Genetic association of urokinase-type plasminogen activator gene rs2227564 site polymorphism with sporadic Alzheimer’s disease in the Han Chinese population☆

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Abstract
A missense C/T polymorphism in exon 6 (the NCBI rsID is rs2227564) of the urokinase-type plasminogen activator gene has been identified as a possible hot spot for Alzheimer’s disease risk. The present study analyzed urokinase-type plasminogen gene polymorphisms of rs2227564 with sporadic Alzheimer’s disease by PCR-restriction fragment length polymorphism. Results showed that CC, CT and TT genotype distribution frequencies had significant differences between sporadic Alzheimer’s disease patients and healthy controls. In-depth analysis of the association between urokinase-type plasminogen gene rs2227564 polymorphisms and sporadic Alzheimer’s disease indicated that people with the C-positive genotype CC + CT were at a higher risk for developing sporadic Alzheimer’s disease. These results support the contribution of the polymorphisms of rs2227564 in the urokinase-type plasminogen gene to the pathogenesis of sporadic Alzheimer’s disease in the Han Chinese population.

Key Words
Alzheimer’s disease; urokinase plasminogen activator; polymorphism; genetic testing; Han Chinese population; neural regeneration

Research Highlights
Polymorphisms of rs2227564 in the urokinase-type plasminogen gene contributed to the pathogenesis of sporadic Alzheimer’s disease in the Han Chinese population.

Abbreviations
Aβ, amyloid β-peptide; SAD, sporadic Alzheimer’s disease; SNP, single nucleotide polymorphism; PLAU, urokinase-type plasminogen activator gene

INTRODUCTION
Currently only apolipoprotein E is a confirmed genetic risk factor for sporadic Alzheimer’s disease (SAD). All other potential risk factors have not shown consistent results[1]. The apolipoprotein E ε4 allele accounts for a fairly small fraction of the incidence of Alzheimer’s disease[2-3], therefore, there may be other as yet unknown risk alleles.

The urokinase-type plasminogen activator (PLAU) gene represents a strong biological and positional candidate for susceptibility for Alzheimer’s disease[4]. A missense C/T polymorphism in exon 6 (the NCBI rsID is rs2227564) which changes proline 141 to leucine (P141L)
in the PLAU gene has been identified as a possible hot spot for Alzheimer's disease risk. Several studies have reported the relationship between the polymorphisms of PLAU and Alzheimer's disease in different ethnic groups, but the results are inconsistent. Based on these results, we hypothesized that PLAU may be associated with SAD. To date, there is little data about SAD in the Han Chinese population. We conducted a case-control study to explore the genetic association between a PLAU single nucleotide polymorphism (SNP) and SAD in the Han Chinese population.

RESULTS

Quantitative analysis of subjects
For this study, 162 Han Chinese SAD patients and 128 healthy controls were recruited. Due to difficulties in obtaining sequences from some samples, in the final analysis of the SNP of rs2227564, there were 157 SAD patients and 128 healthy controls. For the SNP of rs2227562, there were 140 SAD patients and 121 healthy controls. All subjects were involved in the analysis of the results.

A comparison of the baseline data between the SAD group and the control group
The SNPs at rs2227564 and rs2227562 were analyzed, and the comparison of baseline data between the SAD and control groups is shown in Table 1.

No significant differences of gender occurred between the SAD and control groups (P > 0.05), although the SAD patients were older than the control group (P < 0.05). All genotype distributions from rs2227564 and rs2227562 polymorphisms were in Hardy-Weinberg equilibrium in the SAD and control groups (rs2227564: P = 0.110, 0.719; rs2227562, P = 0.970, 0.479).

Sequencing analysis of the PLAU gene SNPs
Four SNPs (rs2227564, rs2227562, rs2227563 and rs12255769) of the PLAU gene were analyzed. The PCR products were determined by direct sequencing (Huada Gene Company, Beijing, China). However, we found only rs2227564 and rs2227562 have SNPs (Figure 1).

The SNP detection at rs2227564 and rs2227562 (Figure 2)
When there was a T allele at rs2227564 of PLAU, the 5’-AG/CT-3’ sequence may be digested by the restriction enzyme, AluI. When there was a C allele at rs2227564 of PLAU, the sequence could not be digested by AluI. There are three possible genotypes: TT, CC or CT. If the genotype was TT, digested PCR products left 33, 203 and 271 bp fragments, of which the 203 and 271 bp fragments were visible after agarose gel electrophoresis. If the genotype was CC, the PCR products could not be digested by AluI, and the intact 507 bp fragment was visible on the agarose gel. If the genotype was CT, after digestion with AluI, three products of 203, 271 and 507 bp fragments were observed (Figure 2).

