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No Evidence for Association Between Norepinephrine Transporter-3081 (A/T) Polymorphism and Attention Deficit Hyperactivity Disorder in Iranian Population

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Background: Attention Deficit Hyperactivity Disorder (ADHD) can lead to drastic problems for the patient and its worldwide prevalence is 5%-12%. It also has many comorbidities with other disorders, and the genetic contribution seems the most significant cause.
Objectives: The current study was conducted to investigate the association between norepinephrine transporter-3081(A/T) polymorphisms and ADHD in Iranian population.
Patients and Methods: Participants were chosen from children and adolescents diagnosed with ADHD referred to Imam Hoseyn Hospital. A child and adolescent psychiatrist confirmed the diagnosis using the Kiddie-Sads-Present and Lifetime Version (K-SADS-PL) semi-structural interview. The control group was from pupils of schools in Tehran (capital city of Iran) who had no history or presence of psychiatric and medical complications. Also, a child and adolescent psychiatrist confirmed their health using the K-SADS-PL semi-structural interview. Genetic examinations were DNA distraction, Polymerase Chain Reaction (PCR), and Restricted Fragment Length Polymorphism (RFLP), which were conducted according to standard protocols. The statistical analysis was performed using chi-square and Fisher's exact test in SPSS version 21.
Results: The percentages of ADHD subtypes for combined, inattentive, and hyperactive/impulsive were 72.2%, 17.2%, and 11.9%, respectively. There was no significant association between norepinephrine transporter polymorphism and ADHD (P = 0.81). Moreover, no significant relationship was found between gender [male (P = 0.92) and female (P = 0.63)] and polymorphism. No significant association was found between subtypes of ADHD [combined (P = 0.46), inattentive (P = 0.41), hyperactive/impulsive (P = 0.32)] and polymorphism SCL6A2. This lack of association can also be seen in gender in every subtype.
Conclusions: The results of the study show no significant association between norepinephrine transporter polymorphism SCL6A2 and ADHD.

Keywords: SCL6A2 Protein; Humans; Attention Deficit Disorder with Hyperactivity; Polymorphism, Genetic; Iran

1. Background

Attention Deficit Hyperactivity Disorder (ADHD), is a syndrome of inattention, disruptive behavior, restless overactivity, impulsivity, and other deficit of executive function (1). Attention deficit hyperactivity disorder is one of the most common childhood onset psychiatric disorders, affecting 5%-12% of children worldwide (2). Since ADHD can cause significant impairment in functioning which interferes with normal development and all areas of functioning in patients of all ages, it became a costly public concern (3). Studies on twins and adopted samples with ADHD made genetics as an influential part of etiology, with heritability of 60% to 90% (4). It suggests a genetic predisposition and has been the incentive for comprehensive research on the molecular genetic basis of this sociologically relevant disorder (5).

Many studies investigating candidate genes encoding effectors of monoaminergic neurotransmission have been conducted (6-8). One promising candidate gene in ADHD is the Norepinephrine Transporter (NET), which is also called Solute Carrier Family 6, Member 2 (SCL6A2) (9).

Norepinephrine is suggested as one of the vital role in the neurotransmitters underlying ADHD pathophysiology (10). Moreover, atomoxetine, a specific norepinephrine reuptake inhibitor, a drug alleviating ADHD symptoms, binds predominantly to NET and blocks its transport function (11). Indeed, SCL6A2 is a noticeable

candidate gene for molecular genetic studies in ADHD. Several studies probed the association between genetic variants of SCL6A2 and ADHD with inconsistent results (12-17); a gene analysis of genome wide association data by Lasky-Su et al. pointed to SCL6A2 as one of the most promising candidate (18).

Recently, Kim et al. (19) indicated a -3081 A to T single nucleotide polymorphism in the promoter region of the SCL6A2 and its association with ADHD. This study was also identified -3081 (A/T) polymorphism as a functional one which can decrease promoter function, and increase the risk of ADHD. There has been no study preformed on Iranian population yet. As a consequence, in this study we focused on the functional relevant Single Nucleotide Polymorphism (SNP) in the promoter of SCL6A2 in Iranian population.

