



Screening of polymorphisms located in the *FGF20* and *TMEM175* genes in North Chinese Parkinson's disease patients

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ABSTRACT. Genome-wide association studies have reported numerous susceptibility loci for Parkinson's disease (PD). However, there have been few replication studies examining these loci in northern Chinese populations. To evaluate the relationships among 3 polymorphic markers located in the fibroblast growth factor 20 and transmembrane protein 175 genes and the genetic susceptibility to PD in northern Chinese subjects, 2 single nucleotide polymorphisms, and 1 insertion/deletion marker (rs591323 in *FGF20*; rs6599388 and rs142821586 in transmembrane protein 175 near the G-associated kinase/diacylglycerol kinase theta region) were investigated in 313 PD patients and 318 matched controls. Mismatched multiplex polymerase chain reaction-restriction fragment length polymorphism analysis as well as sequence-specific primer polymerase chain reaction and restriction fragment length polymorphism assays were performed. The genotypic frequency of rs591323 differed significantly between the patient and control groups;

however, neither rs6599388 nor rs142821586 was associated with PD. We corrected the Hardy-Weinberg disequilibrium for rs6599388, which was previously reported to be common in 4 Asian descent populations into equilibrium status by simultaneously genotyping rs6599388 and rs142821586. In summary, we found that rs591323 was associated with PD but rs6599388 and rs142821586 were not associated with PD in a northern Chinese population.

Key words: Association study; Fibroblast growth factor 20; Insertion/deletion; Parkinson's disease; Transmembrane protein 175

INTRODUCTION

Parkinson's disease (PD) is the second most common neurodegenerative disease, affecting approximately 1% of the population over the age of 50. PD is manifested by resting tremors, bradykinesia, and rigidity caused by the loss of dopaminergic neurons within the substantia nigra. The cause of dopaminergic cell death is not clear, and multiple genetic and environmental factors contribute to disease development in most cases (Schapira and Jenner, 2011). To date, at least 16 PD susceptibility genes have been reported. Additionally, genome-wide association studies and meta-analyses have linked more loci to the risk of PD, and consistently show common variations in synuclein, alpha and an inversion in the region containing the microtubule-associated protein tau gene are associated with PD (Satake et al., 2009; Simón-Sánchez et al., 2009). Simultaneously, some novel loci, such as the gene encoding for fibroblast growth factor 20 (*FGF20*) and cyclin G-associated kinase/diacylglycerol kinase theta (GAK/DGKQ) region, were recently associated with PD (van der Walt et al., 2004; Latourelle et al., 2009; Pankratz et al., 2009).

FGF20 is a member of a highly conserved family of growth factor polypeptides that regulate central nervous development and function (Dono, 2003). *FGF20* enhances the survival of midbrain dopaminergic neurons (Ohmachi et al., 2000). GAK (PARK17)/DGKQ is a particularly promising candidate region because it is one of 137 genes shown to be differentially expressed in PD, with a 1.54-fold change in expression in the substantia nigra pars compacta of PD patients compared with in controls (Grünblatt et al., 2004). The gene encoding human transmembrane protein 175 (TMEM175), located between the GAK and DGKQ loci, is also a member of the transmembrane protein family. It encodes a protein motif that is conserved across a number of hypothetical proteins of unknown function found in eukaryotes, bacteria, and archaea. These may be integral membrane proteins. Genome-wide association and meta-analysis studies on PD have recently identified new susceptibility single-nucleotide polymorphisms (SNPs) both in *FGF20* and the GAK/DGKQ region in western European populations, but few studies have examined different ethnic groups or in different regions (International Parkinson's Disease Genomics Consortium and Wellcome Trust Case Control Consortium 2, 2011; Sharma et al., 2012).

In this study, we conducted multiplexed polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) analysis and sequence-specific primer PCR (SSP-PCR) assays to identify 2 SNPs and an insert/deletion locus in *FGF20* and *TMEM175* to study their possible associations with PD in a northern Han Chinese population.

MATERIAL AND METHODS

Subjects

A total of 313 ethnic Han Chinese patients and 318 control subjects from northern China were included in the study. Patients were diagnosed with idiopathic PD by movement disorder neurologists at the First Affiliated Hospital of China Medical University in Liaoning Province, China. All patients met the criteria for the clinical diagnosis of PD, presenting at least 2 of the 3 cardinal signs for PD (tremor, rigidity, and bradykinesia) and had a positive response to levodopa therapy. Unrelated control participants were recruited from the local community. Control subjects were healthy and had no neurodegenerative diseases. The clinical characteristics of the study population are shown in Table 1. The local Ethics Committees approved the research protocols and all participants signed informed consent forms.

