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Association between NR2B subunit gene (*GRIN2B*) promoter polymorphisms and sporadic Alzheimer's disease in the North Chinese population

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ABSTRACT

N-methyl-D-aspartate (NMDA) receptor plays a crucial role in learning, memory and information processing of human brain. Its dysfunction is related to the pathogenesis of Alzheimer's disease (AD). We detected four polymorphisms of the promoter regions of the human NMDA receptor 2B (NR2B) subunit gene (*GRIN2B*) in 362 AD patients and 334 healthy in North Han Chinese populations, these were −200T/G (rs1019385), −421C/A (rs3764028), −1447T/C (ENS10557853), and −1497G/A (rs12368476). Genetic analysis confirmed that there were significant differences in genotype ($P = 0.029$) and allele ($P = 0.010$) frequencies for −421C/A between SAD and control. In the subjects without *APOE ε4* allele, these difference remained significant (genotype $P = 0.012$, allele $P = 0.004$). The −421CC genotype was about 1.5 fold increasing risk compared with CA + AA genotypes (OR = 1.517, 95% CI 1.077–2.137, $P = 0.017$). Luciferase reporter assay showed a 34.69–39.79% decrease in transcriptional activity for −421C allele of *GRIN2B* promoter compared with −421A in SH-SY5Y and HeLa cell lines. Our study suggests that the −421C allele may induce lower *GRIN2B* transcriptional activity and NR2B protein expression, thus it is associated with AD.

N-methyl-D-aspartate (NMDA) receptor is a subtype of ionotropic glutamate receptors in postsynaptic membrane that is involved in synaptic mechanisms of learning and memory, and mediates excitotoxic neuronal injury [5,39,24]. The receptor interacts with multiple intracellular proteins by way of different subunits (NR1, NR2A, NR2B, NR2C, NR2D, NR3A and NR3B). Previous studies have suggested that NMDA receptor played a crucial role in neuronal development, plasticity and survival [10,9,31]. Disfunctions of the receptor should induce many nervous system diseases, such as seizure disorder [30], Parkinson's disease [13], Huntington's disease [11] and AD [15]. Physiologically, the function of NMDA receptor as highly permeable Ca^{2+} channels by binding glutamate was pivotal for synaptic plasticity, and long-term potentiation (LTP), which was considered to be integral to learning and memory [40]. Thus, some researchers hypothesized that reduction of binding to NMDA receptor or level of the receptor might result in the neurodegeneration that characterizes AD [12]. Presently, many studies have confirmed that lower expression of NMDA receptor isoforms transcripts and proteins was found in susceptible regions of AD brain, in which NR2B subunit was downregulated significantly [14,3]. On the other hand, NMDA receptor has been described as being associated with several pathological circumstances involving excessive stimulation of glutamate receptors and increased intracellular calcium concentration, a process known as excitotoxicity [32]. The pathological effect has been thought to trigger neuronal death via activation of calcium-activated proteases such as calpains or by indirectly activating the apoptosis-related caspases [6].

The NR2B subunit is one important functional isoform of NMDA receptor, on which the glutamate-binding site is located [19]. Some structural properties and functional roles attributed to the NR2B subunit of the NMDA receptor are organized into pharmacological, electrophysiological, and behavioral sections [22]. NR2B subunit of the NMDA receptor in the prefrontal cortex is critically involved in both LTP and contextual memory [42]. Some studies have proposed that NR2B can enhance learning and memory in transgenic mice [7,36,4]. Therefore, the relationship between NR2B subunit gene (*GRIN2B*) variants and AD has attracted the attention of many the studies. In Caucasian AD patients, the distribution of the single-nucleotide polymorphism results was negative including the −200T/G in promoter, 2664A/G in exon 13 and 5072G/T in 3′UTR of *GRIN2B* compared with healthy controls [34], and in Chinese, 2664A/G polymorphism was also not associated with AD [38]. In the present study, we intent to screen the proximal promoter of *GRIN2B* systematically in the North Chinese population,

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Table 1
PCR primer and enzyme for RFLP.

Locus	Primer sequence	PCR product size (bp)	Allele (size in bp)	Mismatched or mutated	Enzyme
Promoter	Forward: (GGGGTACC)AACTGCAAACCTGCCCGATA Reverse: (CTAGCTAG)CACCCTTGACGAGCACTCTC	1798	–	–	Kpn I Nhe I
−200T/G (rs1019385)	Forward: CTGGGAGCAGAAGCAGTATC Reverse: ACACACACAGACACACAGGCAC	98	T (98), G (76, 22)	CA → GG	BshN I
−421C/A (rs3764028)	Forward: CGCTCTCCGTCGGTGCTGTT Reverse: CTGGGGAAGTGGGGTGGTAACG	115	C (115), A (96, 19)	G → C	Tai I
−1447T/C (ENS10557853)	Forward: CTCTTAGAAAAATAGAAAACCAGTA Reverse: ACTTTAGTCTGTAGAAGCCATCA	229	T (205, 24), C (229)	T → G	Sca I
−1497G/A (rs12368476)	Forward: ACCCTGGAGACAAATTAT Reverse: AGAACTGATCTTTCACTACCT	110	G (110), A (87, 23)	T → C	Eco130 I
Site-directed mutagenesis (−421C/A)	Forward: TTCCGTCCCCCTTCCATCC**A**CCTTACCACCCCACTTCC Reverse: GGAAGTGGGGTGGTAAGG**T**GGATGGAAGGGGGACGG	>6000	–	C → A G → T	–

Mismatched sites were underlined; mutagenesis sites were in bold; added restriction sites were in brackets.

detecting possible variants, and then determining whether these variants were associated with sporadic AD (SAD) genetically and functionally.

