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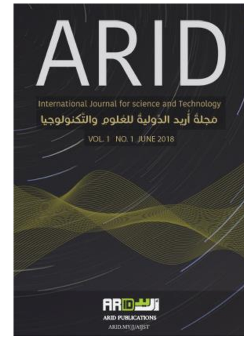

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GENOTYPING OF SEROTONIN TRANSPORTER GENE (*SLC6A4*) OF AUTISTIC CHILDREN IN IRAQI POPULATION

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التنميط الجيني للجين المشفر للناقل السيروتونين (SLC6A4)عندأطفال التوحد في المجتمع العراقي

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ABSTRACT

In present study, the blood samples were collected from 145 children (95 autistic children and 50 healthy children), autistic children were attended to autism unit at Disabled Hospital in Thi-Qar province, Iraq during the period from January to November 2016. The results showed males (81%) more than female (19%) with ratio 4:1 and also results explain the age group of 3-5 years recorded the highest percentage (41.05%). Distribution of autistic children according to sibling showed six were brotherly with occurrence rate (6/95) 6.3%.

The genetic study included using polymerase chain reaction (PCR) for amplification of 5-HTTLPR region in *SLC6A4* gene. The results revealed that seven patients (7.36%) gave positive results for amplification of *SLC6A4* gene by using a specific primer. The results of genotype distribution of the 5-HTTLPR polymorphism in autistic children were L/L, L/S, and S/S with percentage 28.57%, 14.28% and 57.14% respectively. Significant differences were noticed in the distribution of allele frequency among patients were 35.71% for the L allele and 64.28% for the S allele ($p \leq 0.05$). The mutant patients showed significant difference with gender ($p \leq 0.05$), they were 5 male (71.42%) and 2 female (28.57%) children.

الملخص

تم جمع عينات الدم في الدراسة الحالية من 145 طفل (95 طفل مصاب بالتوحد و 50 طفلاً سليما) ، كان أطفال التوحد يراجعون وحدة التوحد في مستشفى المعاقين في محافظة ذي قار خلال الفترة من كانون الثاني إلى تشرين الثاني 2016. أظهرت النتائج أن الذكور (81٪) أكثر من الإناث (19٪) بنسبة 4: 1 وسجلت الفئة العمرية 3-5 سنوات أعلى نسبة أصابة (41.05٪). أظهر توزيع الأطفال المصابين بالتوحد وفقا للأخوة أن ستة منهم كانوا أخوة بمعدل تردد (95/6) 6.3٪.شمات الدراسة الوراثية استخدام تفاعل البلمرة المتسلسل لتضخيم منطقة 5-HTTLPR في جين *SLC6A4* باستخدام بادئ متخصص للطفرة. أوضحت النتائج أن سبعة مرضى (7.36٪) يعانون طفرة. كانت نتائج التنميط الوراثي لتعدد الأشكال L/Lو L/S و S/S بنسبة 28.57٪ و 14.28٪ و57.14٪ على التوالي. لوحظ وجود اختلاف معنوي($p\leq0.05$) في توزيع التردد الاليلي بين المرضى حيث كانت 35.71% للاليل L و64.28% للاليلS. أظهرالمرضى الذين يعانون طفرة اختلافا معنويا مع الجنس ($p\leq0.05$) كان خمسة أطفال ذكور (71.42%)K واثنتان إناث(28.57%). الدراسة الحالية اقترحت أن اطفال التوحد الحاملين للطراز الوراثيS/S ربما لديهم تحسس لظهور علامات مرض التوحد.

1. Introduction:

Autism is neurodevelopmental disorder known as a spectrum of phenotypes that characterized by difficulties in three domains: deviant language, development of social deficits and a restricted range of stereotyped repetitive behaviors, usually happening within the first 3 years of life [1]. The prevalence was significantly higher in boys (23.6 per 1,000) than that in girls 5.3 per 1,000 [2]. Autism disorder known as multi factorial causes, genetic and epigenetic variants, environmental and hormonal factors, contribute to autism risk and phenotypic variability [3]. Family and twines studies explained the repetition rate among siblings with autism is ∼3%, the risk is 50–100 times higher than in the general population, that illuminate genetic role in causes of autism disorder [4].

