Association analysis of **NUCKS1** and **INPP5K** polymorphism with Parkinson’s disease

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Genome-wide association studies have reported numerous candidate loci associated with Parkinson’s disease (PD). **NUCKS1** and **INPP5K** are two such candidate loci, although they have rarely been reported in Asian populations. To explore these potential genes for PD susceptibility, we investigated the association between PD and two SNPs, rs823114 and rs1109303, located on the **NUCKS1** and **INPP5K** genes, respectively, in the Han population of northern China. We genotyped the two SNPs using the multiplex PCR-RFLP (polymerase chain reaction-restriction fragment length polymorphism) technique. A total of 685 subjects including 322 sporadic PD patients and 363 healthy controls were recruited from the population. After Bonferroni correction, our results suggested that there was a significant association of a minor allele (G) in rs823114 with reduced risk of PD development (\(P = 0.017\), OR = 0.768, 95%CI = 0.618 – 0.955), and the difference in genotypes between the PD patients and healthy controls was significant under the dominant model (GA + GG vs. AA). After stratification by gender, males had a lower risk than females (\(P = 0.008\), OR = 0.666, 95%CI = 0.495 – 0.898). However, the distribution of genotype frequency exhibited no significant differences between the PD and control groups (\(P > 0.025\)) in **INPP5K** rs1109303 (\(P = 0.048\), OR = 0.806, 95%CI = 0.650 – 0.998). We conclude that **NUCKS1** rs823114 indicates a decreased risk of susceptibility to PD and shows a male genetic distribution bias in the Han Chinese population.

**Key words:** Parkinson’s disease, single nucleotide polymorphism, association, **NUCKS1**, **INPP5K**

**INTRODUCTION**

Parkinson’s disease (PD) is a progressive neurodegenerative disorder, characterized by a large number of motor and non-motor features that can impact on function to a variable degree, which affects about 1% of individuals over 65 years of age (Spencer et al., 2011). There are four cardinal features of PD that can be grouped under the acronym TRAP: tremor at rest, rigidity, akinesia (or bradykinesia) and postural instability; these are caused by accelerated loss of dopaminergic neurons in the substantia nigra and abnormal formation of Lewy bodies in residual neurons (Jankovic, 2008). The etiology of PD is very complex, probably involving a combination of polygenic inheritance, environmental exposures, oxidative stress, neuroinflammation and apoptosis (Klein and Schlossmacher, 2007; Cicchetti et al., 2009; Schapira and Jenner, 2011). However, increasing knowledge related to the genetic architecture of PD has demonstrated that hereditary factors play an important role in PD pathogenesis (Hamza and Payami, 2010).

The **NUCKS1** (nuclear casein kinase and cyclin-dependent kinase substrate 1) gene in the **PARK16** locus located on chromosome 1q32 was recognized as a new risk factor for PD in a Japanese cohort (Satake et al., 2009). The **NUCKS1** gene, which encodes the nuclear protein casein kinase, plays an important role in the DNA damage response (Parplys et al., 2015) and is also used for DNA repair (Grundt et al., 2004); however, its involvement in the pathogenesis of PD has not been addressed. A recent study has shown that a novel single nucleotide polymorphism (SNP), rs823114, at **NUCKS1** is...
Uric acid (UA) possesses potent antioxidant activity and is the enzymatic end product of purine metabolism in vivo (Weisskopf et al., 2007). UA exerts a neuroprotective effect by reducing oxidative stress and scavenging oxygen radicals against dopaminergic neuron death in PD (Duan et al., 2002). Epidemiological studies have established that high levels of serum urate reduced the risk of PD (de Lau et al., 2005; Ascherio et al., 2009). Serum UA levels associated with the inositol phosphoinositide-5-phosphatase K (INPP5K) gene can be used to predict disease severity and progression in potential PD patients. The interaction between the SNP rs1109303 on the INPP5K gene and serum urate level in PD patients was significant \(P = 2.01 \times 10^{-8}\), and may be associated with the risk of PD (Nazeri et al., 2015).

