The association of CNTNAP2 rs2710102 and ENGRAILED-2 rs1861972 genes polymorphism and autism in Iranian population

Fatemeh Beiranvandia, Mansoureh Akouchekianb,⁎, Gholam Reza Javadia, Hossein Darvishc

a Department of Biology, Science and Research Branch, Islamic Azad university, Tehran, Iran
b Department of Medical Genetics and Molecular Biology, School of Medicine, Iran University of Medical Sciences, Tehran, Iran
c Cancer research center, Semnan University of Medical Sciences, Semnan, Iran

A B S T R A C T

Autism spectrum disorder (ASD) is a highly heritable neurodevelopmental disease characterized by impaired social interactions, communication deficits, restricted interests, stereotyped and repetitive behaviors, which results from the interaction between genetic vulnerability and environmental factors. Our study was aimed to explore the association between CNTNAP2 gene polymorphism (rs2710102 C/T) and ENGRAILED-2 (EN2) (rs1861972 A/G) with the risk of autism in the Iranian population. A total of 67 autism cases and 100 controls were recruited. Single nucleotide polymorphism (SNP) rs2710102 C/T was genotyped by utilizing polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) and SNP rs1861972 A/G was genotyped by using Tetra-primer ARMS-PCR. The results of this study showed that there is no significant association between rs2710102 CNTNAP2 gene polymorphism and autism, but there is a significant association between rs1861972 EN2 gene polymorphism and autism in the studied population. Consequently, our data provide evidence that the EN2 gene may be implicated in the predisposition to autism in the Iranian population.

1. Introduction

Autism spectrum disorder (ASD) is a group of neurodevelopmental behavior disorders identified by impairment language development and restrictive interests. The heritability and genetic basis involved in ASD can be diagnosed in early childhood in severe cases (Pinto et al., 2010). Moreover, the ASD has been increasing with the spreading rate of 0.62% worldwide in the past two decades (Fombonne, 2009; Brugha et al., 2011; Elsabbagh et al., 2012).

Several studies gave information regarding the functions and the phenotypes associated with the Contactin associated protein-like 2 (CNTNAP2) gene. It has been shown that CNTNAP2 has a significant role in brain development. For instance, The behavioral and cognitive dysfunction in the mouse with CNTNAP2 deficiency is similar to the patients with ASD (Peñagarikano and Geschwind, 2012). A recent in vivo study on CNTNAP2 knockout mice suggested that the formation of unstable new synaptic circuitry is related to the lack of CNTNAP2 (Gdalyahu et al., 2015). Furthermore, Lazaro et al. showed the alteration in circuit-level synchronous activity in the medial prefrontal cortex, which is the result of the limited excitatory drive onto pyramidal cells in the loss of CNTNAP2 (Lazaro et al., 2019). Moreover, Gao and coworkers demonstrated the relationship between disruption of CNTNAP2 and alteration of GluA1 quantity and localization which leads to neurodevelopmental diseases (Gao et al., 2019).

Numerous studies investigated the single nucleotide variations (SNVs) and copy number variants (CNVs) of CNTNAP2 in several cohorts of patients with neurodevelopmental disorders including depression, schizophrenia, ASD, intellectual disability, and language delay (Abrahams et al., 2007; Alarcón et al., 2008; Poot, 2015). For example, Bakkaloglu et al. discovered eight nonsense/missense mutation in a study of 635 ASD patients and 942 healthy controls (Bakkaloglu et al., 2008). Moreover, another nonsense/missense mutations in CNTNAP2 were found in Pitt-Hopkins syndrome, childhood apraxia, and Intellectual disability (Poot, 2015). Furthermore, splice site mutations were observed in Intellectual disability and Pitt-Hopkins syndrome (Zweier et al., 2009; Gregor et al., 2011). However, Morduch and coworkers found no significant association between Rare Heterozygous Point Mutations in the CNTNAP2 gene (Murdoch et al., 2015).