Role several genes in psychiatric behavior and mental disorders, extensively studied by many researchers, such as serotonin transporter gene. Serotonin transporter (5-HTT) is coded by a single gene (*SLC6A4*), which is located in human chromosome 17, this protein responsible for the reuptake of serotonin from the synaptic cleft to presynaptic nerve terminals [5]. The promoter region of the 5-HTT gene (5-HTTLPR polymorphism) undergo polymorphism. That, it consists of two common alleles a short (S) allele with 14 copies and a long (L) allele with 16 copies [6]. The polymorphism due to a 44-base pair (bp) insertion or deletion was linked to different protein expression. The S allele (SS or SL genotypes) is associated with lower 5-HTexpression, in this manner resulting in reduced 5-HT reuptake and release capability, whereas the L allele is associated with higher 5-HT transporter expression and threefold increase in gene transcription [7]. 5-HTTLPR is one of the functional polymorphism sites of the 5-HTT gene [8] and it has been implicated in some psychiatric behavioral and mental disorders [9]. The abnormalities in serotonergic systems including an altered developmental trajectory of 5-HT turnover and reduced binding of 5-HT receptors and serotonin transports in autistic individual [10].

So far, the association between 5-HTTLPRgene polymorphism and autistic disorder has not been completely recognized, and there are few studies concentrating on the association between the genetic polymorphism and the clinical characteristics. In the present study, the 5-HTTLPRstatus in the 5-HTT gene was evaluated in order to establish whether it had an association with autism pathogenesis in Thi-Qar populations.

2.Methodology:

2.1 Blood sample collection:

The diagnosed children with an autism were submitted by pediatrician (Dr. NaamaGludKazar Al-Tamimi) manager of the Thi-Qar Center for Autism in the Disabled Hospital in Thi-Qar province/Iraq. The diagnosis of ASD was made in accordance with the standardized criteria provided in the American Psychiatric Association's Diagnostic and Statistical Manual-IV.

Two ml peripheral blood samples were collected from 145children (95 autistic children and 50 healthy children), for both the sexes during the period from January to November 2016, in a sterile K_2EDTA tube and these blood samples were stored at -80˚C until use.

2.2 DNA extraction and polymerase chain reaction (PCR) analysis:

Genomic DNA was isolated from whole blood by using kit gSYNC™ DNA Extraction Kit 100Preps Cat.No.GS100 according to manufacturer's protocol. The quantity and quality of DNA was checked using Nano drop technique and agarose gel electrophoresis:

2.2.1Nano drop spectrophotometer

Nano drop technology performed for masseur of DNA and reveal the potential error rate in the sample from the standard readings of nucleic acid (DNA = ~ 1.8).

2.2.2 Agarose gel electrophoresis

Electrophoresis performed by mixing 5µl from DNA with 2µl of loading dye (bromothymol blue) and loaded into the dedicated wells, then exposed to an electric field 70V for 45-60 min. Amplification primers for the promoter region of the 5-HTT gene that using in current study were in table (1):

Table (1): Primers sequences used for *SLC6A4* gene amplification.

Primer Sequences (5´- 3´)	Product	Reference
F: TCCTCCGCTTTGGCGCCTCTTCC R: TGGGGGTTGCAGGGGAGATCCTG	S(469) bp L(512) bp	[11]

The final volume of reaction tubes is 20µl, consist of 5µl Master Mix, 1.25µl of each forward and reverse of the primers specific for the this gene, 5µl of DNA template and the volume was completed by adding nuclease free water. The thermo cycling program of PCR was mentioned in table (2).

Table (2): Program of amplification *SLC6A4* gene according to [11] with modification.

Step	Temperature(°C)	Time	Cycle
Initial denaturation	95	5 min	1
Denaturation	94	30 sec	35
Annealing	65.5	90 sec	
Extension	72	60 sec	
Final extension	72	10 min	1

The PCR products were separated by 2% agarose gel electrophoresis, and the product were examined under the UV light spread through the gel [12].