In the current study, because rs823114 and rs1109303 have not yet been reported in any Chinese population with reference to PD, we genotyped the NUCKS1 SNP rs823114 and the INPP5K SNP rs1109303 by the multiplex polymerase chain reaction - restriction fragment length polymorphism (PCR-RFLP) technique to assess their association with the risk of PD in the northern Han Chinese population.

**MATERIALS AND METHODS**

**Subjects and sample collection** As PD subjects, a total of 322 ethnic Han Chinese PD patients from northern China were recruited (161 males, 161 females). All patients were diagnosed by neurological specialists from the First Affiliated Hospital of China Medical University according to the guidelines of the British PD Society Brain Bank (Hughes et al., 1992), excluding cerebrovascular diseases, encephalitis, trauma, and other patients with severe systemic disease (Table 1).

For the control group, a total of 363 unrelated healthy participants who did not have a family history of PD were recruited from the local community (205 males, 158 females), and were matched to the PD group for race, age and gender (Table 1).

The current study was approved by the Ethics Committee on Human Research of China Medical University. All participants were provided with an informed consent form.

**DNA extraction and genotyping** Peripheral blood samples were collected from the participants, and genomic DNA was extracted using the sodium dodecyl sulfate - phenol-chloroform method. According to published sequences in GenBank, forward and reverse primers were designed and are shown in Table 2. Multiplex PCR-RFLP was used to identify polymorphisms of the SNPs. A final volume of 20 μl was used, comprising 1 μl genomic DNA (about 20–50 ng/μl), 10 μl 2 × Power Taq PCR MasterMix (Biotek Corporation, Beijing, China), 7 μl ddH₂O and primer concentrations as shown in Table 2. PCR amplification was performed under the following conditions: initial denaturation of 94 °C for 1 min, followed by 33 cycles of 94 °C for 30 s, annealing at 65 °C for 30 s and elongation at 72 °C for 30 s, and a final extension at 72 °C for 5 min. For restriction enzyme digestion, 2 μl PCR product, 5 U HaeIII and BanII (TaKaRa, Dalian, China) were mixed in 10 μl TaKaRa M buffer and incubated at 37 °C for 2 h. Digestions were separated on an 8% polyacrylamide gel, and fragments were visualized by the Molecular Imager Gel Doc XR+ System (Bio-Rad, Hercules, CA, USA) after ethidium bromide staining.

**Statistical data analysis** The data were analyzed using Power Marker v3.25, Statistical Package of Social Sciences v20.0 (IBM, Chicago, IL, USA) and Stata version 10.0 (StataCorp, College Station, TX, USA). A Hardy-Weinberg equilibrium test was performed using a χ² test for both patients and control groups. The strength of

| Table 1. Clinical features of PD patients and healthy controls in the northern Chinese Han population |
|-----------------------------------------------|
| Variable | Controls | Patients with PD |
|---------|----------|-----------------|
| N = 363 | Male 205 (56.47%) | N = 322 (50%) |
| Female 158 (43.53%) | 161 (50%) |
| AAE 67.15 ± 10.60 | 64.09 ± 8.63 |
| AAO NA | 58.62 ± 9.52 |

The sample mean ± SD is given for age at enrollment (AAE) and age at PD onset (AAO); NA = not applicable.

| Table 2. Primer sequences and parameters for PCR amplification of SNPs rs823114 and rs1109303 |
|-----------------------------------------------|
| SNP | Direction | Sequence (5'-3') | Primer concentration (μM) | Restriction enzyme | Allele size (base pairs) |
|-----|------------|-----------------|--------------------------|-----------------|--------------------------|
| rs823114 | forward | ATAGGCTCCACCTTACCCGT | 0.40 | HaeIII | 194 (136, 58) |
| reverse | AAACACCTGAGCAGCGCTGAGC | 0.40 |
| rs1109303 | forward | TGCCCTCCAGAATAAGTAAAGTCCC | 0.60 | BanII | 168 (121, 47) |
| reverse | AAGGTGTTCAGGCAGCAAT | 0.60 |
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the association between the two loci and PD was measured by odds ratios (ORs) with 95% confidence intervals (CIs). We explored the association for a homozygous co-dominant model, a heterozygous co-dominant model, a dominant model and a recessive model. Power analysis was conducted by PS program statistical software. The degree of heterogeneity between males and females was determined by Q-statistic. $P > 0.05$ for the Q-test indicated a lack of heterogeneity and $P < 0.05$ indicated heterogeneity. Probability values were adjusted for multiple comparisons using the Bonferroni correction. The significance threshold for single SNP tests was set as 0.025 (0.05/2) since two independent SNPs were included in the association analyses.