Table 1. Clinical characteristics of patients with Parkinson's disease and normal controls in a northern Han Chinese population.

| Group stage | N | No. of males (%) | AAE (mean \pm SD, years) | AAE (range, years) | AAO (mean \pm SD, years) | AAO (range, years) | Disease duration, (mean \pm SD) | Hoehn-Yahr (mean \pm SD, years) |
|-------------|-----|------------------|----------------------------|--------------------|----------------------------|--------------------|-----------------------------------|-----------------------------------|
| Control | 318 | 211 (66.3) | 69.19 \pm 12.89 | 34-95 | NA | NA | NA | NA |
| Patient | 313 | 161 (51.4) | 64.41 \pm 10.16 | 24-86 | 61.04 \pm 10.07 | 24-83 | 4.88 \pm 3.75 | 1.85 \pm 0.68 |

AAE = age at enrollment, AAO = age at onset, NA = not applicable, PD = Parkinson's disease, SD = standard deviation.

Genotyping

Peripheral blood samples were collected from all participants. Genomic DNA was extracted from leukocytes using the sodium dodecyl sulfate-proteinase K phenol-chloroform method.

We synthetically generated an *MluI* restriction endonuclease site in the amplified product of the rs951323 locus and a *SalI* site in the amplified product of the rs6599388 locus using mismatched PCR primers based on published sequences. Primer sets 1 and 2 used to detect the 2 SNPs are shown in Table 2. Several bases were mismatched in either the forward (5') or reverse (3') primers to produce synthetic recognition sites for the *MluI* or *SalI* restriction enzymes.

Multiplex PCR-RFLP was used to identify SNPs in the DNA samples as described previously (Zhou et al., 2012). PCR was conducted in a final volume of 20 μ L consisting of *rTaq* buffer plus Mg²⁺ containing 40 ng genomic DNA, 200 nmol dNTPs, 0.5 mM of each primer, and 0.5 U *rTaq* DNA polymerase (TaKaRa, Shiga, Japan). PCR amplification was performed with initial denaturing step at 94°C for 1 min, followed by 35 cycles of denaturing at 94°C for 30 s, annealing at 68°C for 30 s, and extension at 72°C for 30 s, and a final extension at 72°C for 5 min. The PCR products were digested at 37°C for 2 h in 10 μ L TaKaRa H buffer containing 3 μ L PCR product and 4 U *MluI* and *SalI* (TaKaRa).

Table 2. Information about the 3 polymorphic markers and parameters for mismatched multiplex PCR amplification.

| Polymorphic marker | Nucleotide changes | Primer sets | Primer direction | Sequence (5'→3') | Fragment size (No. bp) | Primer concentration (μM) |
|--------------------|--------------------|-------------|------------------|--------------------------------|------------------------|---------------------------|
| rs591323 | G>A | Set 1 | Forward | ATAAGTGGGAGCTAAATGGTGATAACgC* | 164 (139, 25) | 0.5 |
| | | | Reverse | CCATGAGGGGTTGTTATATAGATCACCC | | 0.5 |
| rs6599388 | C>T | Set 2 | Forward | TATGGCACTACACCTTATTAAGTGTGCTAA | 121 (100, 21) | 0.5 |
| | | | Reverse | GCGGGGAGTGAGGAGACAGAGtC* | | 0.5 |
| rs142821586 | del TC | Set 3 | Forward | TATGGCACTACACCTTATTAAGTGTGCTAA | | 0.5 |
| | | | Reverse | GCGGGGAGTGAGGAGACAGtC* | | 0.5 |

bp = base pairs. *Small letters indicate mismatched bases.

Digested products were separated using polyacrylamide gel electrophoresis (T = 8%, C = 5%). Electrophoresis was conducted at room temperature for 45 min with a voltage gradient of 10 V/cm. Next, the gel was placed in 20 mL 1X TBE buffer containing 2 μL 10,000X Genefinder (Bio-V, Xiamen, China) for staining for 30 min, and was then washed in distilled water for 1 min. Bands were observed using a UV MiniBis BioImaging System (DNR Bio-Imaging Systems, Jerusalem, Israel).

The rs142821586 locus in the TMEM175 gene is a TC deletion, located 2 base pairs (bp) downstream of the rs6599388 locus. Thus, we designed a reverse primer and used the same forward to amplify the rs6599388 locus to genotype the rs142821586 locus using the SSP-PCR assay. Again, 1 base was mismatched in the reverse (3') primer to produce a synthetic recognition site for the *SalI* restriction enzyme. Primer set 3 is shown in Table 2.