Two different allelic fragments with the lengths of L and S alleles corresponded to 512bp and 469bp fragments, respectively were detected among the PCR products.

2.3 Statistical analysis: Statistical analysis was performed using SPSS 13.0. P<0.05 was considered to indicate a statistically significant difference.

3. Results:

3.1Distributionchildren according to gender, age and sibling

The present study was included 145 children divided on 95autistic children involved 77males (81%) and 18 females (19%), and 50 healthy children as control (28 males and 22 female). Statistically, there were significant differences (P≤0.05) among children according to gender distribution, table (4).

Table (3): Distribution and percentages of autistic children and control according to gender.

Gender	Patient		Control	
	No.	%	No.	%
Male	77	81	28	56
Female	18	19	22	44
Total	95	100	50	100

X^2=15.802, df=1, P.value =.000

All the children whom infected with autism divided according to age as shown in table (5). The age group of 3-5 years recorded the highest percentage (41.05%), followed by age group of 6-8 years (35.78%), less than 3 years group (11.6%), 9-11 years (7.36%), 12-14 years (4.21%) with significant differences ($P \leq 0.05$) between the age groups.

Table (4): Distribution and percentages of autistic children and control according to age group.

Age group (Years)	Patients No.(%)	ControlNo.(%)
Lessthan 3	11(11.6)	1(2)
3 – 5	39(41.05)	11(22)
6 – 8	34(35.78)	13(26)
9 – 11	7(7.36)	13(26)
12 – 14	4(4.21)	12(24)
Total	95(100)	50(100)

X^2=41.543,df=4, P-value = 000

To investigate genetic role in autism, disorder the autistic children distribution according to sibling, the result showed six from 95 patients were brotherly with occurrence rate 6.3%.

3.2 (5-HTTLPR) region in SLC6A4 gene

The results of PCR assay revealed that seven patients (7.36%) gave positive results for amplification of *SLC6A4* gene by using specific primer, the bands showed in fig. (1) illuminate the size of band which was appear in approximately 569 bp corresponding to the long allele of 5-HTTLPR. While, band was appearing in approximately 412 bp revealed the short allele.