RESULTS

Genotyping the two SNPs Multiplex PCR-RFLP analysis was performed to genotype the two SNPs. PCR product sizes were 194 bp (rs823114) and 168 bp (rs1109303). PCR products were simultaneously digested using HaeIII and BanII and separated by electrophoresis. Undigested fragments represent rs823114-A and rs1109303-T alleles, while the fragments of 136 and 121 bp represent rs823114-G and rs1109303-G alleles, respectively (Fig. 1). Fragments shorter than 47 bp ran off the gel under our electrophoresis conditions. These results indicated that the multiplex PCR-RFLP assay was successful for genotyping.

Table 3. Distribution of genotypic and allelic frequencies of SNPs rs823114 and rs1109303 in PD patients and controls in the northern Chinese Han population

| SNP     | Variants | PD (%) | Control (%) | OR (95% CI) | $P$ value | Power |
|---------|----------|--------|-------------|-------------|-----------|-------|
| rs823114 | A        | 407 (63.20) | 413 (56.89) | 0.768 | 0.017 | 0.664 |
|         | G        | 237 (36.80) | 313 (43.11) |              |           |       |
|         | n        | 644     | 726         | (0.618–0.955) |           |       |
|         | AA       | 133 (41.31) | 116 (31.96) |              |           |       |
|         | AG       | 141 (43.79) | 181 (49.86) |              |           |       |
|         | GG       | 48 (14.90) | 66 (18.18)  | NA          | 0.038    |       |
|         | n        | 322     | 363         |              |           |       |
| rs1109303 | G       | 381 (59.16) | 391 (53.86) |              |           |       |
|         | T        | 263 (40.84) | 335 (46.14) | 0.806 | 0.048 | 0.504 |
|         | n        | 644     | 726         | (0.650–0.998) |           |       |
|         | GG       | 119 (36.96) | 114 (31.41) |              |           |       |
|         | GT       | 143 (44.41) | 163 (44.90) |              |           |       |
|         | TT       | 60 (18.63) | 86 (23.69)  | NA          | 0.165    |       |
|         | n        | 322     | 363         |              |           |       |

Two polymorphic markers were in Hardy-Weinberg equilibrium.

OR: odds ratio; CI: confidence interval.

Fig. 1. Genotyping patterns of rs823114 and rs1109303 loci determined by multiplex PCR-RFLP. M: size marker.
and genotypic frequency distribution between patient and control groups (Table 3).

**Male genetic distribution bias and heterogeneity analysis** We performed gender stratification to understand associations between SNPs and PD. We found a substantial difference in the rs823114 G-allele in the male cohort ($P = 0.008$), which establishes that the rs823114 G-allele is a protective factor (Table 4). The value of heterogeneity chi-square was 1.06 and the $P$ value was 0.304 based on the respective allele frequency of both sexes in rs823114. There was no significant difference between the female and male groups. No association between the SNP rs1109303 locus and PD was observed in male or female populations (data not shown).

**Analysis of genotype distributions of rs823114 between PD and control groups under four genetic models** The difference in genotypes between the PD patients and healthy controls was significant under the heterozygous co-dominant model ($P = 0.022$) and the dominant model ($P = 0.011$), but not in the homozygous co-dominant model ($P = 0.045$) or the recessive model ($P = 0.251$) (Table 5). No associations were found among the four genetic models for the INPP5K rs1109303 with PD (data not shown).

**DISCUSSION**

Sporadic PD has a complex etiology, and most studies indicate that the pathogenesis of PD involves interactions between environmental and complex genetic factors (Schapira and Jenner, 2011). Up to now, hundreds of candidate SNPs have been identified in PD susceptibility genes, and their associations with PD have been widely explored. To investigate the association between PD susceptibility and genes, we genotyped two SNPs located on NUCKSI and INPP5K genes in the northern Han...
Chinese population.

