ORIGINAL ARTICLE

Molecular Evaluation of Ex3 VNTR Polymorphism of the DRD4 Gene in Patients With Autism Spectrum Disorder

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Abstract

Objective

Autism Spectrum Disorders (ASDs) are a group of neurodevelopmental disorders that affect social and communication skills. These diseases are characterized by severe communication and social skills disabilities and limited and repetitive activities. The prevalence of these disorders appears to be steadily increasing. It is proposed that the genes involved in the dopamine pathway may play an essential role in the development of autism. In this study, we investigated the possible association between Ex3 VNTR polymorphism of the DRD4 gene and autism spectrum disorders in the Iranian population.

Materials & Methods

In this case-control study, 97 children with autism and 103 healthy individuals from a northwestern area of Iran as the case and control groups, respectively. After genomic xtraction from peripheral blood samples by the proteinase K method, the polymerase chain reaction (PCR) technique was used to determine the polymorphism genotypes. The data were then coded and analyzed using SPSS version 22 software.

Results

The study results showed that the allele frequencies differed in the two groups, some of them being statistically significant. The most common allele in both the ASD and the control group was the 700 bp allele, and its frequency was significantly different in the two groups and was more common in the ASD group (p-value=0.0018). The other allele with a statistically different frequency was the 800 bp allele which was less frequent in the ASD group (p-value=0.0017).

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Conclusion

These results suggest a potential association between Ex3 VNTR polymorphism of the DRD4 gene and autism spectrum disorder in the Iranian population. This necessitates further studies for the evaluation of the DRD4 gene.

Keywords: Autism Spectrum Disorder; EX 3 VNTR Polymorphism; DRD4 Gene
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Introduction

Autism spectrum disorder was first described in 1940 by two psychiatrists, Connor(1) in the United States and Asperger(2) in Australia.

Autism Spectrum Disorders (ASDs) are a group of neurodevelopmental disorders that affect social and communication skills. The prevalence of ASDs appears to be steadily increasing. Still, it is unclear whether this is due to the change in the diagnostic criteria or an actual increase in the number of patients. Recent estimates have shown that the median prevalence estimate of ASD is 62 in 10000. Geographic region, ethnic/cultural, or socioeconomic factors do not appear to impact the prevalence of ASD(3) strongly. Due to the significant difference in concordance rates between monozygotic and dizygotic twins, autism is considered the most heritable neurodevelopmental disorder. Genetic factors seem to have a vital role in this regard (4).

Genetic alterations in ASDs can be divided into different subtypes: individuals with an identifiable Mendelian condition or congenital syndrome (such as Fragile X syndrome, tuberous sclerosis, Rett syndrome, and neurofibromatosis), cytogenetically visible chromosome rearrangements, Rare de novo and some inherited CNVs (which are too small to be seen by karyotyping), and rare penetrant genes(5).

The onset of autism spectrum disorder in early childhood is usually three years old. Studies in different parts of the United States have estimated the current prevalence of autism spectrum disorders (ASDs) from 1 in 150 to 1 in 91(6, 7), with a 4: 1 male-female ratio. Several studies have provided significant evidence confirming the role of genetics (8,9) and environmental factors (10,11) in the development of autism, suggesting that autism spectrum disorder is a multi-factorial disease.

Dopamine receptors are involved in several biological (primarily neurological) processes, including learning, motor control, cognitive memory, and the regulation of endocrine signals. This justifies their association with different neurological and psychiatric disorders (12).

The human dopamine receptor D4 (DRD4) gene, located on chromosome 11p, encodes the dopamine receptor D4 and has many expressed polymorphisms. This gene is well-studied in psychiatric diseases, and a 48-bp Variable Number Tandem Repeat (VNTR) polymorphism occurs in its exon III(13).

