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PDF issue: 2026-11-08

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(Citation)
The Kobe journal of the medical sciences,53(6):327-333

(Issue Date)
2007-12

(Resource Type)
departmental bulletin paper

(Version)
Version of Record

(JaLCDOI)
https://doi.org/10.24546/81000132

(URL)
https://hdl.handle.net/20.500.14094/81000132

KOBE UNIVERSITY

Kobe J. Med. Sci., Vol. 53, No. 6, pp. 327-333, 2007

Lack of Association between the Dopamine Transporter Gene 3'VNTR Polymorphism and Attention Deficit Hyperactivity Disorder in Chinese Han Children: Case-control and Family-based Studies

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Received 22 January 2007 /Accepted 1 May 2007

Key words: attention deficit hyperactivity disorder (ADHD), dopamine transporter (DAT1), haplotype-based haplotype relative risk (HHRR) analysis, polymorphism, transmission disequilibrium test (TDT).

Attention deficit hyperactivity disorder (ADHD) is the most common childhood onset neurodevelopment disorder. The etiology is unclear, but is suspected to involve the dopamine system. In this study we used the haplotype-based haplotype relative risk (HHRR) analysis and the transmission disequilibrium test (TDT) to investigate the potential contribution of dopamine transporter (DAT1) gene variants to ADHD. DAT1 gene polymorphisms were assessed in 54 ADHD Chinese Han children and all 108 of their parents and 66 normal child controls. No differences were found in either genotype or allele distributions. The HHRR analysis of the DAT1 polymorphisms suggests that the transmission of this polymorphism is not significantly associated with ADHD. And the TDT result showed that ADHD was not in linkage with the DAT1 gene. These findings do not support the hypothesis that DAT1 gene variants contribute to the pathogenesis of ADHD in Chinese Han population.

Attention deficit hyperactivity disorder (ADHD) is one of the most common, neurodevelopment disorders with childhood onset. Children with ADHD have impairment in their daily routine, family and peer relationships, and academic performance [1-3], so they often have a severe effect on their families and society. The etiology of ADHD is unknown. Therefore understanding the etiology and pathogenesis of ADHD is a key and important challenge in psychiatry. A number of recent studies, including family, twin and adoption studies, have provided various lines of evidences that genetic factors play a substantial role in the etiology of ADHD [4-6].

Methylphenidate is the most frequently prescribed medicine in the treatment of ADHD [7-9] because of its ability to improve ADHD core symptoms as well as associated features including aggression, social interaction, and academic productivity. Methylphenidate is assumed to increase the concentration of dopamine in the synaptic cleft by blocking the dopamine transporter (DAT1)[10], a solute carrier protein that is responsible for the reuptake of dopamine into the presynaptic neuron [11]. Recently, animal studies with DAT1 gene

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knock-out mice indicated that DAT1 may be involved in the development of ADHD [12,13]. These mice showed hyperactive behavior in the open field, and had difficulty in stopping ongoing behaviors and in learning and memory tasks. In addition, these ADHD-like-behaviors were reduced by treatment of methylphenidate.

Over the past several years, there has been increasing interest in the potential involvement of DAT1 in ADHD. DAT1 is the second gene suspected to be related to ADHD in the field of molecular biology [14]. In 1995, as the pioneers, Cook and colleagues [15] first reported an association between ADHD and DAT1. They found that the 3'untranslated region (3'UTR) of DAT1 has a variable number of tandem repeat (VNTR) polymorphism and that ADHD was associated with a 10-repeat allele (480bp PCR fragment) of the VNTR. Subsequently, several research groups looked for an association between the DAT1 gene and ADHD [16-19]. Some of them used family-based association study method, some used case-control study method, and some used both methods, but the findings were inconsistent.

In China, two studies examined the association of DAT1 with ADHD in Chinese Han children and their findings were also contradictory. One of these studies [20] was conducted in Hong Kong and reported no relationship between DAT1 and ADHD. The other study [21], conducted in Beijing, found that long repeat alleles were associated with the disorder in a case-control analysis, but not family-based analysis, and stated that their positive finding should be interpreted cautiously.

As China has the largest population in the world, thus, further studies are needed to determine if there is a relationship between ADHD and DAT1. Specially, there is a need to replicate the molecular genetic linkage disequilibrium with ADHD in different area of China to establish a role of DAT1 in the etiology of ADHD. The main aim of the present study was to explore the possibility that DAT1 polymorphisms are in linkage disequilibrium with ADHD in Chinese children in Xi'an using a family-based association and case-control study designs.