Regarding the SNPs, rs7794745T and rs2710102C were shown to be associated with the impairment of verbal communication which is the core feature of ASD (Whalley et al., 2011; Eyler et al., 2012). Moreover, rs2710102 which is located in intron 13 consistently correlated with Specific language impairment and ASD with major depression (Newbury et al., 2011). In some studies, we can see a positive link between CNTNAP2 and autism. For instance, a study by Nascimento et al. on the effect of the SNPs (rs7794745 and rs2710102) in the CNTNAP2 gene suggested an association in the Brazilian population (Nascimento et al., 2016). We can see the same results in the Autism Genetic Research Exchange ( AGRE) cohort (Alarcón et al., 2008). However, some studies revealed contradictory results and...
demonstrated no association such as the Autism Genome Project (AGT) cohort (Anney et al., 2012). Consistently, Jonsson et al. Study on CNTNAP2 (rs7794745 and rs2710102) suggested no major associated with Autistic-like traits (ALTs) (Jonsson et al., 2014). Moreover, a recent study by Zhang and coworkers in the Chinese Han population combined with an updated meta-analysis revealed that the CNTNAP2 may not be connected to autism (Zhang et al., 2019). Specifically, Toma et al. examined the most comprehensive sample in ASD and schizophrenia, which is the major genetic study ever conducted for CNTNAP2 in ASD. This study implied that the CNTNAP2 gene is not a primary risk gene for psychiatric disorders. They also concluded that the rare variants of the CNTNAP2 gene are not involved in autism and schizophrenia. (Toma et al., 2018).

EN2 gene, a homeobox transcription factor which identified as an autism candidate gene because it influences the development of the midbrain and cerebellum in mice parallels neurodevelopment abnormalities seen in individuals with autism (Sen et al., 2010). Many studies demonstrated the crucial role of EN2 in cerebellum development. The EN2−/− mice show some of the autistic phenotypes such as multiple shifts in social and motor behaviors. One of the regular sites of neuroanatomic abnormalities in autism is the cerebellum including loss of Purkinje cells and hypoplasia, which has been demonstrated by the functional MRI studies in autism patients. Moreover, the expression of the EN2 gene in the postnatal cerebellar development reduced the cerebellum size and decrease the number of Purkinje cells by more than one-third compared to normal conditions. Furthermore, the mutation in EN2 in primary cortical cultures reduces the neural differentiation which related to the number of neurons that express BIII-tubulin (Fatemi et al., 2002; Palmen et al., 2004).

Petit et al. conducted the first case study in 1995 which demonstrated the significant association between case and controls in EN2 rs34808376 polymorphism of 200 people. Conversely, Gharani et al. and Zhong et al. in 2 different studies failed to show any relationship between rs3735653 in EN2 and autism. Although, Gharani and coworkers detected the meaningful association between rs1861972 and rs1861973 SNPs with autism (Petit et al., 1995; Zhong et al., 2003; Gharani et al., 2004).

Herein, we investigated the association between CNTNAP2 gene polymorphism (rs2710102 C/T) and EN2 (rs1861972 A/G) with the risk of autism in the Iranian population. We first examined the CNTNAP2 gene SNP rs2710102 with RFLP-PCR and subsequently SNP rs1861972 was genotyped by utilizing the Tetra-primer ARMS-PCR. Finally, the genotype frequencies were calculated and the statistical analysis performed by the chi-squared test Table 1.

2. Materials and methods

2.1. Patients and controls

The case-control study performed from 2017 to 2018. The current study included a total of 167 individuals with 100 disease-free control subjects and 67 patients with ASD. All the patients were selected from 3 to 7 years old and diagnosed with ASD by well-trained psychiatrists according to the Diagnostic and Statistical Manual of Mental Disorders criteria. Afterward, the selected patients in the first phase of diagnostics were investigated in terms of developing certain conditions associated with ASD such as Fragile X, Tuberous Sclerosis, Non-diagnostic Phenylketonuria (PKU), and Metabolic Problems. We selected 67 patients with ASD and we excluded any positive cases with the aforementioned conditions. All subjects provided written informed consent for participation in this study.

