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Genetic analysis of DNA methylation and hydroxymethylation genes in Parkinson's disease

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ABSTRACT

DNA methylation is an important regulatory mechanism of Parkinson's disease (PD). To investigate the relationship between DNA methylation and hydroxymethylation genes and PD, we performed gene-targeted sequencing using molecular inversion probes in a Chinese PD population. We sequenced 12 genes related to DNA methylation and hydroxymethylation in 1657 patients and 1394 control subjects. We conducted genewise association analyses of rare variants detected in the present study and identified the *TET1* gene as important in PD ($p = 0.0037738$, 0.013, 0.019521 (b.collapse test, variable threshold test, and skat-o test, respectively; sex + age as covariates). However, no positive results were observed when conducting association analyses on common variants in these genes. We performed a comprehensive analysis of associations between variants of DNA methylation and hydroxymethylation genes and PD, resulting in determination that *TET1* might play a role in PD.

1. Introduction

Parkinson's disease (PD) is one of the most common neurodegenerative diseases. The major motor symptoms of PD include bradykinesia, resting tremors, rigidity, and postural instability. Genetic factors, environmental factors, and their interactions with aging increase susceptibility to PD (Ross and Smith, 2007). Currently, most PD cases are sporadic and candidate genes are associated with risk of sporadic PD (Guo et al., 2015; Li et al., 2015; Liu et al., 2015; Peeraully and Tan, 2012). Several environmental factors are also positively associated with risk for the development of sporadic PD. Pesticides (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) and heavy metals (manganese, iron, and so forth) are representative environmental toxicants associated with development of PD (Fleming, 2017).

Epigenetic modifications are hereditary, do not change DNA sequences, and can affect gene expression. These modifications may act as bridges between genetic and environmental factors (Marques et al., 2011). DNA methylation is one of the most important epigenetic modifications. It occurs when a methyl group is covalently bonded to the C5 position of a cytosine residue in a DNA sequence (Feng and Fan, 2009). In the nervous system, dynamic regulation of DNA methylation plays a critical role in sustaining alterations in brain functions, such as formation of memories in the hippocampus.

Previous studies have observed changes in methylation levels in the brains and peripheral blood of patients with PD (Wen et al., 2016). Many PD-related genes (*SNCA*, *PARKIN*, and so forth) have been found to be either hypermethylated or hypomethylated in PD (Ai et al., 2014; Cai et al., 2011). These DNA methylation modifications and subsequent changes in gene expression may contribute to onset and progression of PD. DNA is methylated primarily by the DNA methyltransferases (DNMTs) DNMT1, DNMT3A, and DNMT3B. These DNMTs are involved in neurodevelopmental processes in adult and postnatal developing rat brains (Simmons et al., 2013). DNMT1 levels were decreased in

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adult PD brains and PD cell models in which neuronal cells overexpress alpha-synuclein, resulting in global DNA hypomethylation (Desplats et al., 2011). Recently, association analyses between DNMT gene polymorphisms and PD were performed in different cohorts, including Chinese. Although several variants of DNMT3B were reported to be associated with PD, the results of these studies were not consistent because of small sample sizes (Chen et al., 2017; Pan et al., 2018; Pezzi et al., 2017).

Methylated DNA affects gene expression by recruiting DNA methyl binding proteins such as methyl-CpG-binding domain (MBD) family members (MBD1–4) and methyl-CpG-binding protein 2 (MECP2) (Feng et al., 2015). Ubiquitin like with PHD and ring finger domains 1 plays an important role in maintaining DNA methylation by interacting with DNMT1 (Harrison et al., 2016). MECP2 was shown to be associated with brain functions such as neural plasticity (Fasolino and Zhou, 2017). A previous report showed that mutations in MECP2 led to Rett syndrome, which is a progressive neurodevelopmental disorder (Amir et al., 1999).