2. Objectives

This study aimed to investigate the association between NET-3081 (A/T) polymorphism and ADHD in Iranian population.

3. Patients and Methods

A case-control study was conducted to examine the association between the functional SNP rs28386840 located in promoter region of SCL6A2 and ADHD. This study was approved by ethics committee of Shahid Beheshti University of Medical Sciences, Tehran, Iran

3.1. Participants

There were 151 participants in the ADHD group and 151 participants in the control group with 26 female and 125 male in each group. In the ADHD group, participants were recruited from outpatient's individuals seeking treatment at the Child and Adolescent Psychiatric Clinic of Imam Hosein Hospital in Tehran, Iran.

Patients with the age range of 7-12 years old (Mean = 9.2 ± 2.1) and a primary diagnosis of ADHD according to the DSM-IV-TR criteria were included in the study. From a total of 225 patients entered to the study, 151 patients (26 females and 125 males) were selected using the semi-structured Interview Schedule for Affective Disorders and Schizophrenia for School-Age Children-Present and Lifetime Version (K-SADS-PL). The male/female ratio was due to studies in which they determined the gender proportion of ADHD patients in large populations (20); so, our participants can be a representative of ADHD population. Exclusion criteria were bipolar disorder, mental retardation and Tourettes syndrome. The participants' blood samples were taken after obtaining a written informed consent from both parent and children.

In the control group, participants were recruited from elementary school students matched in terms of gender, racing and socio-economic factors with the ADHD group. Mean age for the control group was 2 years higher than the ADHD group for decreasing the risk of latent ADHD. Health history files of 280 males/110 females were reviewed that 220 males/65 females were selected and assessed using the K-SADS-PL semi-structured interview, medical examination and parental interview about family history of psychiatric and medical disorders. Finally, after obtaining the informed consent from both parents and children, 125 males/26 females were enrolled for blood sampling; be completely healthy without any history of medical and psychiatric disorder up to 3 generation; and be 7-12 years old (Mean = 11.1 ± 2.3). To determine the sample size in this research, the following formula was used (21):

$$n = \frac{p(1-p)\times(1.96)^2}{d^2} \quad (1)$$

Since allele prevalence is unknown in population, the p number is 0.5, d is 0.08 and confidential interval is 95%. According to this formula, the minimum sample size should be 150 individuals.

3.2. Genotyping

Genomic DNA that was extracted from whole-blood sample following a standard SNP rs28386840 (A/T) was genotyped through the Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP) method. Polymerase chain reaction primers were as follows: forward: 5'AGGACAGGCTCATCCCTCTT3', and reverse: 5'GAGATAATCCTGGAAGCAATCG3', (annealing at 56°C; fragment size, 268 bp); and the restriction enzyme BsrI was used to digest the PCR products. Digestion was electrophoresed on 3% agarose gels to visualize the band of 268 bp (allele T) and 268 bp + 164 bp + 104 bp (allele A). The accuracy of the genotyping method was confirmed by sequencing the PCR fragments.

3.3. Statistics

Data were analyzed using the openEpi software (Tehran, Iran) . Chi-square and Fisher's exact test were used to compare the frequency of genotype and ADHD and ADHD subtypes (combined, inattentive, and hyperactive) in the ADHD/control groups.

4. Results

A case-control study was conducted to examine the association between the functional SNP rs28386840 located in a promoter region of SCL6A2 and ADHD. There was no significant difference in genotype distribution (P = 0.81, $\chi^2 = 0.406$).

The mean age of the ADHD group was 9.2 ± 2.1 years; the mean age of the control group was 11.1 ± 2.3 years, both consisting of 125 males (82.8%) and 26 females (17.2%). Groups were matched in accordance with gender, race and socio-economic factors (Table 1).