For the rs2227562 SNP of PLAU, digesting with AlwNI could distinguish between the three possible genotypes. When there was an A allele at rs2227562, the 5’-CAGNNN/CTG-3’ sequence could be digested by AlwNI. When there was a G allele, the rs2227562 SNP could not be digested by AlwNI. Thus, if the genotype was AA, 102 and 405 bp fragments were obtained, although only the 405 bp fragment was visible on the agarose gel. If the genotype was GG, the PCR product could not be digested by AlwNI, and the intact 507 bp fragment was visible on the agarose gel. If the genotype was AG, 405 and 507 bp fragments were observed on the gel (Figure 3).

The correlation between the PLAU rs2227564 polymorphisms and SAD

For the rs2227564 SNP, the frequencies for the CT and TT genotypes were significantly different between the SAD patients and controls ($\chi^2 = 7.349, P = 0.007$), with the CT genotype carriers at higher risk of developing SAD versus the TT genotype carriers (odds ratio (OR) = 3.083, 95% confidence interval (CI): 1.333 - 7.132). Further analysis demonstrated that the CC + CT genotypes were also associated with SAD, and the frequencies for the CC + CT and TT genotype were significantly different between the SAD patients and the controls ($\chi^2 = 5.540, P = 0.019$), with CC + CT genotype carriers versus TT genotype carriers having a higher risk of developing SAD (OR = 2.562, 95% CI 1.146 - 5.730; Table 3).

The strength of this distribution difference (CC + CT genotypes versus TT genotype) remained after controlling for age. Also, statistical analysis demonstrated that the CC + CT genotypes were significant predictors of disease status, with an OR for the risk of SAD for CC + CT carriers of 2.997 (95% CI 1.144 - 7.851, Wald = 4.992).

**DISCUSSION**

Alzheimer’s disease is the most common form of dementia. The presence of large numbers of extracellular senile plaques in the cerebral neocortex and hippocampus is a pathologic characteristic of Alzheimer’s disease[15]. Senile plaques are mainly composed of the 40-42 amino acid amyloid β-peptide (Aβ) which is derived from endoproteolytic processing of the amyloid precursor protein[16]. Aβ is constantly anabolized and catabolized in the brain, and the steady-state of Aβ level is physiologically determined by the metabolic balance between the anabolic and catabolic activities[17-18]. Many peptidases have been proposed as Aβ-degrading enzymes, such as plasmin[19], neprilysin[20] and insulin-degrading enzyme[21-22].

In the past years, the plasmin proteolytic cascade has been recognized as playing a critical role in fibrinolysis, inflammation, cell migration and tumor metastasis[23-26]. More recently, the plasmin proteolytic cascade was found to play an important role in AD[27-29]. Plasmin can not only degrade monomeric and oligomeric forms of Aβ1-40, but also can degrade Aβ1-42, which blocks Aβ1-42 aggregation[30-31].
Urokinase-type plasminogen activator is an activator of plasmin. Urokinase-type plasminogen activator is encoded by PLAU and is critical for Aβ clearance [32-33]. Through the plasmin proteolytic cascade, urokinase-type plasminogen activator can significantly decrease the injury to neurons induced by aggregated Aβ [34-36].

A missense C/T polymorphism in exon 6 of PLAU (the NCBI rsID is rs2227564), which changes proline to leucine (P141L), has been identified as a possible hot spot for AD risk on chromosome 10. The P141L changes a pleated sheet. The P141 zymogen binds fibrin aggregates less efficiently than the L141 zymogen [37], suggesting the possibility of altered PLAU stability, or modified ability to degrade extracellular Aβ.

In our study, we directly sequenced the PLAU gene and identified four SNPs (rs2227562, rs2227563, rs2227564 and rs12255769) according to the public SNP databases. Only the rs2227564 and rs2227562 SNPs were found to have polymorphisms in the Han Chinese population. We focused our attention on the relationship between SAD and the C/T polymorphism of P141L (rs2227564), and discovered a correlation between the frequency of the C-positive genotype of the PLAU P141L gene and the risk of SAD. While the C-positive genotype may be a risk genotype for SAD, the TT genotype appeared to be a protective factor against SAD. Nevertheless, neither C nor T allele frequencies were shown to have a significant association with SAD in our study.