The DNA demethylation process is catalyzed by the 10–11 translocation (TET) family of enzymes, including TET1, TET2, and TET3, which can demethylate 5-methylcytosine to 5-hydroxymethylcytosine (5hmC), 5-formylcytosine, and 5-carboxylcytosine through oxidative processes (Wu and Zhang, 2017). Hydroxymethylation was identified several decades ago but began to receive greater attention when 5hmC was detected in the brain (Kriaucionis and Heintz, 2009). In our previous work, we sequenced the exomes of 16 patients from 8 Chinese PD families. Three patients from different pedigrees shared the single nucleotide polymorphism rs150689919 of TET1. However, we failed to confirm an association between this single nucleotide polymorphism and sporadic PD in 514 sporadic Chinese patients with PD and 529 normal control subjects (Liao et al., 2013).

Variants in genes encoding these enzymes and binding proteins could disrupt methylation and demethylation processes and might be associated with PD risks. Because of a lack of comprehensive analyses of DNA methylation and hydroxymethylation genes in PD, we conducted genetic analysis to determine relationships between DNA methylation and hydroxymethylation genes and PD in a relatively large Chinese population using targeted sequencing with molecular inversion probes (MIPs).

2. Materials and methods

2.1. Subjects

Basic clinical information was collected from 1692 sporadic PD patients and 1419 neurologically normal control subjects of Han Chinese ethnicity at the outpatient ward of Xiangya Hospital, Central South University. We collected 10 mL of peripheral blood drawn into an EDTA anticoagulant tube from all individuals. Using principal component analysis with EIGENSOFT package (Price et al., 2006), we detected 60 outliers. After removal of outliers, our subjects included 1657 patients and 1394 control subjects. The patient group contained 906 males and 751 females. The mean age at onset of the patients was 48.69 ± 12.36. The control group comprised unrelated individuals without neurologic diseases. In total, 713 males and 681 females were included in the control group, and their mean age was 48.59 ± 16.32. The patient and control groups were matched for age and sex (Supplementary Table 1). PD was diagnosed according to the UK PD Society Brain Bank Clinical Diagnostic Criteria (Hughes et al., 1992) by at least 2 experienced neurologists. All data is publically available through the Parkinson's disease Multicenter database and Collaboration network in China (PD-MDCDC) website (pd-mdcnc.com). Using a standard phenol-chloroform extraction and ethanol precipitation method, genomic DNA was extracted from leukocytes in the peripheral blood drawn from patients and control subjects at the Center for Medical Genetics at Central South University. The Ethics Committee of Xiangya Hospital approved this study and written informed consent was collected from all participants.

2.2. Targeted sequencing using MIPs

An outline of the MIP workflow is listed subsequently. Detailed information can be found in a previous publication (Nuttle et al., 2014). In total, 494 MIPs for targeted sequencing were designed to capture exons and exon-intron boundaries (±50 bp) of the following 12 candidate genes: *DNMT1*, *DNMT3A*, *DNMT3B*, *MBD1*, *MBD2*, *MBD3*, *MBD4*, *MECP2*, *TET1*, *TET2*, *TET3*, and *UHRF1* (Table 1). MIPs were mixed and activated by phosphorylation. These pooled probes were then hybridized to capture DNA sequences and reverse complementary genomic DNA sequences. Exonuclease types I and III were used to split the single strand circles from the former step into a combined single chain consisting of probe sequences and complementary DNA sequences. Quantitative polymerase chain reaction (PCR) was performed using forward and reverse primers to amplify the captured DNA sequences. The following standard amplification protocol was used on a light thermocycler: 98 °C for 30 seconds, then 30 cycles at 98 °C for 10 seconds, 60 °C for 30 seconds, 72 °C for 20 seconds, plate reading, 72 °C for 10 seconds, 72 °C for 2 minutes, and a final hold at 4 °C. After the PCR procedure, target sequences were amplified and sample-specific indices (reverse barcode primers) were introduced. PCR products were cleaned up using magnetic beads and 70% ethanol. Next, we pooled the cleaned PCR products. The amplified samples were sent for next generation sequencing using the Illumina platform (Illumina HiSeq2000) with traditional pipelines.

2.3. Quality control

The mean sample depth for all included genes was >3000×, and the average coverage was >95%. Q30 (indicates the probability of an incorrect base call of 1 in 1000) was >90%. All samples were qualified for subsequent data analysis. Hardy-Weinberg equilibrium ($p < 1 \times 10^{-5}$) was tested in both patients with PD and control subjects.

