a 2.5 MHz phased-array transducer was used to the TCS examination for PD patients. The probe was placed consecutively at bilateral temporal bone windows, scanning supratentorial and infratentorial brain areas in axial planes. For this study, we mainly paid special attention to the mesencephalic brainstem. In general, hyper-echogenicity of the SN is stated if the SN shows a pathological signal at least on one side. An area of echogenicity [20] <0.18 cm^2 was classified as normal (**Figure 1a**) and areas of echogenicity ≥ 0.18 cm^2 was classified as hyperechogenic in either one or two sides in our research (**Figure 1b**). The examiners were ultrasound specialists blind to the clinical diagnosis.
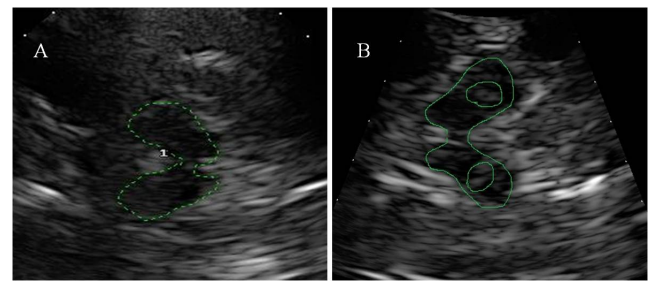

Figure 1 Echogenicity examined by TCS in SN area. A. Midbrain was lined out. No obvious hyperechogenicity was detected in SN area. B. Peripheral line circled out midbrain. Inner line circled out hyperechogenicity in SN area.

Genetic analysis

Genomic DNA was extracted from peripheral blood leukocytes through standardized phenol/chloroform extraction method. Primers were designed using the Primer software. Genotyping for the *DMT1* polymorphisms or mutations was conducted by polymerase chain reaction–restriction fragment length polymorphism (PCR–RFLP) method, alleles and genotypes frequencies were determined by direct sequencing. The PCR amplification was performed in a total volume of 20 μl reaction mixture, after an initial denaturation of 95 for 5 min, amplification was performed for 35 cycles at 95°C for 30s, 59°C for 30s and 72°C for 7 min and 16°C forever. After amplification, the PCR products were sequenced directly. The primers for each SNPS or mutation are listed below:

- IVS4+44C/A:
- The sense primer5'-GACACATGCAATATCTGACATTG-3'
- The antisense primer5'-AGGCTACTATCCAACATGCAG-3'
- The product length is 351bp.
- 1254T/C and 1303C/A:
- The sense primer 5'-ATTCTTCTGAGGTCTCTC-3'
- The antisense primer 5'-AGACCACAACCATGCCTCTG-3'
- The product length is 362 bp.

Statistical analysis

All statistical analysis was performed using Statistic Package for the Social Science (SPSS) version 17.0 for Windows. Goodness-of-fit to the Hardy–Weinberg equilibrium was examined by chi-square test, and differences in genotype and allele frequencies between the SN+ and SN- groups were calculated and compared by Chi-squared or Fisher's exact test. Exact logistic regression adjusted for age and gender was also used to analyze differences in genotype and allele distributions between two groups. Linkage disequilibrium (LD) and haplotype frequencies were analyzed by SHEsis software (http://analysis.bio-x.cn/myAnalysis.php). The P-value, odds ratios (OR) and 95% confidence intervals (CI) were estimated for the association between *DMT1* polymorphisms and iron accumulation in PD. The statistical significance level was set at P value <0.05 for all the tests.

Results

Clinical characteristics were shown in **Table 1**. SN+ PD patients and SN- PD patients were well matched in age (P=0.915), gender (P=0.772), age of onset (P=0.88), UPDRS III score (P=0.106), and Hoehn-Yahr stage (P=0.77).

Table 1 Clinical characteristics of PD patients.