The NUCKS1 gene is located in the PARK16 locus together with four other genes, RAB7L1, SLC41A1, SLC45A3 and PM20D1, and the RAB7L1, SLC41A1 and NUCKS1 genes are located on the same linkage disequilibrium block (Tucci et al., 2010). The PARK16 locus has been found to be associated with risk of PD and shows population differences (Gan-Or, 2015), but the role of the susceptibility region within PARK16 in the pathogenesis of PD is still a mystery. Analysis of CpG methylation in the frontal cortex and cerebellum tissues revealed that NUCKS1 and RAB7L1 were promising candidate genes for PD (Plagnol et al., 2011). Although RAB7L1 gene polymorphisms in relation to PD have been studied extensively (Khaligh et al., 2017), few studies have examined the association between the NUCKS1 gene and PD in Chinese populations. In our previous study, we investigated SNPs located in the susceptibility region of PARK16, including rs823076 on the SLC41A1 gene, rs708723 on the RAB7L1 gene and rs4951261 on the NUCKS1 gene, but none of these SNPs were associated with PD (Cui et al., 2013). The rs823114 locus on the NUCKS1 gene was initially found to be closely related to the transcript levels of NUCKS1 (P = 2.7 × 10^{-3}) (Liu et al., 2011). We observed the mutation in the NUCKS1 rs823114 locus as a protective factor for PD (P = 6.12 × 10^{-4}) (Li et al., 2011). We observed the mutation in the NUCKS1 rs823114 locus as a protective factor for PD (P = 0.017, OR = 0.768, 95% CI = 0.618−0.955), which is consistent with the Ashkenazi Jewish population (P = 2.76 × 10^{-6}, OR = 0.75, 95% CI = 0.68−0.83) (Vacic et al., 2014). Based on the available data, we also performed a meta-analysis (Supplementary Fig. S1). However, it is known that sample size and race background for population studies can interfere with a meta-analysis result. Thus, further association studies in China and Asia will undoubtedly be helpful. In this study, we found that the G-allele of rs823114 on the NUCKS1 gene was significantly associated with reduced risk of PD in our subject population. However, the genotype of rs823114 did not remain associated after Bonferroni correction. In addition, results for the heterozygous codominant model (AG vs. AA) and dominant model (GA+GG vs. AA) demonstrated that the G-allele of rs823114 played a protective role for PD patients. Besides that, the result from gender stratification showed that rs823114 was strongly associated with PD in males (P = 0.008). Previous studies have reported that the prevalence of PD in males is higher than in females (Benito-Leon et al., 2003; Van Den Eeden et al., 2003), so this study provides meaningful data for the association of NUCKS1 gene polymorphism with PD in a Chinese Han population, especially by suggesting that male genetic distribution bias on NUCKS1 occurs in PD patients. Hyperuricemia has a protective effect on the development and progression of neurodegenerative diseases such as PD and Alzheimer’s disease (Mandal and Mount, 2015). Large prospective studies suggested that higher uric acid levels could reduce the risk of PD (Weisskopf et al., 2007) and slow down disease progression (Ascherio et al., 2009). In PD-related cell culture and animal model studies, treatment with uric acid can reduce oxidative stress levels and protect DA neurons (Cipriani et al., 2012; Chen et al., 2013). The expressed product of the INPP5K gene inhibits the PI3K/Akt pathway by hydrolyzing intracellular phosphatidylinositol (3,4,5)-trisphosphate, and the PI3K/Akt pathway has been demonstrated to be neuroprotective in PD (Nakano et al., 2017). The SNP rs1109303 on INPP5K was found to cause this inhibition, leading to a risk of PD occurrence (Gong et al., 2012). A recent study has shown that a relationship between rs1109303 on the INPP5K gene and serum urate level in patients with PD was significant (Nazeri et al., 2015). Unfortunately, studies on INPP5K genetic polymorphisms in PD have rarely been reported among Asian populations, especially in Chinese populations. Identification of this genetic polymorphism will support the use of urate-based intervention in PD (The Parkinson Study Group SURE-PD Investigators, 2014). In this study, the allelic and genotypic frequencies of rs1109303 were not significantly different between the PD and control groups (P > 0.025). Therefore, we hypothesized that the SNP rs1109303 shows no association with PD in the Han population of northern China. One possible reason is the limited experimental sample size enrolled in this investigation. Thus, increasing sample size and selecting more populations in mainland China are required to reasonably assess whether there is a significant association between PD and this SNP in future studies.