2.2. Genotyping

The peripheral blood samples were taken from patients and controls which poured into the tube containing EDTA as an anticoagulant agent. Afterward, the DNA extraction performed by utilizing the DNA extraction kit (MBST, Iran) according to the manufacturer's instructions. A SNP within the CNTNAP2 (rs2710102) was targeted and genotyped employing the Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP).

The primers used for PCR-RFLP were designed by Oligo primer analysis software which included a forward primer (5′-ATGGATGGAC TGACCGATTG-3′) and Reverse primer(5′-TTGTGTTGGTGGCCAT GAT-3′). The conditions of PCR are as follows, initiation step of PCR was performed at 94–96 °C for 1–9 min, 94 to 98 °C for 20–30 s as the denaturation step, the annealing phase was conducted at 50–65 °C for 20–40 s, extension step at 75–80 °C for 45 s and final extension conducted at 70–74 °C for 5–15 min. Eventually, the restriction enzyme digestion was accomplished utilizing Aval (Eco881) (Thermo scientific) which cut the sequence into 198-bp fragments with T nucleotide in the rs2710102 SNP, but the T to C substitution creates the 43-bp and 155-bp fragments. The digestion carried out in the hot plate at 36 °C for 16 h and the digestion products visualized with agarose gel electrophoresis.

Moreover, the SNP of the rs1861972 in the EN2 gene was investigated using the Tetra primer amplification refractory mutation system (Tetra-primer ARMS-PCR). In this technique, four primers were designed using Oligo primer analysis software including two inner primers and two outer primers. The information about Primers and restriction enzymes was depicted in Table 2. The PCR conditions for the EN2 gene were the same as PCR-RFLP for the CNTNAP2 gene except for the annealing phase which conducted at 70 °C in Tetra-primer ARMS-PCR. Finally, the PCR products visualize by the agarose gel electrophoresis.

2.3. Statistical analysis

The statistical analysis of genotype frequencies in this study performed by the chi-squared test utilizing Med Calc ver. 12.1.4 Software. The Hardy-Weinberg equilibrium assumption was evaluated and the logistic regression procedure was utilized to achieve the adjusted odds ratio (OR) for genetic polymorphism. The P-value of less than 0.05 was considered statistically significant.

3. Results

This study included 167 subjects with 100 healthy persons as the control group and 67 with autism patients in Iran. The DNA obtained from the subjects characterized by utilizing agarose gel electrophoresis. The undigested PCR product size was 198 bp base pairs for rs2710102 in the CNTNAP2 gene (Fig. 1). The Aval (Eco881) restriction enzyme products including the T base is 198 bp fragment. Although, for the genotype which has the C base, the restriction enzyme produces 2 DNA fragments with 155 bp and 43 bp. As shown in Fig. 2 the heterozygous CT showed two bands in 155 bp and 43 bp. Moreover, the homozygous CC and TT have one band in 155 bp and 198 bp respectively. The frequency of the rs2710102 in the CNTNAP2 gene was analyzed. All information for alleles and genotype frequency and patients and controls is presented in Tables 3 and 4. The C and T allele frequencies of this polymorphism were calculated 50%, 50% for control and 44.8%, 55.2% for patients respectively. Consequently, there was no significant association in CNTNAP2 gene rs2710102 polymorphism was seen between patients and control groups.

The relation of the rs1861972 in the EN2 gene for the cases and
control was calculated. As shown in Table 2, the AA, AG, and GG genotype frequencies were calculated 30.0%, 61.0%, 9.0% for the control group and 3.0%, 74.6%, 22.4% for patients respectively which revealed the significant difference between the rs1861972 alleles between control groups and cases (Tables 3 and 4).

