Autism in Iraqi Children: Investigating the CNTNAP2 rs7794745 Polymorphism in the Middle Euphrates
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Abstract
Objectives: This study aims to determine if the CNTNAP2 gene polymorphism at the rs7794745 locus is associated with autism spectrum disorder (ASD) by comparing its frequency in autistic children and healthy controls.
Methods: The case-control study involved a total of 150 samples, comprising 80 individuals diagnosed with autism spectrum disorder (ASD) and 70 healthy children. These participants, both male and female, had an average age of 3.5 ± 3 years for autistic individuals and 4.2 ± 3 years for healthy children. Genomic analysis was performed by amplifying and scrutinizing DNA sequences through the utilization of polymerase chain reaction (PCR) and restriction enzymes. Specifically, the genotyping of the CNTNAP2 gene at the rs7794745 locus was accomplished using the PCR-RFLP method. To facilitate genotyping, the isolation of DNA from peripheral blood cells sourced from healthy children and patients.
Results: Our findings indicated a statistically non-significant low-frequency distribution (P-value > 0.05) of the rs7794745 SNP in ASD patients compared to healthy children.
Conclusion: The study’s findings propose that there is no correlation between the rs7794745 polymorphism and autism spectrum disorder (ASD).
Keywords: ASD, CNTNAP2, SNP rs7794745, gene polymorphism, PCR-RFLP, PCR, Iraqi children, autism

Introduction
Autism spectrum disorder (ASD) is a neurodevelopmental condition marked by social interaction difficulties and repetitive behaviors. ASD encompasses various disorders such as autistic disorder, Rett syndrome, Asperger syndrome, and pervasive developmental disorder. Individuals with ASD struggle with both verbal and nonverbal social communication and often face cognitive and physical challenges. They also exhibit unusual sensory responses, intense passions, and repetitive behaviors.1 Over the past two decades, the prevalence of autism has significantly increased, with an estimated global prevalence of 0.62%.2 Males are approximately four times more likely to be diagnosed with ASD than females, with a sex ratio of 4.2:1.3

Despite extensive research, no single cause of ASD has been identified. However, evidence suggests that a combination of genetic, environmental, and neurobiological factors may contribute to ASD by impacting brain development.4 The higher incidence of autism among siblings and the greater concordance rate in monzygotic twins compared to dizygotic twins support the notion that 80–90% of ASD may have a genetic basis.5 Several critical genes are involved in the formation and functioning of synapses. Genes such as CNTNAP2, NLGN3, and NLGN4X are implicated in neuron-glia adhesion processes.6 The CNTNAP2 gene, expressed in brain regions associated with ASD, has been studied for its potential role in the disorder. CNTNAP2, one of the largest mammalian genes, belongs to the neurexin superfamily, spans 2.3 megabases on chromosome 7q, and comprises 24 exons as shown in Figure 1.7

CNTNAP2 encodes contactin-associated protein-like 2 (Caspr2), which is highly expressed in the developing brain and spinal cord. This protein is crucial for language development and is linked to language impairments in ASD. Variants of the CNTNAP2 gene, both rare and common, are associated with ASDs, seizures, and intellectual disability.3 The SNPs rs7794745 and rs2710102, located in the 2nd and 13th introns of chromosome 7q respectively, are notable non-coding variants. The A/T variant in rs7794745 is particularly significant, as it affects the brain’s response to human voices and increases the risk of ASD in the Chinese Han population.4

This study aims to investigate the association between the rs7794745 polymorphism of the CNTNAP2 gene and the risk of autism in children from Iraq’s Middle Euphrates region.

Study Objective
This research aims to investigate the potential linkage between the single nucleotide polymorphism (SNP) rs7794745 within the CNTNAP2 gene and the susceptibility to autism among children residing in the Middle Euphrates region of Iraq.