2.4. Calling variants, single variant association, and genewise association

The raw fastq files generated from high-throughput sequencing were mapped to the human genome (hg19) with BWA v0.7.12 (Li and Durbin, 2010). GATK3.6 (McKenna et al., 2010), HaplotypeCaller, and GenotypeGVCFs were used to call variants and they were annotated with Annovar (Wang et al., 2010). Data analyses were performed depending on the minor allele frequency (MAF) of detected variants in control subjects. Exome Aggregation Consortium (ExAC, http://exac.broadinstitute.org/) and 1000 Genome (http://www.1000genomes.org) were used to infer the frequency of rare variants identified in this study in public databases.

If MAF ≥0.01, we defined the variants as common variants and conducted association studies between the variants and PD risk by calculating *p* values using the allelic model with plink 1.07 (Purcell et al., 2007) and logistic regression analysis (sex + age as covariates). Bonferroni and false discovery rate corrections were made after modeling.

If MAF <0.01, variants were defined as rare. We used prediction tools including sorting intolerant from tolerant, Polymorphism Phenotyping v2 (PolyPhen-2) to predict pathogenicity of variants.

The gene-based burden test was performed using EPACTS 3.2.6 (Kang et al., 2010) to analyze genewise associations by combining all rare variants of a specific gene.

Table 1
The genewise association analyses based on rare variants of 12 genes

Gene	Genomic location (hg19)	Numbers of variants	B.collapse test			Variable threshold test			Skat-o test		
			p value (no covariate)	*p* value (sex covariate)	*p* value (sex + age covariates)	*p* value (no covariate)	*p* value (sex covariate)	*p* value (sex + age covariates)	*p* value (no covariate)	*p* value (sex covariate)	*p* value (sex + age covariates)
TET1	chr10:70320117-70454239	86	**0.003135**	**0.003175**	**0.003774**	**0.011**	**0.0122**	**0.013**	**0.015696**	**0.015876**	**0.019521**
TET2	chr4:106067842-106200960	90	0.3823	0.38329	0.40117	0.7	0.63	0.67	0.62353	0.61783	0.57977
TET3	chr2:74273405-74335302	118	0.092329	0.091783	0.093767	0.12	0.16	0.11	0.099016	0.098589	0.097907
DNMT1	chr19:10244022-10305755	115	0.61065	0.60229	0.55807	0.69	0.62	0.51	0.60958	0.60359	0.60761
DNMT3A	chr2:25455830-25564784	63	0.79256	0.79753	0.76173	0.52	0.58	0.63	0.65776	0.66433	0.62153
DNMT3B	chr20:31350191-31397162	74	0.10031	0.10069	0.11726	**0.00094**	**0.00096**	**0.00124**	0.10304	0.10278	0.11774
MBD1	chr18:47795216-47808144	66	0.27354	0.27764	0.24376	0.37	0.45	0.35	0.29524	0.29727	0.30404
MBD2	chr18:51677971-51751158	10	0.073721	0.075267	0.066057	0.16	0.19	0.15	0.07602	0.076812	0.061663
MBD3	chr19:1576678-1592652	36	0.25709	0.25416	0.27063	0.27	0.22	0.19	0.37203	0.36773	0.38931
MBD4	chr3:129149787-129159022	35	0.074284	0.074968	0.08346	0.21	0.16	0.18	0.10545	0.10662	0.12036
MECP2	chrX:153287264-153363188	27	0.87932	0.89823	0.89394	0.92	0.93	0.89	0.38662	0.37846	0.38462
UHRF1	chr19:4909510-4962165	68	0.87645	0.87875	0.87267	0.85	0.91	0.87	1	1	1

The positive results are presented in bold text.

2.5. Statistical analysis

Statistical analysis was performed using R (version 3.5.0). Fisher's exact test on a 2 × 2 contingency table was used to test significant differences in gender between PD cases and control subjects. Student's *t* test was used to test significant differences in age between PD cases and control subjects. Two-sided *p* values <0.05 were considered statistically significant.