To date, several studies have reported the relationship between the PLAU rs2227564 polymorphism and Alzheimer’s disease in different ethnic groups, but the results have been inconsistent [14]. Finckh et al [5] noted that the homozygous CC genotype and the C allele of PLAU rs2227564 were increased risk factors for Alzheimer’s disease in their series of patients from Germany, Switzerland and Italy. In contrast, Ertelkin-Taner [51] meta-analyzed the PLAU rs2227564 SNP in six case-control series, and indicated that the rs2227564 T-positive genotype is associated with a modest but marginally significant increase in risk for Alzheimer’s disease.

| SNP (rs2227564) | Genotype | Allele |
|-----------------|----------|--------|
|                 | n        | CC (%) | CT (%) | TT (%) | P       | C (%) | T (%) | P       |
| SAD group       | 157      | 61 (38.8) | 86 (54.8) | 10 (6.4) | 0.019 | 208 (66.2) | 106 (33.8) | 0.655 |
| Control group   | 128      | 56 (43.8) | 53 (41.4) | 19 (14.8) |       | 165 (64.5) | 91 (35.5) |       |

Pearson chi-square tests were used to compare SAD patients with controls for distributions (%) of alleles and genotypes. Direct counting was used to count the numbers of alleles and genotypes. SAD: Sporadic Alzheimer’s disease; SNP: single nucleotide polymorphism.

| SNP (rs2227562) | Genotype | Allele |
|-----------------|----------|--------|
|                 | n        | AA (%) | AG (%) | GG (%) | P       | A (%) | G (%) | P       |
| SAD group       | 140      | 11 (7.9) | 54 (38.6) | 75 (53.5) | 0.299 | 76 (27.1) | 204 (72.9) | 0.510 |
| Control group   | 121      | 7 (5.8) | 58 (47.9) | 56 (46.3) |       | 72 (29.8) | 170 (70.2) |       |

Table 2 Comparison of the allele and genotype distributions of PLAU gene polymorphisms in SAD patients and the control group.

Table 3 SAD risk analysis among SAD patients and controls.

| Risk analysis | \( \chi^2 \) | df | P    | OR    | 95% CI |
|--------------|-------------|----|------|-------|-------|
| CC vs. TT    | 2.899       | 1  | 0.089| 2.070 | 0.887-4.829 |
| CT vs. TT    | 7.349       | 1  | 0.007| 3.083 | 1.333-7.132 |
| CC+CT vs. TT | 5.540       | 1  | 0.019| 2.562 | 1.146-5.730 |

The relative risks estimated by odds ratios (OR) and P values for CC, CT or CC + CT genotypes versus TT genotype are shown. Pearson chi-square tests and multiple logistic regression analyses were used to compare SAD patients with controls for the distributions of alleles and genotypes. Direct counting was used to count the numbers of alleles and genotypes. There were 157 SAD patients and 128 healthy controls analyzed in this study. SAD: Sporadic Alzheimer’s disease; CI: confidence interval.

Interestingly, conflicting to the above two investigations, we found the C-positive genotype was associated with an increased risk of SAD. Besides these findings of positive correlations, three other groups failed to observe significant associations of the PLAU rs2227564 genotype or allele frequencies with Alzheimer’s disease in their study populations [11-13]. Such discrepancies between our study and others are possibly due to the different distribution of the polymorphism in the control group in our investigation compared with the control groups previously reported. We found a higher frequency (14.8%) of the PLAU rs2227564 TT genotype in the control group in our study compared with the TT genotype in previously reported control groups (3.0-11.0%). Ethnic differences among populations likely explain the discrepancies in these findings. It is also possible that people with familial Alzheimer’s disease and SAD may express different
Since SAD is more common than familial Alzheimer's disease, we focused on SAD for this study. In addition to rs2227564, we studied the relationship between SAD and the A/G SNP of rs2227562, which is 140 base pairs upstream from rs2227564 and located in intron 5. However, we failed to observe significant associations of either genotypic or allelic frequencies with SAD.

In summary, the present study shows that PLAU is a promising new candidate gene for SAD risk in the Han Chinese population, with the C-positive genotype of rs2227564 as a recessive risk factor while the TT genotype offers protection from SAD. These results support the contribution of the variation in the PLAU P141L gene to the pathogenesis of SAD. The rs2227564 SNP in the PLAU gene appears to be a prognostic marker of SAD. While this polymorphism could be a useful genetic marker, the complex mechanisms relevant to this process await further exploration.

Recently it was reported that tert-butylhydroquinone dramatically reduced brain Aβ load and was accompanied by increases in the activities of PLAU[38]. Evidence suggests that the plasminogen activator/plasmin system is a potential therapeutic target in Alzheimer's disease[39-40]. Continuing our studies of the PLAU polymorphism and its connection to SAD in other ethnic groups will further clinical investigations and may offer new treatment options for SAD.

SUBJECTS AND METHODS

Design
A case-control study regarding gene polymorphisms.