Because the reverse primer used to amplify the rs6599388 locus spanned the 'TC deletion', some DNA samples could not be efficiently amplified by primer set 2 on the rs6599388 locus. This indicated that there was a TC deletion genotype at the rs142821586 locus. Therefore, we first performed an SSP-PCR assay on samples that did not produce PCR products and with a 'homozygous genotype' at the rs6599388 locus. We then carried out further RFLP analysis of the amplified PCR products from the SSP-PCR assay. PCR was conducted under same conditions as the multiplex PCR-RFLP assay described above.

Statistical analysis

The results were analyzed using SPSS version 13.0 (SPSS, Inc., Chicago, IL, USA) and PLINK-1.07 version for Windows (<http://pngu.mgh.harvard.edu/purcell/plink/>). A 2-tailed P value ≤ 0.05 was considered to be significant.

RESULTS

Genotyping of 3 polymorphic markers

We performed mismatched multiplex PCR amplification and a subsequent RFLP analysis. The results showed that the mismatched sequences containing the *MluI* recognition site at the rs591323 locus were specifically amplified in all samples. In addition, sequences containing the *SalI* site at the rs6599388 locus were also amplified, except in 4 PD patients and 2 controls. The sizes of the amplified PCR products were 164 bp (rs591323) and 121 bp

(rs6599388) (Figure 1). PCR products were simultaneously digested using *MluI* and *SalI* and separated by electrophoresis. Undigested fragments that remained originated from the A allele of rs591323 and the C allele of rs6599388, while fragments of 139 and 100 bp originated from the G allele of rs591323 and the T allele of rs6599388, respectively (Figure 1). Fragments shorter than 25 bp ran off the gel under the electrophoresis conditions used.

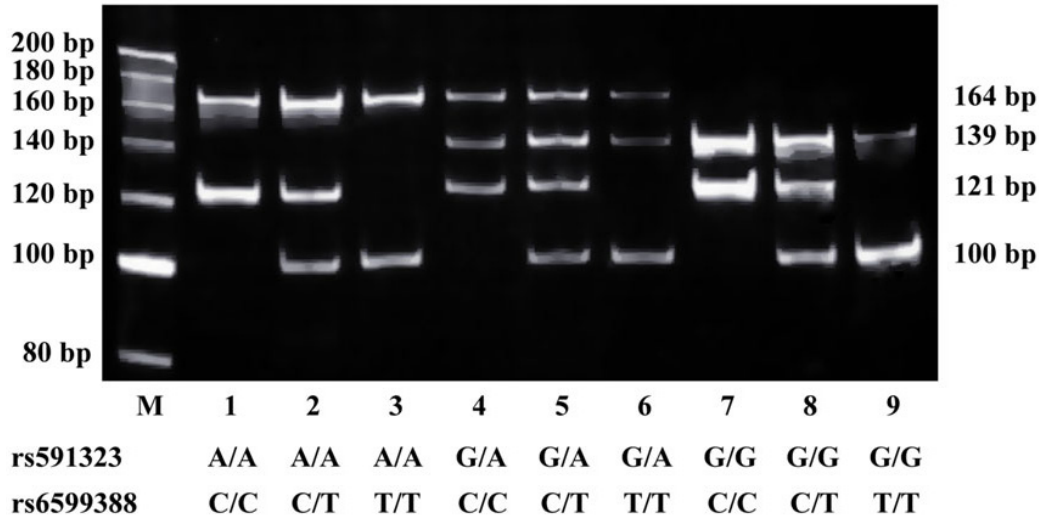


Figure 1. Electrophoretic pattern following mismatched multiplex PCR-RFLP analysis of the 2 SNPs in *FGF20* and *TMEM175* in a polyacrylamide gel stained by Genefinder. Lane M = DNA ladder ranging from 80-200 bp as a molecular size marker. Lanes 1-9 = all genotype combinations at the rs591323 and rs6599388 loci found in this study.

Second, we carried out SSP-PCR amplification of samples without PCR products and with a ‘homozygous genotype’ at the rs6599388 locus based on the results of multiplex PCR-RFLP analysis. We not only obtained target products from the previously unamplified samples using primer set 3, but also extracted the insert/deletion alleles from the ‘homozygous genotype’. DNA samples that were only amplified with primer set 3 showed the del TC(-/-) genotype at the rs142821586 locus, those only amplified with primer set 2 showed the del TC(+/+) genotype, and those amplified with both sets 2 and 3 had the del TC(+/-) genotype (Table 3). Furthermore, the PCR products from SSP-PCR amplification were digested by *SalI* and analyzed by RFLP analysis to determine whether a T allele was present at the rs6599388 locus in the unamplified samples. However, we did not detect this allele, suggesting that there is no linkage between a T allele at the rs6599388 locus and del TC allele at the rs142821586 locus. These results indicate that mismatched multiplex PCR-RFLP combined with SSP-PCR and RFLP assays can successfully identify all genotypes at the 3 polymorphic loci.