3. Results

3.1. Rare variants of TET1 were associated with PD

We detected 788 rare variants in the exons and exon-intron boundaries of 12 individual genes (Table 1). We further conducted genewise association analyses of these rare variants using b.collapse test, variable threshold test, and skat-o test. The gene-based burden test of the *TET1* gene was positive on all 3 tests ($p = 0.0031352$, 0.011, and 0.015696, respectively). After using sex and age as covariates, the gene-based burden test of *TET1* remained positive ($p = 0.0037738$, 0.013, and 0.019521, respectively). As shown in Supplementary Table 2 variants of *TET1* detected in our cohorts included 2 frameshift variants (c.762_763insGACCG and c.5158_5159del), 45 nonsynonymous variants, and 27 synonymous variants in the *TET1* exonic region, and 12 variants in the *TET1* intronic region, resulting in a total of 86 rare variants.

We also observed positive results for the *DNMT3B* gene in variable threshold test using sex and age as covariates ($p = 0.00094$ and 0.00124). However, we did not find any gene associations between patients with PD and control subjects when we performed genewise association analyses on other genes.

3.2. Common variants detected in PD cohorts

Common variants detected in 12 genes in our cohorts are listed in Supplementary Table 3. Association analyses on single variants using an allele model (adjusted by Bonferroni and false discovery rate corrections) with sex and age as covariates did not produce positive results for any common variants.

4. Discussion

PD is a complex neurodegenerative disease associated with multiple factors, including genetic factors, environmental factors, and aging. Many PD-related genes (*SNCA*, *PARKIN*, and so forth) have been found to be either hypermethylated or hypomethylated in PD, resulting in altered gene expression level. Genes encoding DNA methylation and hydroxymethylation have been demonstrated to be involved in PD pathogenesis. However, comprehensive analyses of DNA methylation and hydroxymethylation genes in PD cohorts have not been performed.

Targeted sequencing using MIPs has a simple workflow for clinical use. This method has the advantage of identifying low frequency variants (lower than 1% frequency) with deep sequencing depth. In studies with large samples, this method is robust to low sample quality and quantity, and is relatively inexpensive (Hiatt et al., 2013). Our group has previously used targeted sequencing with MIPs to identify disease-associated genes in PD (Xu et al., 2017). In our study, we analyzed 12 genes encoding DNA methylation and hydroxymethylation-related core proteins in Chinese PD cohorts by targeted sequencing combined with MIPs. We found a positive result in gene-burden analysis on rare variants of the *TET1* gene. None of the common variants in the 12 genes evaluated were associated with PD.

The *TET* gene family encodes TET enzymes that play a prominent role in the DNA demethylation process. 5hmC is enriched in the central nervous system and is approximately 40% as abundant as 5-methylcytosine in Purkinje neurons in the cerebellum (Kriaucionis and Heintz, 2009). In response to oxidative stress, modulation of the DNA demethylation process, including 5hmC by TET1, was found to be able to cause neuronal cell death, providing a potential mechanism for development of neurodegenerative diseases (Xin et al., 2015). Researchers have found that TET1 could be associated with multiple neurodegenerative diseases such as AD and Huntington's diseases by modulating 5hmC levels and subsequent gene expression levels (Wang et al., 2017). In the present study, 86 rare variants were detected in the *TET1* gene and gene-based burden test of *TET1* showed a positive result. This indicated that *TET1* is associated with PD. We speculated that it might contribute to PD risk by modulating levels of 5hmC, resulting in changes in gene expressions.

Our study had some limitations. Although we had a relatively large Chinese sample, the lack of replication cohorts could influence the generalizability of the results. Validation studies in a larger Chinese cohort and other populations are needed to confirm the results.

In conclusion, by screening for common and rare variants of DNA methylation-related genes with targeted sequencing using MIPs, we found that the *TET1* gene might be associated with PD in a Chinese population. Although our study is an initial study on methylation process-related genes in PD, we identified *TET1* as potentially associated with PD, which may allow for future studies that contribute to PD diagnosis and treatment.

Disclosure

The authors have no actual or potential conflicts of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.neurobiolaging.2019.02.025.

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