Several studies have demonstrated that DRD4 (especially its 7R allele) is associated with

several psychiatric disorders, including ADHD, pathological gambling, substance use, alcoholism, bulimia nervosa, schizophrenia, and autism(14-20). A possible positive association between autism and the 7R allele of the DRD4 gene was suggested by a study designed to evaluate gene polymorphisms involved in the dopaminergic and serotonergic pathways in a group of Spanish children with autism (21). Nonetheless, another study that assessed the DRD4 gene and its associated polymorphisms cast doubt on any possible association between exon III polymorphism and autism (22).

Since the association between DRD4 gene variants and autism spectrum disorder in the Iranian population has not been investigated hitherto, the current study was designed to evaluate the possible association between Ex3 VNTR polymorphism of the DRD4 gene and autism spectrum disorder in a cohort of residents of a northwestern region of the country.

Materials & Methods

Samples collection

A total of 97 cases with ASD (97 boys aged between 3-9 years) from a Northwest region of Iran, who were referred to a psychiatric clinic and diagnosed with ASD by pediatrics and adolescent psychiatrists, constituted the case group. As the control group, 103 healthy children with similar demographic features, such as mean age, sex, and gender, were selected.

The convenience sampling method was used to select the participants who were referred to the psychiatric clinic of the children's hospital. The case and control groups were formed according to the exclusion and inclusion criteria. After being briefed about the study and its objectives and procedures, informed consent was obtained from all participants and their parents.

Inclusion criteria were participants' willingness to participate in the study and confirmed autism spectrum disorder cases. The main exclusion criterion for both groups was being diagnosed with other neurological diseases, fragile X syndrome, ADHD, and tuberous sclerosis.

Molecular evaluation

First, 3-5 ml of peripheral blood was collected from the participants in sterile conditions. DNA was extracted from blood samples of patients with autism spectrum disorder and the control group. The Ex3 VNTR fragment of the DRD4 gene was amplified using specific primers by the conventional PCR method, and two alleles of the abovementioned polymorphisms were examined using the acrylamide gel method. Primer sequences were designed using Primer 3 software version 4. The frequency and distribution of the variants were calculated using POPGENE software version 1.32.

DNA extraction

DNA extraction was performed utilizing the proteinase K method. In the next step, polymerase chain reaction (PCR) was performed using specific primers (Table-1), which were designed to study the polymorphism of the target area. DNA markers were used to determine the participants' genotypes. For this purpose, each individual's PCR product was loaded on a 2% agarose gel with a marker, and the size of the product was determined using the marker.

PCR technique

The VNTR locus was amplified by polymerase chain reaction (PCR) using primers shown in Table 1. To evaluate the PCR process, gel electrophoresis was used (to separate PCR products and assess the fragments' size).

Statistical Analysis

We manually entered and coded the data and statistically analyzed them in SPSS software (version 22.0; IBM Corp, Armonk, NY, USA). Mean values and standard deviations were calculated. We analyzed the categorical variables using descriptive statistics (frequency and percentage) and used range, mean, and standard deviation to analyze and describe continuous data. Pearson's Chi-square test and Fisher's exact test were used to analyze the categorical data and to compare the frequency of different alleles between the two groups to determine possible associations. A P value of less than 0.05 was considered significant.

Results

The frequency and percentage of DRD4 gene alleles in the subjects in both patient and healthy groups are presented in Table 2 and Figure 1. The allele frequencies differed in these two groups; some were statistically significant. The 700 bp allele was the most common in both groups and was significantly more frequent in the ASD group (p-value=0.0018). The 800 bp allele was significantly less frequent in the ASD group (p-value=0.0017).

Table 1. Primer sequence for DRD4 gene polymorphism

Product size PCR (bp)	Desired primer sequence	Primer
2 rep: 470 bp 3 rep: 518 bp 4 rep: 566 bp 5 rep: 614 bp 6 rep: 662 bp 7 rep: 710 bp 8 rep: 758 bp	GCTGCTGCTCTACTGGGGCCA GTGCACCACGAAGAAGGGCG	Forward Reverse