Variables	SN+ PD patients	SN- PD patients	P value
Total sample	67	53	
Male	50	40	
Female	17	13	0.915
Age (year)	65.3 ± 10.5	64.8 ± 10.9	0.772
Age of onset (year)	59.4 ± 10.9	59.1 ± 11.3	0.88
UPDRS score	24.72 ± 12.9	28.72 ± 13.7	0.106
Hoehn and Yahr stage	2.09	2.13	0.77

Distributions of genotypes and allele frequencies of 1254T/C, IVS4+44C/A were in Hardy-Weinberg equilibrium (P>0.05). As shown in **Table 2**, no C→ A mutation at 1303 nucleotide was found in our study.

Genotypes and allele frequencies of 1254T/C and IVS4+44C/A didn't show significant difference between SN+ and SN- PD groups. Haplotypes were analyzed between

1254T/C and IVS4+44C/A (D'=0.91, r^2=0.77) and didn't reveal any potential risk factor for abnormal iron accumulation as shown in **Table 3**.

Table 2 Distribution of genotype and allele frequencies of the *DMT1* gene mutations/polymorphisms in PD patients.

Variables	Genotype (n, %)			P	Allele (n, %)		P
1303C/A	CC	CA	AA		C	A	
SN+ group	67 (100)	0	0	-	134 (100)	0	-
SN- group	53 (100)	0	0		106 (100)	0	
1254T/C	TT	TC	CC		T	C	
SN+ group	52 (77.6)	15 (22.4)	0	0.18	119 (88.8)	15 (11.2)	0.98
SN- group	43 (81.1)	8 (15.1)	2 (3.8)		94 (88.7)	12 (11.3)	
IVS4 + 44C/A	CC	CA	AA		C	A	
SN+ group	51 (76.1)	16 (23.9)	0	0.2	118 (88.1)	16 (11.9)	0.94
SN- group	4279.2	9 (17)	2 (3.8)		93 (87.7)	13 (12.3)	

Table 3 Distribution of haplotypes in SN+ and SN- PD patients.

Haplotype	SN+ (%)	SN- (%)	χ²-test single statistics		χ²-test global statistics		OR [95%CI]
			χ²	p	χ²	p	
CA	15 (11.2)	10 (9.4)	0.21	0.65	4.4	0.22	1.22 [0.52-2.83]
CC	0 (0)	2 (1.9)	2.61	0.11	--	--	--
TA	1 (0.7)	3 (2.9)	1.62	0.2	--	--	0.25 [0.03-2.47]
TC	118 (88.1)	91 (85.8)	0.27	0.61	--	--	1.22 [0.57-2.60]

Since age is an important factor in abnormal iron metabolism and accumulation, we tried to analyze our data by classifying PD patients into early onset Parkinson's disease (EOPD, age of onset ≤ 50 years) and late onset Parkinson's disease (LOPD, age of onset >50 years) respectively [21]. EOPD subgroup only has 15 SN+ patients and 13 SN- patients, thus distribution of genotypes and allele frequencies were analyzed in LOPD patients only. No significant difference was found between SN+ LOPD patients and SN- LOPD patients as shown in **Table 4**.

Exact logistic regression also showed that the results were not influenced by age, gender (P=0.845, P=0.637,

respectively). Thus, our results didn't reveal *DMT1* as a risk factor for abnormal iron accumulation in PD patients.

Discussion

Role of abnormal iron metabolism in PD arises from two lines of evidence. First, iron plays a key role in many crucial neuronal functions, such as oxygen transport, mitochondrial energy production, DNA synthesis and mitochondrial respiration [22]. Iron-induced oxidative stress, inflammatory stimulation, mitochondrial impairment, ubiquitin-proteasome system (UPS) dysfunction and alpha-synuclein aggregation are

important factors to the nigral dopaminergic neuron degeneration [23].

Table 4 Allele and genotype frequencies of 1254T/C, IVS4 + 44C/A in LOPD.