ADHD individuals were classified into 3 subtypes of combined, inattentive, and hyperactive/impulsive according to the DSM-IV-TR (1) criteria, the percentages of three subtypes of combined, inattentive, and hyperactive/impulsive were 72.2%, 17.2% and 11.9%, respectively. Also, the percentages of comorbidities were 34.4% for Learning Disorder (LD), 23.8% for enuresis, 31.1% for anxiety, 13.2% for Obsessive-Compulsive Disorder (OCD), and 41.1% for disruptive behavioral disorder (Table 2).

Regarding the comparison between the ADHD and control groups (P = 0.81), there was no significant association between NET polymorphism and diagnosis of ADHD, Also, there was no relationship between gender and NET polymorphism in the two groups [male (P = 0.92), female (P = 0.63)] (Table 3).

Indeed, no significant association was found between subtypes of ADHD [combined (P = 0.46), inattentive (P = 0.41), hyperactive/impulsive (P = 0.32)] and control group in polymorphism SCL6A2.This lack of association can also be seen in gender in every subtype [combined: male (P = 0.23), female (P = 0.26)], [inattentive: male (P = 0.22), female (P = 0.63)], and [hyperactive/impulsive: male (P = 0.39), female (P = 0.22)] (Table 4).

Table 1. Discrepancy of Gender and Age in the Attention Deficit Hyperactivity Disorder and Control Groups [a]

	Male [b]	Female [b]	Total [b]	Mean Age (SD)
ADHD	125 (82.8)	26 (17.2)	151 (100)	9.2 ± 2.1
Control	125 (82.8)	26 (17.2)	151 (100)	11.1 ± 2.3

[a] Abbreviations: ADHD, attention deficit hyperactivity disorder.
[b] Values are presented as No (%).

Table 2. Clinical Characteristics of the Attention Deficit Hyperactivity Disorder Group [a,b]

Variables	Values
ADHD subtypes	
Combined	109 (72.2)
Inattentive	26 (17.2)
Hyperactive/impulsive	16 (10.5)
Comorbidities	
LD	52 (34.4)
Enuresis	36 (23.8)
Anxiety disorder	47 (31.1)
OCD	20 (13.2)
Disruptive behavior disorder	62 (41.1)

[a] Abbreviations: ADHD, attention deficit hyperactivity disorder; LD, learning disability; OCD, obsessive-compulsive disorder.
[b] Data are presented as No. (%).

Table 3. Comparison of Alleles Between Attention Deficit Hyperactivity Disorder and Control Group [a]

Variables	A/A [b]	A/T [b]	T/T [b]	P Value [c]
ADHD				
Male	51 (33.8)	64 (42.4)	10 (6.6)	0.92
Female	10 (6.6)	10 (6.6)	6 (3.3)	0.63
Total	61 (40.4)	74 (49)	16 (9.9)	0.81
Control				
Male	40 (26.3)	72 (47.6)	13 (8.6)	
Female	15 (9.9)	9 (5.9)	2 (1.3)	
Total	55 (36.2)	81 (53.5)	15 (9.9)	

[a] Abbreviation: ADHD, attention deficit hyperactivity disorder.
[b] Values are presented as No (%).
[c] P ≤ 0.05 is significant.

Table 4. Gender-Based Frequency and Comparison of Allele in Attention Deficit Hyperactivity Disorder Subgroups and Control [a]

Variables	A/A	A/T	T/T	P Value [b]
Combined				
Male	38 (34.8)	45 (41.3)	10 (9.2)	0.23
Female	2 (2)	5 (5)	3 (3)	0.26
Total	40 (36.8)	50 (46.3)	13 (12.2)	0.46
Inattentive				
Male	6 (23)	12 (46)	0 (0)	0.22
Female	2 (8)	4 (16)	2 (8)	0.63
Total	8 (31)	16 (62)	2 (8)	0.41
Hyperactive/Impulsive				
Male	7 (43.7)	7 (43.7)	0 (0)	0.39
Female	0 (0)	2 (12.5)	0 (0)	0.22
Total	7 (43.7)	9 (52.5)	0 (0)	0.32

[a] Values are presented as No (%).
[b] P ≤ 0.05 is significant.