Table 2. Frequency of the DRD4 gene alleles in the two study groups

Product size PCR (bp)	Desired primer sequence	Primer
2 rep: 470 bp 3 rep: 518 bp 4 rep: 566 bp 5 rep: 614 bp 6 rep: 662 bp 7 rep: 710 bp 8 rep: 758 bp	GCTGCTGCTCTACTGGGGCCA GTGCACCACGAAGAAGGGCG	Forward Reverse

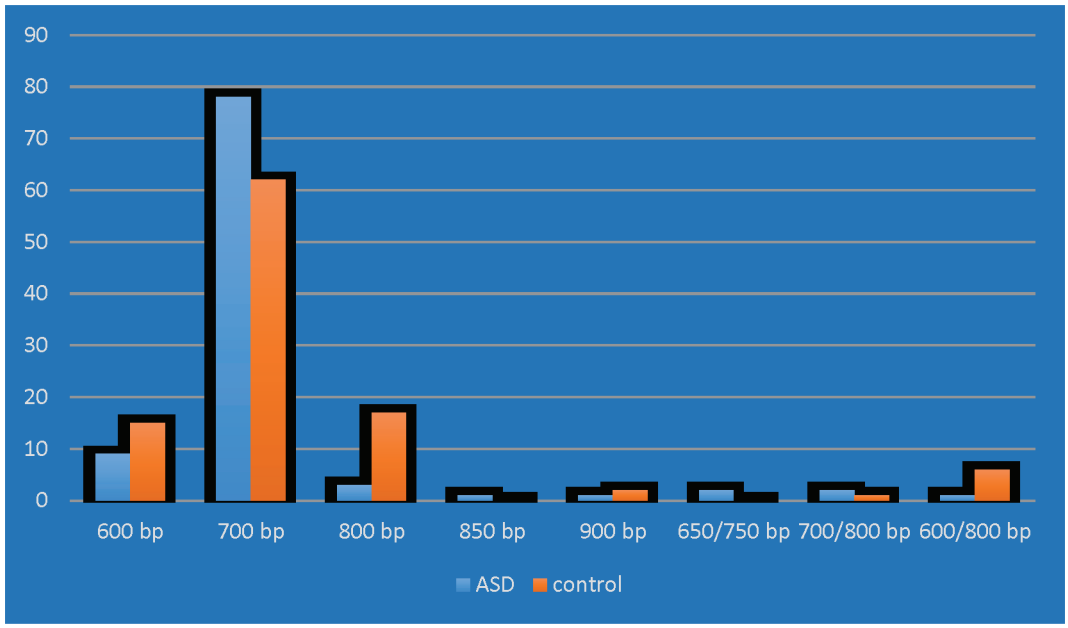

Figure 1. *shows the frequency of DRD4 gene alleles in the two study groups. As shown in the diagram, the 700 bp allele was the most common in both groups.*

Discussion

ASD develops due to several interactions between genetics and environmental factors and has a complex genetic architecture. Several genes may be involved in the development of this disease, including SHMTMTHFR, MTRR, SHANK3, NRP2, SHMT1, MTR, CNTNAP2, and DRD4(23) As discussed previously, dopamine receptors are involved in several neurological processes and are associated with many psychiatric and neurological disorders. However, few studies have investigated the relationship between the DRD4 gene and ASD. Therefore, as no such research has been shown in Iran hitherto, and considering the importance of the issue, we designed and carried out this study to investigate the Ex3 VNTR polymorphism of the DRD4 gene in autistic patients and compared them to a control group comprising healthy children.

In this regard, 200 children (comprising 97 children with ASD and 103 healthy participants) who met the inclusion criteria were investigated for the expression of the Ex3 VNTR polymorphism of the DRD4 gene. According to the study results, the allele frequencies differed in the two groups, some statistically significant. The most common allele in the ASD and the control group was the 700 bp allele, and its frequency was significantly different in the two groups, being more common in the ASD group. The other allele with a statistically different frequency was the 800 bp allele which was less frequent in the ASD group. Few studies have been conducted on the association between DRD4 and ASD. However, the association between the DRD4 gene and other neurological disorders has been investigated in numerous studies, and some of them have confirmed the link. But others have reported none such significant association.