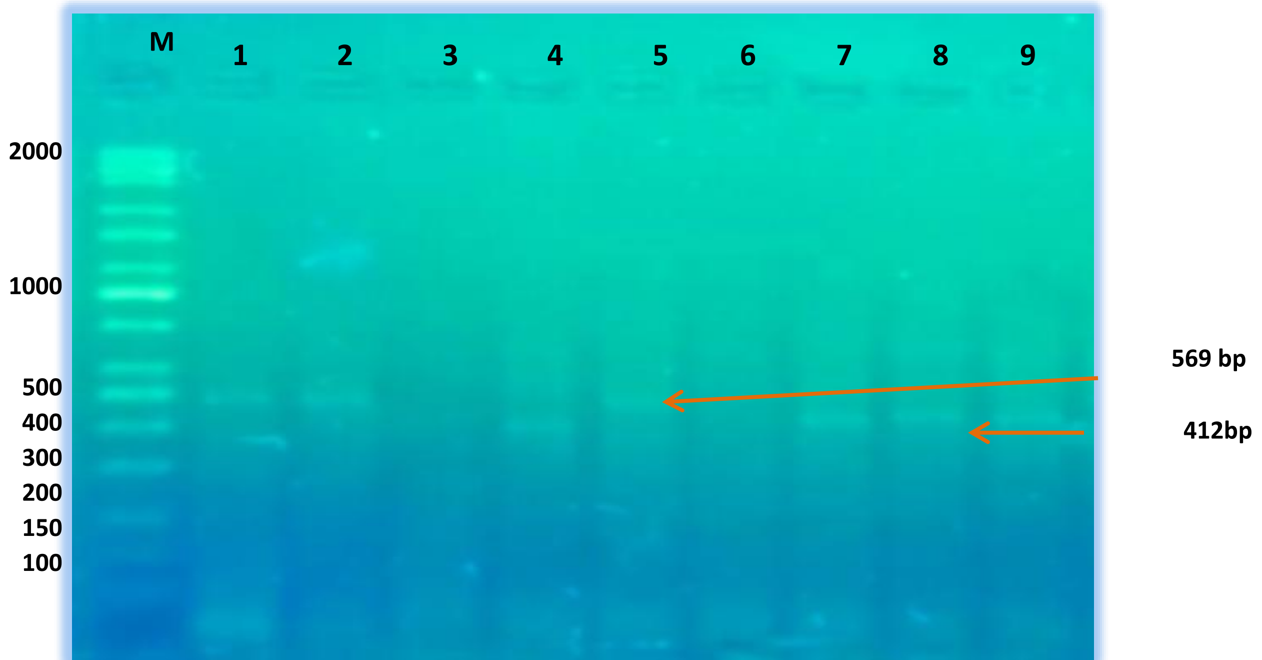

Figure (1): Agarose gel electrophoresis of *SLC6A4* gene amplicon on 2% agarose gel at 70 voltages for one hour, where M:DNA ladder (100bp) (150-2000bp). 1,2 (L/L); 5 (L/S) ; 4,7,8,9 (S/S); patients : 3,6 negative control.

The genotype distribution of the 5-HTTLPR polymorphism in autistic children were L/L, L/S, and S/S with percentage 28.57%,14.28% and 57.14% respectively. While the distribution of allele frequency among patients were 35.71% for the L allele and 64.28% for the S allele with significant difference ($p \leq 0.05$), as presented in table (6).

The mutant patients showed significant difference with gender ($p \leq 0.05$), they were 5 male (71.42%) and 2 female (28.57%) children.

Table (5): 5- HTTLPR genotype and allele frequencies among autistic children.

Marker	Autistic children	%
5- HTTLPR genotype		
S/S	4/7	57.14%
L/S	1/7	14.28%
L/L	2/7	28.57%
5- HTTLPR allele frequency		
L	5/14	35.71%
S	9/14	64.28

5-HTTLPR: Serotonin transporter linked polymorphic region; S: short allele ; L: long allele

4. Discussion:

4.1Distribution of autistic children according to sex, age and sibling:

In present study, the results had shown that males (81%) were more likely to had autism symptoms than females (19%) with a prevalence ratio of 4:1 this result agree with many previous studies [13; 14; 15], the cause for this difference is not well understood but several theories had been suggested. Molecular evidence confirm of sex-biased genetic effects by displaying highly significant association driven by families with only affected males, and abnormalities of the sex chromosomes are associated with ASD as X-linked intellectual disability (XLID) as etiology of ASD [16]. Additional, possibility of sex-differences belong to degree of genetic abnormality that associated with autism [17]. This gave initial evidence of abnormalities and sex-specific

differences in the brain structure of females with autism disorder [18]. Moreover, sex differences in symptom stages may contribute to gap in identification of autism traits for both sexes [19].

In the current study, data associated with distributing of the autistic children according to age documented that the age group 3-5 years had the highest percentage (41.05%), followed by the age group of 6-8 years (27.05%) when compared with other groups.

Comparison the present study with other studies was difficult because varied in design and circumstance-ascertainment strategies, but data from a Centers for Disease Control (CDC) pilot project, suggest that progress has been made in identifying autistic children at younger ages. Preschool-aged children identified with ASD were more likely to have an intellectual disability than school-aged children with ASD [20].

In current study, distribution of autistic children according to sibling explain six children were brothers with percentage 6.3%.

The risk of developing autism can now be estimated for family members and siblings were 6 to 8% [21], the danger increased about 25-fold in general population [22]. This supporting the evidence for an increased frequency of autism among siblings and showing heritable role in autism as etiology according to many studies that proven an average autism inheritance of 90% [23 ; 24] indicates that autism is among the most genetic of neuropsychiatric diseases [25]. It is now well known that the same genetic lesion can lead to different behavioral and mental phenotypes within the same family [26]. And the possibility to infection, increased in families have more than one autistic child, because the presence of above one older affected sibling causes a two-fold rise in the risk of autism in the next children [27].