Genetic parameters and association analysis

The genotypic frequencies of the 3 polymorphic markers located in the 2 genes were investigated in 313 patients and 318 control participants. All 3 polymorphic markers were in

Hardy-Weinberg equilibrium (HWE). The related genetic parameters for each polymorphic site are shown in Table 3. Results from linkage disequilibrium analysis of the 2 polymorphic sites located on the same gene showed that rs6599388 and rs142821586 were in linkage disequilibrium. Subsequent association analyses of the 3 polymorphic markers showed that the genotype distribution of rs591323 was significantly different between the 2 groups ($P = 0.0195$; odds ratio = 0.7679, 95% confidence interval: 0.6151-0.9585), while the distributions of the remaining 2 loci were not significantly different between groups ($P > 0.05$). Association tests of rs591323 by gender revealed a significant difference between patients and controls in the male group ($P = 0.0021$; odds ratio = 0.6319; 95% confidence interval: 0.4714-0.8469).

Table 3. Genotypes of 3 polymorphic markers and statistical parameters.

| | Genotype | No. patients | Frequency | No. controls | Frequency | Association with PD |
|--|----------|--------------|-----------|--------------|-----------|----------------------|
| Parameters of three polymorphic markers | | | | | | |
| rs591323 | GG | 101 | 0.323 | 81 | 0.255 | OR = 0.7679 |
| | GA | 155 | 0.495 | 159 | 0.5 | 95%CI: 0.6151-0.9585 |
| | AA | 57 | 0.182 | 78 | 0.245 | P = 0.0195 |
| rs6599388 | CC | 106 | 0.338 | 121 | 0.381 | OR = 1.18 |
| | CT | 152 | 0.486 | 153 | 0.481 | 95%CI: 0.9415-1.478 |
| | TT | 55 | 0.176 | 44 | 0.138 | P = 0.1508 |
| rs142821586 del TC | +/+ | 239 | 0.763 | 243 | 0.764 | OR = 1.033 |
| | +/- | 70 | 0.224 | 73 | 0.23 | 95%CI: 0.7383-1.446 |
| | -/- | 4 | 0.013 | 2 | 0.006 | P = 0.8484 |
| Parameters of rs591323 in the male group | | | | | | |
| rs591323 | GG | 55 | 0.342 | 47 | 0.223 | OR = 0.6319 |
| | GA | 80 | 0.497 | 107 | 0.507 | 95%CI: 0.4714-0.8469 |
| | AA | 26 | 0.161 | 57 | 0.27 | P = 0.0021 |

Genotypes of 3 polymorphic markers were in Hardy-Weinberg equilibrium. CI = confidence interval, OR = odds ratio.

DISCUSSION

Some genes have been found to contribute to Mendelian forms of PD; however, mutations have been identified in fewer than 5% of patients, suggesting that additional genes contribute to disease risk. Unlike previous studies that focused primarily on sporadic PD, *FGF20* and the *GAK/DGKQ* region were recently identified as PD susceptibility loci based on genome-wide association studies and a meta-analysis of studies in PD patients. *FGF20* is a member of the highly conserved family of growth factor polypeptides that regulate central nervous development and function (Dono, 2003). *FGF20* promotes the differentiation of Nurr1-neural stem cells into tyrosine hydroxylase-positive neurons and additional factors are required for the efficient differentiation of dopaminergic neurons in the adult brain (Grothe et al., 2004). *FGF20* is selectively expressed in the calbindin-negative dopaminergic neurons of the substantia nigra, which are preferentially lost in PD, promoting the survival of these neurons *in vitro* and *in vivo* (Ohmachi et al., 2000; Murase and McKay, 2006; Sleeman et al., 2012). Thus, genetic variations in *FGF20* may influence PD occurrence and development. van der Walt et al. (2004) identified a highly significant association between PD and 3 SNPs, including rs1989754, rs1721100, and rs12720208, in *FGF20* based on the pedigree disequilibrium test. However, a subsequent case-control study suggested that *FGF20* was not a major risk factor for sporadic PD in Finnish and Greek patients (Clarimon et al., 2005). A study combining association analysis and functional evidence showed that the T allele of rs12720208 altered PD risk by increasing *FGF20* and α -synuclein protein levels (Wang et al., 2008); the authors suggested that a 3'-untranslated region SNP affects miRNA binding and results in the