CONCLUSIONS

This study showed an association between NUCKS1 rs823114 and sporadic PD in a Chinese population for the first time, but a lack of association with PD for the rs1109303 locus of INPP5K. Meanwhile, the genetic parameters and association analysis between NUCKS1 rs823114 and PD also displayed a male genetic distribution bias in the northern Han Chinese population.

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CONFLICTS OF INTEREST

The authors declare no conflict of interest.
REFERENCES

Ascherio, A., LeWitt, P. A., Xu, K., Eberly, S., Watts, A., Matson, W. R., Mauras, C., Kieburtz, K., Rudolph, A., Bogdanov, M. B., et al. (2009) Urate as a predictor of the rate of clinical decline in Parkinson disease. Arch. Neurol. 66, 1460–1468.

Benito-León, J., Bermejo-Pareja, F., Rodríguez, J., Molina, J. A., Gabriel, R., Morales, J. M., and Neurological Disorders in Central Spain (NEDICES) Study Group. (2003) Prevalence of PD and other types of parkinsonism in three elderly populations of central Spain. Mov. Disord. 18, 267–274.

Chen, X., Burdett, T. C., Desjardins, C. A., Logan, R., Cipriani, S., Xu, Y., and Schwarzschild, M. A. (2013) Disrupted and transgenic urate oxidase alter urate and dopaminergic neurodegeneration. Proc. Natl. Acad. Sci. USA 110, 300–305.

Cicchetti, F., Drouin-Ouellet, J., and Gross, R. E. (2009) Environmental toxins and Parkinson's disease: what have we learned from pesticide-induced animal models? Trends Pharmacol. Sci. 30, 475–483.

Cipriani, S., Desjardins, C. A., Logan, R., Cipriani, S., Xu, Y., and Schwarzschild, M. A. (2012) Urate and its transgenic depletion modulate neuronal vulnerability in a cellular model of Parkinson's disease. PLoS One 7, e37331.

Duan, W., Ladenheim, B., Cutler, R. G., Kruman, I. I., Cadet, J. L., and Mattson, M. P. (2002) Dietary folate deficiency and elevated homocysteine levels endanger dopaminergic neurons in models of Parkinson's disease. J. Neurochem. 80, 101–110.

Gong, L., Zhang, Q. L., Zhang, N., Hua, W. Y., Huang, Y. X., Di, P. W., Huang, T., Xu, X. S., Liu, C. F., Hu, L. F., et al. (2012) Neuroprotection by urate on 6-OHDA-lesioned rat model of Parkinson's disease: linking to Akt/GSK3β signaling pathway. J. Neurochem. 123, 876–885.

Grundt, K., Haga, I. V., Aleporou-Marinou, V., Drosos, Y., Wanvik, B., and Østvold, A. C. (2004) Characterisation of the NUCKS gene on human chromosome 1q22.1 and the presence of a homologous gene in different species. Biochem. Biophys. Res. Commun. 323, 796–801.

Hamza, T., and Payami, H. (2010) The heritability of risk and age at onset of Parkinson's disease after accounting for known genetic risk factors. J. Hum. Genet. 55, 241–248.

Hughes, A. J., Daniel, S. E., Kilford, L., and Lees, A. J. (1992) Accuracy of clinical diagnosis of idiopathic Parkinson's disease: a clinico-pathological study of 100 cases. J. Neurol. Neurosurg. Psychiatry 55, 181–184.

Jankovic, J. (2008) Parkinson's disease: clinical features and diagnosis. J. Neurol. Neurosurg. Psychiatry 79, 368–376.

Khaligh, A., Goudarzian, M., Moslem, A., Mehrtash, A., Jamshidi, J., Darvish, H., and Emamalizadeh, B. (2017) RAB7L1 promoter polymorphism and risk of Parkinson's disease; a case-control study. Neurol. Res. 39, 468–471.