MATERIALS AND METHODS

Subjects

The present study was approved by the Ethics Committee of Xi'an Jiaotong University. Written informed consent was obtained from the patients' parents. The current study included 54 children with ADHD (9.4 ± 2.2 years), consisting of 42 boys and 12 girls, aged 6-16 years, and their biological parents, who visited the outpatient clinic of Child Development and Behavior Institute of the Second Affiliated Hospital, Xi'an Jiaotong University in Xi'an, PRC. Children were diagnosed as ADHD if they met DSM-IV criteria [1] for ADHD: six symptoms of inattention and/or hyperactivity-impulsivity either in the home or school setting determined by clinical interview, evidence of pervasiveness defined as a minimum of four symptoms in the non-criterion setting, and onset of symptoms before 7 years of age. Thirty-seven (68.5%) children met the criteria for the ADHD combined type (ADHD-C), 5 (9.3%) met the criteria for the ADHD hyperactive-impulsive type (ADHD-HI), and 12 (22.2%) met the criteria for the ADHD inattentive type (ADHD-I).

In addition to a family-based design, we included a control group to allow for a case-control design. The control group included 66 healthy individuals (8.6 ± 2.2 years), consisting of 46 boys and 20 girls, aged 6-14 years, who visited the hospital for health examinations, and were confirmed to have no evidence of medical or psychiatric illness.

All subjects involved in this study were Han Chinese. Interviews were conducted by at least two child psychiatrists (two of whom were Drs.Y.P.Wang and Z.H.Wang) with the clinical description of the patients' parents and teachers. The intelligence quotient (IQ) of subjects was tested using the Chinese-Wechsler Intelligence Scale for Children (C-WISC),

which was standardized by Gong Yaoxian. Subjects were excluded if their IQ scored below 70, or showed evidence of neurological or chronic medical illness, bipolar affective disorder, psychotic symptoms, Tourette syndrome, or chronic physical disability. Children were free of medication for a minimum of 24hr before their assessment.

DAT1 genotyping

Peripheral blood was drawn from the ante cubical vein, and genomic DNA was extracted from whole blood using a standard DNA isolation procedure from 54 ADHD cases and 108 their biological parents, in addition to 66 controls. DNA was resuspended in 20 µl of water and stored at 4°C (concentration range 25-100 ng L^{-1}).

The primer sequence used to amplify the 40-bp sequence of the VNTR polymorphic loci was as follows [15,18,22]: PCR amplification of the DAT1 40-bp VNTR in the 3'-UTR was carried out using the following pair of primers, upstream: 5'-TGT GGT GTA GGG AAC GGC CTG AG-3' and downstream: 5'-CTT CCT GGA GGT CAC GGC TCA AGG-3'. Forty cycles were conducted consisting of denaturation at 95°C for 30 sec, annealing at 68°C for 30 sec, and extension at 72°C for 90 sec. An initial denaturing step at 95°C for 5 min and a last extension step at 72°C for 7 min were also added. The reactions were 25 µl that consisted of 100 ng genomic DNA, 10 pmol of each primer, 20 mM dNTPs, 2 U of Taq polymerase, 10×Taq buffer, and distilled water. 2 µl of PCR products were run on 2% agarose gel. A 50-bp DNA ladder was used to identify the various repeat alleles by size: 7-repeat (360bp), 8-repeat (400bp), 9-repeat (440bp), 10-repeat (480bp), and 11-repeat (520bp).

Statistical analysis

The allele frequencies were estimated by counting, and the Hardy-Weinberg equilibrium was calculated based on these allele frequencies, using the Chi-square test. The association of DAT1 with ADHD was tested by two methods; in the case-control association analysis, allele and genotype frequencies in different groups of subjects were compared using the Chi-square test. For the family-based analysis, we used the transmission disequilibrium test (TDT)[23,24], and the haplotype-based haplotype relative risk (HHRR)[15] method to avoid any potential population stratification. All statistical tests were carried out using SPSS for Windows (release 11.0).

RESULTS

For this study, 54 ADHD cases and all 108 of their parents as well as 66 controls were successfully genotyped. Among all subjects (ADHD cases, parents and controls), five DAT1 alleles were identified: 7-repeat, 8-repeat (only in parents group), 9-repeat, 10-repeat and 11-repeat alleles. The genotype distribution of DAT1 gene was in Hardy-Weinberg equilibrium for each of the groups. Tables 1 and 2 show allele and genotypes frequencies, respectively, of the DAT1 40-bp VNTR for both ADHD and control subjects. There were no significant differences between the ADHD cases and the controls (Table1: χ^2=0.588, df =3, p=0.899; Table 2: χ^2=0.635, df =3, p=0.888).

Table 1. Allele frequencies for DAT1 in ADHD and control group (100%)

	7-Repeat	9-Repeat	10-Repeat	11-Repeat	Total
ADHD	2 (1.9)	5 (4.6)	100 (92.6)	1 (0.9)	108 (100)
Controls	4 (3.0)	7 (5.3)	119 (90.2)	2 (1.5)	132 (100)
P value	NS	NS	NS	NS	

NS: no significance

Table 2. Genotype frequencies for DAT1 in ADHD and control groups

	10/7	10/9	10/10	11/10	Total
ADHD	2 (3.7)	5 (9.3)	46 (85.2)	1 (1.9)	54 (100)
Controls	4 (6.1)	7 (10.6)	53 (80.3)	2 (3.0)	66 (100)
P value	NS	NS	NS	NS	