differential allele-specific expression of *FGF20*. However, Wider et al. (2009) examined the association between *FGF20* and PD risk in a 4 patient-control series and measured FGF20 and α -synuclein protein levels in brain samples. They found no association between *FGF20* variability and PD risk, and no relationship among the rs12720208 genotype, FGF20, and α -synuclein protein levels. This inconsistency may be explained by the overlap of samples and differences in statistical approaches. Furthermore, to assess the genetic association between *FGF20* and PD in the Asian population, 3 and 2 SNPs in *FGF20* were investigated in Japanese and Chinese populations (Satake et al., 2007; Pan et al., 2012), respectively. Interestingly, only the rs1721199 polymorphism was found to be a risk factor for PD in both Asian populations, implying that *FGF20* may be a susceptibility gene for PD. In the present study, we selected the SNP rs591323 in *FGF20* for an association study because it was previously associated with PD in familial PD samples and these results have never been replicated in a case-control study (van der Walt et al., 2004; Wang et al., 2008). Our findings further support an association between PD and this locus in the Han Chinese population; however, whether this SNP functionally affects the *FGF20* transcript remains unclear.

Initial analyses found that the *GAK/DGKQ* region on chromosome 4p16 considered was a risk factor for PD based on genome-wide association studies (Latourelle et al., 2009; Pankratz et al., 2009). *GAK*, a serine/threonine kinase, is involved in the cell cycle and in the maintenance of proper centrosome maturation and mitotic chromosome congression (Kimura et al., 1997; Shimizu et al., 2009). *GAK* has been found to play a role in clathrin-mediated endocytosis/vesicle trafficking in the cytoplasm and in the nucleus (Sato et al., 2009). *DGKQ* contains 3 cysteine-rich zinc-binding domains, an N-terminal proline- and glycine-rich region, and a RAS-association domain (Houssa et al., 1997). *DGKQ* is highly expressed in the cerebellum and hippocampus. However, little is known about the function of *DGKQ*. Although many PD susceptibility loci were previously found in both *GAK* and *DGKQ*, *TMEM175* has not been thoroughly described. To date, only 3 SNPs in *TMEM175*, rs6599388, rs6599389, and rs2290403, have been reported to be associated with PD. The rs6599389 and rs2290403 loci were identified from a web-based genome-wide association study (Do et al., 2011), whereas rs6599388 was identified as a risk factor for PD in a meta-analysis of genome-wide association studies (International Parkinson Disease Genomics Consortium et al., 2011). Thereafter, only a single large-scale replication study of the rs6599388 locus that was defined as a SNP in the *GAK* gene has been performed; this study spanned a total of 21 sites and represented 19 countries across 4 continents. Interestingly, the genotype distribution of rs6599388 in samples from 4 of 5 Asian sites showed a departure from HWE, and this locus was excluded from analyses in the Asian population, but a significant association with PD was found in populations of Western European descent. In the present study, in addition to the rs6599388 locus located in intron 1 of *TMEM175*, we also selected the rs142821586 locus to analyze the possible association with PD in a northeast Chinese population. However, no data have been reported regarding the association between PD risk and these 2 loci in the northeast Chinese population, suggesting that ethnic factors have important implications in genetic analysis.

We investigated whether the rs6599388 locus was associated with PD in a northeast Chinese population. As previously reported for 4 Asian populations (Sharma et al., 2012), our data showed a departure from HWE either because of excessive homozygous genotypes or because there were only a few samples without a PCR product. Therefore, we redesigned the reverse primer to replace the original mismatched primer to successfully obtain PCR products from the samples. Sequence information from the newly amplified fragments revealed

a 2-base deletion at the rs142821586 locus located only 2 bp downstream of the rs6599388 locus. Thus, we genotyped the samples without a PCR product and those with a ‘homozygous genotype’ at the rs6599388 locus by combining SSP-PCR and RFLP. After complementary analysis, all genotypes at the rs6599388 locus were not only determined, but also HWE was restored. Additionally, we investigated the genotypic distribution of the rs142821586 locus in a northeastern Chinese population. More significantly, based on the results of our study, the previous investigation of the rs6599388 locus in Asian populations may have produced similar phenomena, showing a deviation from HWE, and the authors were unable to evaluate the association with PD. Therefore, complementary analysis is necessary when using data from a primary assay for genotyping, such as gene chip data, which show a deviation from HWE.

Conflicts of interest

The authors declare no conflict of interest.

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