### Table 2
Primer design information for rs1861972.

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Primer sequence (5′-3′)</th>
<th>Tm</th>
<th>GC%</th>
<th>Product size</th>
</tr>
</thead>
<tbody>
<tr>
<td>Forward inner primer</td>
<td>CAGAGGCCGAGGTCACTCCTCGCAAA</td>
<td>70.98</td>
<td>60.7</td>
<td>197</td>
</tr>
<tr>
<td>Reverse inner primer</td>
<td>GTGGGGGAAAGGGGCGAAGGCAAC</td>
<td>71.11</td>
<td>65.4</td>
<td>250</td>
</tr>
<tr>
<td>Forward outer Primer</td>
<td>TGACTTCCTGACCTGAGGGGGTCGA</td>
<td>71.04</td>
<td>63</td>
<td>393</td>
</tr>
<tr>
<td>Reverse outer primer</td>
<td>CTGCCAGTTTTGAGAGGGTGGCT</td>
<td>71.04</td>
<td>63</td>
<td>393</td>
</tr>
</tbody>
</table>

### 4. Discussion
We intended to investigate the association between CNTNAP2 and EN2 gene polymorphism and autism. Moreover, the interactions between these two genes investigated with the case-control study (Fatemi et al., 2002; Palmen et al., 2004; Petit et al., 1995). Previous in vivo experiments showed that genetic mutations in the CNTNAP2 genes result in severe neurological diseases. However, there was no significant relationship between CNTNAP2 genetic variation and Autistic-like traits in the Swedish population (Jonsson et al., 2014). Moreover, a large case-control association study revealed that the common variants of FOXP2 protein and two SNPs of CNTNAP2 (rs2710102 and rs7794745) do not correlate with autism (Toma et al., 2013).

A further major and conclusive study by Toma et al. showed that CNTNAP2 variants do not play any significant role in psychiatric disorders. The authors investigated the association of various SNPs of CNTNAP2 in ASD patients, in particular rs1770073 and rs2710102, which were reported associated with ASD in previous studies. Using the Psychiatric Genomics Consortium (PGC) data sets they reported that these two SNPs were not associated with any psychiatric phenotype. They also examined 6171 families with autism and 2163 control families for de novo variants and reported no impact of this class of mutations in ASD. Finally, they investigated the pathogenic effect of CNTNAP2 ultra-rare variants (URV) in 4483 patients with ASD. They...
could not detect any significant difference in the number of URVs between ASD patients and controls (Toma et al., 2018). We are interested to note that our study does not suggest evidence for the association of rs2710102 variants of CNTNAP2 with autism. In conclusion, we believe it is unlikely that the CNTNAP2 gene is a primary risk factor for ASD. In 2008, Pinchen Yang and coworkers examined the rs1861972 and rs1861973 intron polymorphisms of the EN2 gene and autism susceptibility. In a sample-control study in the Chinese population (Yang et al., 2008). Moreover, in another study conducted in 2010, the allele frequency and genotype distribution of rs1861972 polymorphism were investigated in the Chinese population which revealed that The A-C haplotype of rs1861972 and rs1861973 plays a protective role against autism. Besides, the G-A-C haplotype was less frequent in males than in controls (38.64% vs. 52.51%), which may increase the vulnerability to autism. In both the aforementioned studies, there was no significant difference between case and control groups (Yang et al., 2010).

In contrast, our study demonstrated the significant difference between control and cases. Moreover, the G allele was considered the risk factor. Consequently, individuals with AG and GG genotypes are more susceptible compared to AA genotype. Choi, et al. showed that the A-C haplotype (rs1861972, rs1861973 A-C) is an expression activator and EN2 gene is a susceptibility gene which related to the higher risk for ASD (Choi et al., 2011).

Declaration of Competing Interest

The authors certify that they have NO affiliations with or involvement in any organization or entity with any financial interest, or non-financial interest in the subject matter or materials discussed in this manuscript.

References