The case group was consisted of 362 unrelated SAD patients (165 men and 197 women; mean age of 70.4 ± 6.8 years; mean age at onset of 62.3 ± 5.5 years) from the Xuan Wu Hospital of Capital Medical University, the Beijing Senile Hospital and several other hospitals in Beijing City between 2000 and 2007. Probable AD patients were diagnosed clinically according to the criteria of National Institute of Neurological and Communicative Disorders and Stroke, and the Alzheimer's Disease and Related Disorders Association (NINCDS-ADRDA) [25]. None of these patients reported a family history of AD. The control group was consisted of total 334 subjects (142 men and 192 women; mean age of 68.4 ± 8.1 years), who were sourced from the outpatient department of Xuan Wu Hospital of Capital Medical University, underwent regular health examinations, and were confirmed healthy and neurologically normal by Mini-Mental State Examination (MMSE), Hachinski Ischaemic Score and general examinations. All subjects were representative of the North Han Chinese populations. Informed consent was obtained for the participants, and this study was approved by the Ethics Committee.

PCR primer was designed *GRIN2B* proximal promoter fragment, from −1621 to +177 relative to the translation start site (TSS) (Table 1). The TSS of human *GRIN2B* has been determined in previous study [26]. Systematic screening of *GRIN2B* promoter was performed using standard PCR and direct sequencing in 25 randomly selected controls and 25 SAD patients. The products of PCR were sequenced by the Gene Center of North China. The primers (Table 1) were designed by Primer Premier 5 according to the polymorphisms in *GRIN2B* promoter. All variants were genotyped by PCR-restriction fragment length polymorphism (PCR-RFLP) using enzymatic digestion. Fragments were separated by polyacrylamide gel electrophoresis (PAGE) on 20% gel. According to the methods described previously [37], all subjects were genotyped for apolipoprotein E (*APOE*).

The 1798 bp promoter fragments (−1621 to +177 relative to the TSS) were PCR-amplified using the promoter primers (Table 1) which included Kpn I and Nhe I restriction sites. The genomic DNAs with identified genotype were used as templates that were homozygote of each allele according to wild-type (WT) −200T/−421C/−1447T/−1497G (named TCTG) haplotype. The PCR products with TCTG haplotype were cloned into pGL3-Basic vector (Promega) upstream of the firefly luciferase gene. The mutant-type (MT) TATG haplotype was generated by using a QuickChange® Site-Directed Mutagenesis Kit (StrGATAen). The site-directed mutagenesis primer (Table 1) was designed based on pGL-TCTG (pLG-WT) plasmid.

Human neuroblastoma (SH-SY5Y) cells (Cell Center of Institute of Chinese Academy of Medical Sciences, Beijing, China) and human epithelial carcinoma (HeLa) cells (Cell bank of Shanghai Institute of Cell Biology, Chinese Academy of Sciences, Shanghai, China) were propagated in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen, CA, USA) with 10% fetal bovine serum (Invitrogen), 10 mmol/L 2-[4-(2-Hydroxyethyl)-1-piperazinyl]ethanesulfonic acid (HEPES) (Sigma, Louis, MO, USA), 200 IU/ml penicillin and 200 μg/ml streptomycin (Invitrogen). For transient transfection, SH-SY5Y and HeLa cells were seeded in 96-well culture dishes at 2×10^4 and 1×10^4 cells/well, respectively, and allowed to recover for 24 h. A pRL-TK expression plasmid (Promega, Madison, USA) containing the herps simplex virus thymidine kinase promoter upstream of the renilla luciferase gene (Promega) was used as internal standard. Then, cells were co-transfected with 4 ng of pRL-TK plasmid and 200 ng of either one of the *GRIN2B* promoter constructs or one of the control plasmids, using Lipofectamine 2000 (Invitrogen) as described in the manufacturer;s protocol. Empty pGL3-Basic vector was used as a negative control, and pGL3-Control (Promega) containing the SV40 promoter and enhancer sequence was applied as a positive control. Each construct was transfected in at least three different experiments, in six wells. Transfected cells were cultured for 48 h, washed twice with 200 μl of phosphate-buffered saline (PBS) (Sigma), and lysed with passive lysis buffer (Promega). Firefly luciferase activities (LAF) and renilla luciferase activities (LAR) were measured sequentially using a Dual-Luciferase reporter assay system (Promega) and a model GloMax™ 96 Microplate Luminometer (Promega). To correct for transfectional efficiency, DNA uptake and expression efficiency, the relative luciferase activity (RLA) was calculated as: RLA = LAF/LAR.

In genetical study, Hardy–Weinberg equilibrium was tested for each group by SHEsis (http://analysis.bio-x.cn/myAnalysis.php) [35]. The Chi-square test was performed allele and genotype distribution in patients and controls in SPSS 13.0. Linkage disequilibrium was also analyzed in SHEsis [35]. The normalized linkage disequilibrium coefficient D' and the squared correlation coefficient r^2 were also calculated. Haplotype frequencies were estimated and differences in distribution were evaluated by maximum likelihood estimation based on expectation maximization (EM) algorithm on same website. We calculated all alleles or genotypes per Odds Ratio (OR) with 95% confidence intervals. Logistic regression was used to adjust sex, age, and *APOE*. In functional study, the differences of TCTG and TATG haplotypes RLA were determined by *t*-test using SPSS 13.0.

The proximal promoter of *GRIN2B* had four polymorphisms detected by sequencing, which were −200T/G (rs1019385), −421C/A (rs3764028), −1447T/C (ENS10557853), and −1497G/A