Method
Patients, Materials, and Methods
This study included one hundred and fifty children, comprising eighty children with autism (both males and females, mean age 3.5 ± 3 years) and seventy healthy individuals (control group, mean age 4.2 ± 3 years) recruited from visitors to
Al-Sadr Teaching Hospital. The diagnosis of autism was made by well-trained psychiatrists using the Diagnostic and Statistical Manual of Mental Disorders, 4th edition (DSM-IV) criteria, based on available historical information from interviews and clinical records. All participants and their families were informed about this research and gave their consent to participate.

The Ethical Committee Approval
Approval from the Ethical Committee (in the Faculty of Pharmacy /Kufa University) was taken for the protocol of the study.

DNA Extraction and Genotyping
Blood samples were acquired from both children diagnosed with autism spectrum disorder (ASD) and unaffected children. Specifically, a single nucleotide polymorphism (SNP) located within the CNTNAP2 gene, denoted as rs7794745. Subsequently, the genomic DNA present in these samples was isolated utilizing the AddPrep Genomic DNA Extraction Kit. The genotyping process of the SNP of interest was carried out employing the polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) technique, adhering to the instructions provided by the manufacturer.

DNA Amplification and Genotyping
A fragment of 315 bp that contains the desired SNP was amplified by the PCR-utilized special primers shown in Table 1.

The reaction required to amplify the DNA fragments containing the targeted SNP was adjusted using:

1. Primer volume (8 µl F, 8 µl R)
2. DNA volume (8 µl)
3. Gradual temperature increment for annealing (56°C)

The reaction volume as shown in Table 2 was placed in a 0.5 ml tube of PCR (at room temperature), then followed by 2000 xg centrifugation for fifty seconds, after that the product was transmitted to the thermo-cycler.

IBM SPSS
Statistical analysis was conducted using IBM SPSS version 26.0 software (SPSS Inc., Chicago, IL). The mean levels of each characteristic by genotype were compared using the student t-test for continuous variables and analysis of variance (ANOVA) for comparison among multiple groups. The chi-square test was utilized to examine categorical data, including alleles and genotypes. A p-value less than 0.05 was considered statistically significant.

Results
Throughout our investigation, a total of one hundred and fifty participants were examined, with the control group comprising seventy healthy children and eighty children diagnosed with autism. The outcomes obtained from the MluI enzyme analysis revealed distinctive patterns in the PCR products of both patients and control individuals. Specifically, individuals lacking the A→T mutation in the CNTNAP2 gene (genotype AA) exhibited a PCR product fragment size of 315 base pairs. Conversely, those with the heterozygous genotype (AT) manifested three distinct bands in their PCR product, spanning 95, 315, and 220 base pairs. Furthermore, individuals, regardless of health status, harboring the homozygous genotype (TT) and possessing the A→T mutation, displayed two fragments, measuring 95 and 220 base pairs, respectively (refer to Figure 2 for a visual representation).

All pertinent data regarding genotype and allele frequencies, along with their associated odds ratios (95% confidence intervals), are comprehensively documented in Table 3. This table presents a detailed overview of the genotype distributions of the rs7794745 A→T polymorphism within the CNTNAP2 gene, contrasting cases of autism with control subjects. Notably, no significant association was discerned from these genotype distributions (95% odds ratio, confidence interval). Furthermore, the frequencies of the A and T alleles among cases were determined to be 108 % and 52%, respectively, while in the healthy group, they were 88% and 52%, respectively (p-value = 0.9).

Table 1. Sequence of the primers used to amplify the DNA fragments containing the targeted SNP

<table>
<thead>
<tr>
<th>Target gene</th>
<th>Sequence (5’-3’)</th>
<th>Ta (°C)</th>
<th>Product size</th>
<th>Accession number</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>CNTNAP2</td>
<td>F AATACGGACCAAGATACCAAC</td>
<td>54</td>
<td>315 bp</td>
<td>NG_007092.3</td>
<td>Zare et al., 2017</td>
</tr>
<tr>
<td></td>
<td>R TTCAGACCAACAGTCCTT</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 2. PCR solution preparation