Klein, C., and Schlossmacher, M. G. (2007) Parkinson disease, 10 years after its genetic revolution: multiple clues to a complex disorder. Neurology 69, 2093–2104.

Liu, X., Cheng, R., Verbitsky, M., Kisseleff, S., Browne, A., Mejia-Santanana, H., Louis, E. D., Cote, L. J., Andrews, H., Waters, C., et al. (2011) Genome-wide association study identifies candidate genes for Parkinson's disease in an Ashkenazi Jewish population. BMC Med. Genet. 12, 104.

Mandal, A. K., and Mount, D. B. (2015) The molecular physiology of uric acid homeostasis. Annu. Rev. Physiol. 77, 323–345.

Nakano, N., Matsuda, S., Ichimura, M., Minami, A., Ogino, M., Murai, T., and Kitagishi, Y. (2017) PI3K/AKT signaling mediated by G protein-coupled receptors is involved in neurodegenerative Parkinson's disease (Review). Int. J. Mol. Med. 39, 253–260.

Nazeri, A., Roostaei, T., Sadaghiani, S., Chakravarty, M. M., Eberly, S., Lang, A. E., and Voinoyskos, A. N. (2015) Genome-wide variant by serum urate interaction in Parkinson's disease. Ann. Neurol. 78, 731–741.

Parpols, A. C., Zhao, W., Sharma, N., Grosser, T., Liang, F., Maranon, D. G., Leung, S. G., Grundt, K., Dray, E., Idate, R., et al. (2015) NUCKS1 is a novel RAD51AP1 paralog important for homologous recombination and genome stability. Nucleic Acids Res. 43, 9817–9834.

Plagnol, V, Nalls M. A., Bras, J. M., Hernandez, D. G., Sharma, M., Sheerin, Ü. M., Saad, M., Simón-Sánchez, J., Schulte, C., Lesage, S., et al. (2011) A two-stage meta-analysis identifies several new loci for Parkinson's disease. PLoS Genet. 7, e1002142.

Satake, W., Nakabayashi, Y., Mizuta, İ., Hirota, Y., Ito, C., Kubo, M., Kawaguchi, T., Tsunoda, T., Watanabe, M., Takeda, A., et al. (2009) Genome-wide association study identifies common variants at four loci as genetic risk factors for Parkinson's disease. Nat. Genet. 41, 1303–1307.

Schapira, A. H., and Jenner, P. (2011) Etiology and pathogenesis of Parkinson's disease. Mov. Disord. 26, 1049–1055.

Spencer, A. H., Rickards, H., Fasano, A., and Cavanna, A. E. (2011) The prevalence and clinical characteristics of pungding in Parkinson's disease. Mov. Disord. 26, 578–586.

The Parkinson Study Group SURE-PD Investigators (2014) Inosine to increase serum and cerebrospinal fluid urate in Parkinson disease: a randomized clinical trial. JAMA Neurol. 71, 141–150.

Tucci, A., Nalls, M. A., Houlden, H., Revesz, T., Singleton, A. B., Wood, N. W., Hardy, J., and Paisán-Ruiz, C. (2010) Genetic variability at the PARK16 locus. Eur. J. Hum. Genet. 18, 1356–1359.

Vacic, V., Ozelius, L. J., Clark, L. N., Bar-Shira, A., Gana-Weisz, M., Gurevich, T., Gusev, A., Kedmi, M., Kenny, E. E., Liu, X., et al. (2014) Genome-wide mapping of IBD segments in an Ashkenazi PD cohort identifies associated haplotypes. Hum. Mol. Genet. 23, 4693–4702.

Van Den Eeden, S. K., Tanner, C. M., Bernstein, A. L., Fross, R. D., Leimpeter, A., Bloch, D. A., and Nelson, L. M. (2003) Incidence of Parkinson's disease: variation by age, gender, and race/ethnicity. Am. J. Epidemiol. 157, 1015–1022.

Weisskopf, M. G., O'Reilly, E., Chen, H., Schwarzschild, M. A., and Ascherio, A. (2007) Plasma urate and risk of Parkinson's disease. Am. J. Epidemiol. 166, 561–567.