Variables		Genotype (n, %)		P	Minor allele frequency (n, %)	P
1254T/C	TT	TC	CC		C	
SN+ LOPD	39 (75)	13 (25)	0		13 (12.5)	
SN- LOPD	33 (82.5)	6 (15)	1 (2.5)	0.23	8 (10)	0.6
IVS4 + 44C/A	CC	CA	AA		A	
SN+ LOPD	38 (73.1)	14 (26.9)	0		14 (13.5)	
SN- LOPD	32 (80)	7 (17.5)	1 (2.5)	0.26	9 (11.3)	0.65
LOPD: Late Onset Parkinson's Disease.						
SN+ LOPD: Late Onset Parkinson's Disease with SN Hyperechogenicity						
SN- LOPD: Late Onset Parkinson's Disease without SN Hyperechogenicity						

Second, dysregulation of iron homoeostasis has been identified in PD. Mounting evidence shows that iron content dramatically increases in the substamtia nigra pars compacta (SNpc) rather than in other brain regions in idiopathic PD [6,24].

However, the mechanism for abnormal iron accumulation in SN is still not clearly understood so far. Dysregulation of influx proteins [25], blockade of axonal transport [26,27] and mitochondria abnormalities [25] could all contribute to abnormal iron metabolism. Related researches also proved that *DMT1* involved in the process of iron accumulation in SN [14,15]. Up to now, five single nucleotide mutations or polymorphisms were identified within the *DMT1* gene. Mutation 1303C→A occurs in the coding region of *DMT1* and results in an amino acid change from leucine to isolecine. 1254T/C also occurs in the coding region of *DMT1* but does not cause an amino acid change. The other three polymorphisms are within introns (IVS2 + 11A/G, IVS4 + 44C/A, and IVS6 + 538G/Gdel) [28]. The previous functional and genetic studies of *DMT1* make its polymorphic variants attractive candidates for the study of iron accumulation, but earlier reports failed to reveal *DMT1* as strong risk factors in both PD and RLS patients. One reason for failing to find the association should be considered is that abnormal iron accumulation is not a universal pathogenetic mechanism to these diseases. Thus, it is reasonable to explore association of *DMT1* and iron accumulation between PD patients with abnormal iron accumulation and PD patients without abnormal iron accumulation. Hyperechogenicity examined by TCS highly indicated iron overload [11,12]. In our study, one single nucleotide mutation (1303C/A) and two polymorphisms within *DMT1* (1254T/C and IVS4+44C/A) were tested in PD SN+ patients and PD SN- patients. We did not find any significant association of *DMT1* variants between patients with SN hyperechogenicity and those without SN hyperechogenicity in either genotypes or alleles frequencies of the three-tested locus. There was not any C →A mutation at 1303C/A nucleotide site detected in the present study. These results found in our study were consistent with previous study about *DMT1* polymorphism and risk of Parkinson's disease in Northern Han Chinese PD patients except that we didn't reveal CC haplotype as a possible risk factor in haplotype analysis of 1254T/C and IVS4+44C/A sites as indicated by the above study [16]. As a matter of fact, CC haplotype is very rare in both SN+ PD paitents (0%) and SN- PD patients (1.9%) in our study, which is much lower than the above study conducted in Northern Han Chinese patients, 18.2% in PD patients and 11.4% in control. Regional difference (Southern vs Northern part of China) might be one reason for lower CC haplotype in our study. Another possible reason for failing to find correlation of *DMT1* with abnormal iron accumulation might be that the sample size is not big enough in our study, but such a low CC haplotype in our study subjects largely limits its potential clinical importance.

Conclusion

In conclusion, our present data do not support the relationship between 1303C/A mutation or 1254T/C, IVS4+44C/A polymorphism within *DMT1* and abnormal iron accumulation indicated by SN hyperechogenicity in idopathic PD patients from Southern China. How *DMT1* affects the process of iron accumulation, and whether it plays a key role in pathogenesis of PD still needs further study.

Ethics Approval and Consent to Participate

The study was approved by the Ethics Committee of Rujin Hospital. The consents to participate are stored in Ethics office.

Consent for Publication

Not relevant.

Availability of Data and Materials

All the data mentioned in this article are available on published article.

Competing Interests

The authors declare they have no competing interest.

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Authors' Contributions

Wei M did the genetic analysis, statistical analysis and wrote the manuscript. Hu YY and Zhan WW did the transcranial sonography. Lou Y was responsible for the patient recruitment and data integration. Tan YY and Xiao Qin were responsible for study design and manuscript revision. All authors read and approved the final manuscript.

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