<table>
<thead>
<tr>
<th>Components</th>
<th>Concentration</th>
<th>Volume (100 µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GoTaq® G2 Green Master Mix</td>
<td>2X</td>
<td>50 µl</td>
</tr>
<tr>
<td>Forward primer</td>
<td>20 µM/µl</td>
<td>8 µl</td>
</tr>
<tr>
<td>Reverse primer</td>
<td>20 µM/µl</td>
<td>8 µl</td>
</tr>
<tr>
<td>ddH2O</td>
<td>–</td>
<td>26 µl</td>
</tr>
<tr>
<td>DNA</td>
<td>80 ng</td>
<td>8 µl</td>
</tr>
</tbody>
</table>

Fig. 1 CNTNAP2 gene in genomic location (https://www.genecards.org/).
Table 3. The occurrence rates of each allele and genotype for the rs7794745 polymorphism were assessed in both the patient group and the control group

<table>
<thead>
<tr>
<th>Alleles (A→T)</th>
<th>Controls (n = 70)</th>
<th>Autism cases (n = 80)</th>
<th>OR (95% CI)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>88</td>
<td>108</td>
<td>1.00</td>
<td>1.03</td>
</tr>
<tr>
<td>T</td>
<td>52</td>
<td>52</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Genotypes (A→T)</th>
<th>Controls (n = 70)</th>
<th>Autism cases (n = 80)</th>
<th>OR (95% CI)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>AA</td>
<td>32</td>
<td>42</td>
<td>1.00</td>
<td>1.30</td>
</tr>
<tr>
<td>AT</td>
<td>24</td>
<td>24</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TT</td>
<td>14</td>
<td>14</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

95% CI: 95% confidence interval; OR is an odd ratio, and p > 0.05 is statistically insignificant.

Discussion

Autism spectrum disorder (ASD) is a neurodevelopmental condition characterized by its highly heritable and multifactorial nature. Both genetic and environmental factors contribute to its etiology through a complex interplay. The genetics of ASD are intricate, involving several genes, each playing a critical role in the development of neural structures in children. Our research aimed to investigate whether there is an association between polymorphisms in the CNTNAP2 gene and autism. In Iraqi children diagnosed with autism, previous studies have established correlations between gene polymorphisms in GABRB3, MTR, and MTHFR and the risk of autism.1-11 CNTNAP2, also known as NRXN4, is a protein primarily located in the postsynaptic membrane, where it serves as a critical scaffolding component.12 The CNTNAP2 gene plays a pivotal role in proper cerebral development, and any disruption to its function significantly increases the risk of neurological impairments. Deficiencies in CNTNAP2 have been linked to autism spectrum disorder (ASD)-related behaviors such as hyperactivity and epilepsy.13 For instance, an Amish family with autistic traits, cortical dysplasia, and focal epilepsy was found to harbor a mutation in the CNTNAP2 gene, rendering it less active.14 Furthermore, studies have indicated that uncommon variations in CNTNAP2 may contribute to the pathogenesis of ASD.15 The rs7794745 CNTNAP2 gene polymorphism was investigated in a case-control study involving 50 children with autism and 40 controls. Our findings indicated no significant association of the rs7794745 A→T polymorphism with autism (p-value 0.9), and likewise, no positive association was observed between the CNTNAP2 polymorphism and ASD.16 However, previous studies have reported associations between rs7794745 and autism in various populations, including Brazilians and Iranians,17 respectively. A recent study in the Pakistani community strongly linked rs7794745 to ASD,18 and additional research has shown a direct association between rs7794745 and ASD in the Han Chinese population.19

The variability in these findings could be attributed to several factors. Firstly, genetic diversity between populations may have influenced the results of association studies. Secondly, larger sample sizes could reduce sampling error and increase the reliability of findings. Thirdly, ASD is a highly heterogeneous condition, and previous research has primarily focused on recruiting patients with ASD. To mitigate variability, our study only included families with typically developing autistic children.

Conclusion

The Middle Euphrates Iraqi ASD patients did not show any significant correlation between the specific CNTNAP2 gene variations rs7794745 and autism.

Conflict of Interest

None.
References


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