NS: no significance

Table 3. HHRR analysis of DAT1 polymorphism in nuclear families with transmitted cases

Allele	10-Repeat	No 10-Repeat	Total
Transmitted	100(92.6)	8(7.4)	108(100)
No-transmitted	94(87.0)	14(13.4)	108(100)
Total	194(89.2)	22(11.2)	216(100)

χ^2=1.82, df=1, p=0.177

The VNTR variants were divided into 10-repeat allele and no 10-repeat alleles (7-, 9-, and 11-repeats) for statistical analyses, since the sample size was small and the 10-repeat allele is the most common suspected allele in some studies. We used the Haplotype-based haplotype relative risk (HHRR) design to evaluate the association between ADHD and transmission of genetic polymorphisms. This robust statistical method uses the non-transmitted parental alleles as 'controls' for evaluating transmission of alleles and thus prevents spurious associations due to population stratification. The HHRR analysis of DAT1 polymorphism (Table 3.) suggests that the transmission of 10-repeat allele polymorphism of the DAT1 gene is not significantly associated with ADHD in the studied cases (χ^2 =1.82, df =1, p>0.05).

The transmission/disequilibrium test (TDT) is a useful method to detect linkage disequilibrium between a disease and genetic markers in nuclear families consisting of parents and one or more affected offsprings. In our study, the multiple-allele TDT test was employed for the analysis of linkage between ADHD and DAT1. All homozygotes were ignored and only information from heterozygous parents was used for calculation. Twenty parents were informative, thirteen 10-repeat (480bp) alleles were transmitted to children, and seven were not. The calculated TDT chi-square was 1.8 (df=1, p>0.05). The result did not support that ADHD was in linkage disequilibrium with any polymorphism of the DAT1 40-bp VNTR locus.

DISCUSSION

The human dopaminergic system figures prominently in the fields of neurology, psychiatry, and pharmacology.

The dopamine transporter (DAT1) gene maps to the chromosome 5 (5p15.3) and includes a variable number of tandem repeat (VNTR) polymorphisms of 40 bp in the 3'-non-coding region [25]. DAT1 plays an important role in controlling blood levels of dopamine [11,26]. It is not clear how the polymorphisms of VNTR could affect DAT1 function in the human brain. Because the VNTR locates in the 3'-non-coding region, allelic variants cannot result in

structural differences in human DAT protein. VNTR may function as transcriptional regulators [27]. In this study, we investigated the relationship of ADHD and the DAT1 gene polymorphism through family-based and case-control association studies, focusing on variation in the 3'untranslated region. Consistent with previous findings, we didn't find an association for the Chinese Han samples. This was true for both case-control and family-based analyses.

Cook et al. [15] originally reported that the 10-repeat allele of VNTR was associated with ADHD. Attempts to replicate these results were made in different populations [22,28,29]. However, in Asia, two Korean and two Chinese studies denied the association, especially using TDT [16,18,20,21]. The diverse ethnic backgrounds of individuals in the population and the clinical heterogeneity of ADHD itself may account for non-replications of earlier findings to some extent. The 10 repeat allele of the DAT1 gene polymorphism is significantly higher in populations in Mongolian, Japanese and Chinese than in Europeans [30,31]. In the present study, the high frequency of the 10-repeat allele of DAT1 and extremely low frequency of the 7-repeat allele of DAT1 are consistent with previous findings in the Chinese population [30,31], which supports the conclusion that there was no significant sampling bias in this study population.

The cause of ADHD is thought to involve a complex mix of multiple genes and multiple specific environmental risks [32,33]. The past decade of research has revealed just how difficult it is to find genes that contribute to variance in complex traits.

Based upon these data, there are some limitations to our study. Our sample size is not large enough to detect a weak association; large numbers of subjects in different races of Chinese from different areas should be further studied. Furthermore, though we analyzed only one gene in our research, to the best of our knowledge, for complex disorders, gene-gene interactions and gene-environment interactions should be considered in future studies in same sample at once time. Analyses of different genes involved in the etiology of ADHD enhance our understanding on the genetic contributions to the etiology of ADHD, but not understanding on the role of DAT1 itself. To enhance our understanding on the role of DAT1, we need to perform more biological studies on the effects of different polymorphisms on the gene and protein functions, and to study other genetic and environmental modifiers of the DAT1 gene. Apart from sample size, clinical homogeneity of subjects is also crucial for genetic association study of complex disease. This should be addressed. Further studies should employ a more homogenous sample, e.g., same diagnostic subtype or same phenotype as defined by psychometric tests.

In summary, our results do not support the possibility that DAT1 gene 3'VNTR polymorphism contributes to ADHD, although further studies are needed to confirm this in different populations in different environments.

ACKNOWLEDGMENTS

We thank the patients and family members who participated in this study, as well as the teachers. We also extend our appreciation to Dr. Jianghua Lai and Ms. Junhai Zhao for their technical advices. This research was supported by Shaanxi Province Nature Science Foundation (No. 99sm57) and the Public Health Bureau of Shaanxi Province (No. 9931).

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