In a study of ADHD patients in an Asian population, two markers within the DRD4 gene (the exon three variable number tandem repeat (VNTR) and a 5' 120 base-pair duplication) were investigated. They found no association between the incidence or severity of ADHD and any of the mentioned polymorphisms (24).

In a study conducted in China, Qian et al. reported that the long-repeat alleles of DRD4 and DAT1 were more frequent in patients with ADHD compared to the controls ($P < 0.05$). However, there was no significant allelic association when the two groups were analyzed separately (25).In a study by Leung et al., which was designed to evaluate the 7-repeat (7R) allele of the DRD4 gene in ADHD patients, the authors reported an increase in the prevalence of the 2R allele and hypothesized that this was consistent with the 7R allele hypothesis of ADHD in European native children (26)

However, this relationship has been shown in several other studies. Curran et al., in a study investigating the 7-repeat allele of a 48 bp repeat polymorphism (DRD4-7) in exon 3 of the DRD4 gene in children with ADHD, concluded that there was a significant relationship between the polymorphism of the gene and the severity of the inattention scores evaluated by patients' parents. (27).

Tabatabaei et al. investigated the possible association between VNTR polymorphisms in exon 3 of the DRD4 gene and ADHD. They reported a significant difference in the frequency of 4R alleles of DRD4between the two groups. The predominant alleles detected in this study were 4R, 5R, and 6R, of which 4R repeats were the most common (present in 76.2% of patients with ADHD and 53.8% of controls ($P = 0.004$) (28).

As mentioned earlier, a possible positive association between autism and the 7R allele of the DRD4 gene was reported in a study that evaluated gene polymorphisms involved in dopaminergic and serotonergic pathways in Spanish children with autism (21). However, another study evaluating the DRD4 gene and its associated polymorphisms reported that the exon III polymorphism is unlikely to be associated with autism (22).

These contradictory results may be attributed to different gene pools, sample sizes, and study methods. These results may change with increased sample size and other populations (because each population has a different gene pool). Naturally, various factors can affect gene functions and research outcomes.

As discussed, although many studies have confirmed the association between the DRD4 gene and several psychiatric disorders, few studies have investigated its association with ASD, and mainly, no study has investigated the EX3 VNTR polymorphism of the DRD4. This makes it difficult to compare and evaluate the result of our research. Nevertheless, our finding that different allele frequencies in two groups (such as the 700 bp allele) were significantly more frequent in the ASD group and the 800 bp allele was considerably less frequent in them may suggest a possible association between Ex3 VNTR polymorphism of the DRD4 gene and ASD. This should encourage other researchers to design and conduct further studies on the DRD4 gene and ASD.

In Conclusion

Our study showed that the allele frequencies differed in the two groups, and some of these differences were statistically significant. The most common allele in both the ASD and the control group was the 700 bp allele, and its frequency was significantly different in the two groups and was more common in the ASD group. (p-value=0.0018). The other allele with a statistically different frequency was the 800 bp allele which was less frequent in the ASD group (p-value=0.0017).

These findings suggest a possible association between Ex3 VNTR polymorphism of the DRD4

gene and ASD and encourage further research regarding the association between the DRD4 gene and ASD.

One possible limitation of our study was probably the relatively small sample size, which indicates the need for more studies with larger sample sizes. Therefore, it is proposed that similar studies in different regions of the country should be carried out to evaluate this association in more depth. The evaluation of other polymorphisms of the DRD4 gene is also recommended since they seem a promising research topics.

Acknowledgment

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Author's Contribution

Amiri S and Shekari khaniani M designed the study. Shafiee-Kandjani AR, Mohammadi A, Asadian M contributed to the implementation of the research. Mehdizadeh Fanid and Shekari Khaniani performed the laboratory tests. Amiri S prepared the manuscript draft. Shafiee-Kandjani AR reviewed the paper. All read and approved the final version of the manuscript.

Conflict of interest

The authors declare that there is no conflict of interest.

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