5. Discussion

Attention deficit hyperactivity disorder is a heritable neurodevelopmental disorder that can lead to drastic problems for the patients and their family (2). It also has many comorbidities with other disorders such as disruptive behavioral disorder (22), conduct disorder (23), learning disorder (24), and anxiety and mood disorders (1). Its male/female ratio is 4:1 (1).

Although several factors have been mentioned in the etiology of ADHD, the genetic contribution seems the most significant one and finding the main cause of the disorder can improve the prevention, diagnosis, and treatment strategies (25). The purpose of this study was to examine any association between NET-3081 (A/T) polymorphism and ADHD in Iranian population.

The results of this study showed no significant association between NET polymorphism and ADHD. Moreover, we found no significant difference between gene and gender. We observed no association between there subtypes of ADHD (combined, inattentive, and hyperactive/impulsive) and gender. Thus, the result supports no significant difference for SCL6A2 and ADHD (subtypes and gender).

Our findings are in consistent with the findings of many of prior studies. For instance, in a meta-analysis review (26), no association was seen. Another study on Korean population by Xu et al. (17) demonstrated no evidence for association between these two factors. In the earlier studies in 2002 in Canada (27) and in Ireland (28), no association between NET polymorphism and ADHD had been found.

Moreover, there are some studies which indicated the association between NET polymorphism and ADHD. In Chinese population, Guan et al. (29), and Korean population Joung et al. (16) found an association between aforementioned factors. In 2008, Biederman et al. (30) conducted the same study in USA on gender differences, in which the factors appeared stronger in female than male.

According to Gizer et al. (26), it is beneficial to explore association between some particular genes with ADHD subtypes and gender of patients. Therefore, the current study also determined this association between subtypes and gender of Iranian ADHD population.

In conceptualization of polymorphism, a particular gene can represent various polymorphisms in different populations and polymorphism is one of the strains of every population type. There is no evidence for exception in Iranian population.

The item made the current study eminent is that this study is the first one conducted on Iranian population. Besides, subtypes of ADHD (combined, inattentive, and hyperactive/impulsive) and gender differences had been investigated and compared with the control group.

In summary, the current study is the first study conducted on Iranian population about the association between NET-3081 (A/T) polymorphism and ADHD, ADHD subtypes, and gender. The result of the study -no evidence of association- that is consistent with American (30), Canadian (27), Irish (28) and Korean (17) population, and inconsistent with Chinese (29) population.

The limitation of the current study was difficulties in finding pure ADHD without any comorbidity. Maybe for more precise investigation, it would be advantageous to find absolutely pure ADHD participants. Although we considered bipolar disorder and Tourette syndrome as exclusion criteria, in future studies the relationship of comorbidity of learning disorder and bipolar with ADHD and SCL6A2 can be explored. Increasing the number of participants in future studies can improve the validity of the results. Henceforward, comparing comorbidities with polymorphism might be inspiring. Finally, the most consequential follow-up of our study could be assessing the treatment outcomes of pharmacotherapy (stimulant, atomoxetine) with genotypes.

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Authors' Contributions

Study concept and design: Mohammad Reza Eslami Amirabadi. Study supervisor: Katayoon Razjouyan. Acquisition of data: Mohammad Reza Eslami Amirabadi, and Hossein Darvish. Analysis and interpretation of data: Mojgan Khademi. Drafting of the manuscript: Mohammad Reza Eslami Amirabadi. Critical revision of the manuscript for important intellectual content: Sepideh Rajezi Esfahani. Statistical analysis: Sepideh Rajezi Esfahani. Administrative, technical, and material support: Rozita Davari-Ashtiani. Study supervision: Hossein Darvish, and Abolfazl Movafagh.

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