4.2 Polymorphism in (5-HTTLPR) of SLC6A4 gene

In the recent study, genomic analysis for the long and short allele variants of the 5-HTTLPR polymorphism of *SLC6A4* gene showed three genotypes: L/L, L/S, and S/S with percentage 28.57%,14.28% and 57.14% respectively. Statistically, 5-HTTLPR alleles showed significantly increased S allele in autistic children compared to controls ($P \leq 0.05$). The obtained results indicate an association between autism and S/S genotype as a risk factor.

Despite, serotonin transporter gene polymorphism is one of the most extensively studied in psychiatric behavioral genetics [28]. The present study considers the first study that demonstrated a link between the 5-HTTLPR polymorphism and autism in Thi-Qar province /Iraq.

The genes that involved in early neural development could contain polymorphisms or mutations contributing to the disease development. The polymorphisms phenomenon in *SLC6A4* gene consider as the striking risk of autism [29].

The role of S allele of 5-HTTLPR as a risk factor for autism supported by many studies [30; 31]. But, the absence of successful association between studies and small sizes of samples has reduced estimation of the role that genetic variation that plays in causing autism [32]. Generally, S allele associated with reduction of gene expression [33], anxiety-related behavior [34], hopeless behavior [35] and enhanced neural mechanisms [36] in autistic individuals.

However, other studies support preferential transmission of the L allele [37]. Another hand, some studies found no association of the short or long allele with autistic disorder [38; 39; 40].

The variation of genotype in current study, when compare to other studies, may belong to differences in geographical diversity, population size, and ethnic background. Such as L/L genotype were observed with higher rates among Caucasian. While, S/L and S/S genotypes were higher frequencies in east populations [41]. The negative association could also be the result of a

practical mistake of comparing the L/L and S/L genotypes together, with S/S genotype subgroup allele [42].

The evidence on the interactions between genotype and phenotype variations, was found the L/L genotype being valued as more severe on the stereotyped and repetitive motor activities and on an aggression degree [43], and the S/L or S/S genotypes being valued as more severe on the failure to use nonverbal communication to control social interaction [44].

In current study, application of PCR assay by using specific primer proved seven (7.36%) from autistic children suffer mutation. This result similar to study by [45] whom were detected *de novo* mutation in 7% of autistic individuals. Genomic differences that related to *de novo* mutation can be detected in 7–10% of idiopathic autism individuals [46].

So that, the risk of autism could contribute to the point mutations in protein-coding regions of gene, such as fragile X syndrome and tuberous sclerosis complex and identified metabolic conditions [46; 47; 48].

Five from this mutant child were male 5/7 (71.42%), this gave evidence for male-biased genetic effects at chromosome17q that found a nearby the *SLC6A4* locus [29].

The negative results were acquired in the present study could mainly be attributed to the fact that autism is a complex difference with a multifactorial trigger. Signs of the disease in some individuals could be due to some environmental factors during their embryogenesis or during their development. Patient samples also reflect on difference in selection of patients and etiological heterogeneity.

As well as, the gene-environment interaction between 5-HTTLPR and exposure to environmental difficulty were involved [49], and increased activity of genotypes are associated with environmental interference [50].

A series of studies have shown that the 5-HTTLPR consistently interacts with mistreatment in the childhood and medical disorder to believe depression [51].

5. Conclusions:

The results revealed that seven patients (7.36%) gave positive results for amplification of *SLC6A4*. The Significant differences in the distribution of allele frequency among patients were 35.71% for the L allele and 64.28% for the S allele ($p \leq 0.05$). The mutant patients showed significant difference with gender ($p \leq 0.05$), they were 5 males (71.42%) and 2 females (28.57%) children. The results suggest that patients with autism carrying the homozygous S/S genotype may be susceptible to autism symptoms.

List of abbreviation

Abbreviation	Meaning
5-HTT	Serotonin transporter
5-HTTLPR	Transporter linked polymorphic region
DNA	Deoxi ribonuclelotid nucleic acid
L	Long allele
PCR	polymerase chain reaction
S	Short allele
Ultra violet	UV light

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