Time and setting
The experiment was performed at the Neurology Laboratory, Xuanwu Hospital of the Capital Medical University, from February 2005 to March 2006.

Subjects
In this study, 162 ethnic Han Chinese SAD patients who were outpatients of the Department of Neurology, Xuanwu Hospital of Capital Medical University, and 128 healthy controls were recruited from February 2005 to March 2006. All the subjects entered the study voluntarily.

SAD group
Inclusion criteria: All SAD patients were diagnosed according to the criteria of National Institute of Neurological and Communicative Disorders and Stroke, and the Alzheimer's disease and Related Disorders Association for 'probable Alzheimer's disease'[41].

Exclusion criteria: Patients were excluded if they had systemic disorders or other brain diseases that could account for progressive deficits in memory and cognition, or had disturbances of consciousness. Patients with evidence of vascular dementia, 'mixed' dementia, or a family history of Alzheimer's disease were excluded also.

Control group
Inclusion criteria: Controls were healthy check-out staff from the Xuanwu Hospital of the Capital Medical University in China. They underwent regular health examinations, and were confirmed healthy with Mini-Mental Test scores > 24 points.

Exclusion criteria: All subjects who had a history of poison contact, or hereditary disease were excluded. Also, anyone showing signs of dementia, anxiety, depression, or other mental or physical disease were excluded according to the Administrative Regulations on Medical Institutions[42], formulated by the State Council of China, informed consent was obtained from the participants, or their respective caretakers if the patients were not capable.

Methods

SNP screens of PLAU relevant to this study were performed by direct sequencing

Genomic DNA was extracted from peripheral blood leukocytes (cubital vein blood) using standard phenol/chloroform extraction methods[43]. Using data from the National Center for Biotechnology Information SNP consortium database (http://www.ncbi.nlm.nih.gov/SNP/), we analyzed four SNPs by directly sequencing the DNA. For convenience, these SNPs are termed PLAU 1, 2, 3, and 4. The analysis of 48 unrelated individuals revealed that PLAU 3 and 4 were not polymorphic and were ruled out from our study. The details of the SNPs are shown in Table 4.

Analysis of PLAU genotypes by PCR-restriction fragment length polymorphism assay

The regions of the PLAU gene containing rs2227564 and rs2227562 were amplified by PCR. All PCR primers were designed using Primer 5 (Premier, Canada) for the two
SNPs, and the expected product size was 507 bp (GenBank Accession No. AF377330). Forward and reverse amplification primers were 5’TGGGGGACCAAC-3’ and 5’CTTAAAAGCCGGGCTTCAAG-3’, respectively. A total volume of 25 μL for the PCR reaction mixture was used which consisted of 0.625 μL Taq DNA polymerase (TaKaRa Bio Inc, Shiga, Japan), 1.5 μL dNTPs (50 mM), 0.3 μL (20 μM) of each primer, and 100 ng DNA template. After an initial denaturation step at 94°C for 5 minutes, PCR was carried out for 30 cycles at 94°C for 30 seconds, 61.1°C for 30 seconds, and 72°C for 30 seconds, followed by a final extension at 72°C for 5 minutes. A volume of 5 μL of PCR products was digested with the restriction endonucleases, Aul or AlwNI (New England Biolabs, Beverly, MA, USA) for the SNPs of rs2227564 and rs2227562 respectively, then visualized with ethidium bromide after electrophoresis in 2% agarose gels.

**Statistical analysis**

All statistical analyses were carried out with SPSS version 11.5 software (SPSSS, Chicago, IL, USA). Genotypic and allelic frequencies were estimated by direct counting, and were compared between patients and controls by means of a two-sided Pearson chi-square test ($\chi^2$ test). The relationship of gene polymorphism with Alzheimer’s disease was evaluated by a Pearson $\chi^2$ test with OR for the CT or CC + CT genotypes versus the TT genotype. Multiple logistic regression models were used to adjust for age. Two-sample t-tests were used to compare the ages between the two groups. The Pearson $\chi^2$ test was used to compare the genders between the two groups. Pearson $\chi^2$ tests were used to determine whether the observed genotypic frequencies deviated from Hardy-Weinberg equilibrium in the case and control samples. Any $P$ value less than 0.05 was considered statistically significant.

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**Author contributions:** Jianping Jia guided the experiments and obtained funding. Xuelian Ji designed the study, wrote the manuscript, implemented all experimental procedures, contributed to the conceptualization of the study, and reviewed several manuscript drafts. Longfei Jia performed the PCR assays. Li Qi undertook the statistical analysis, and manuscript drafting and text revisions.

**Conflicts of interest:** None declared.

**Ethical approval:** The study was approved by the Ethics Committee of the Capital Medical University in China.

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