POLYMORPHISMS OF (RS7794745) IN THE*CNTNAP2* GENE AS BIOMARKER OF AUTISTIC PATIENTS IN THI-QARPROVINCE/ IRAQ

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Abstract -
Aim: The role of SNP polymorphism in the *CNTNAPA2*gene as a possible risk factor for autism is still a topic of much investigation, and research on genes related to autism susceptibility has been rather challenging. Present study aimed to investigate the possible association of *CNTNAPA2*polymorphism and autism in a Thi-Qar populations.
Methodology: Autistic children were diagnosed by child psychiatrists according to DSM-IV and DSM-V criteria. 95 children diagnosed as autistic children and 50 age and sex-matched children as control were tested for *CNTNAPA2* polymorphism. This polymorphism was studied by using polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) methods.
Results: After, DNA was extracted from peripheral blood cells, analyzed the SNPs (rs7794745) in the *CNTNAP2* gene of 95 ASD patients and 50 non-autistic individuals by polymerase chain reaction-restriction fragment length polymorphism(PCR-RFLP).The results of digestion by restriction enzyme (MICUI) for patients and control samples, showed the frequencies of the AA, AT and TT genotypes of rs7794745 were 25.58%, 60.46% and 13.96% in patients and 38.90%, 44.44% and 16.66% in controls, respectively. Thus, the significant association was observed in genotypes distributions of rs7794745 *CNTNAP2* gene polymorphism between autism patients and controls ($P \leq 0.05$). The frequencies of A and T allele patients were 55.81%, 44.19% and the control group were 61.11% and 38.89% respectively ($P \leq 0.05$). There were no significant differences in allele frequencies between the two groups. This study showed that there is a significant relationship between rs7794745 *CNTNAP2* gene polymorphism and autism in Thi-Qarpopulation.
Conclude: This study conclude that *CNTNAPA2*polymorphisms such as other SNP may be studied to show their possible role in autism.

I. INTRODUCTION

Autism Spectrum Disorder (ASD) is a heterogeneous neurobiological condition and symptoms occur in the first three years of age. It is characterized by severe impairments in social relationships, communication and behavior, that associated with restriction in interests and extreme attachment to routine or to repetitive or perseverative behaviors (APA,2013).The diagnosis of ASD is primarily based on behavioral criteria, rather than physical examination findings or laboratory tests (APA, 2013). The prevalence of children with ASD continues to increase all over the world (Bjorklund and Chartrand, 2016). ASD occurs in all ethnic and social groups, with males being more predominately affected, with a ratio of 4:1 (Lai *et al.*, 2014).

The etiology of autism is largely unknown, but it has been accepted that genetic and environmental factors may both be responsible for the disorder (Sener *et al.*, 2014; Karimi*et al*., 2017).Based on a series of studies have revealed that the genes involved in theinitiatedof autism behaviors,such as impairment in social interaction and verbal communication as well as genetic differentiation in repetitive behaviors indicates that different features in autistic disorder may be caused by different genes associated with distinct (Yoo, 2015).
CNTNAP2, is one of the genes with the strongest evidence of autism susceptibility be influenced by multiple lines of genetic evidence (Sampath *et al*., 2013).In mice lacking *CNTNAP2* showed signal similarity to the main deficits of behavioral and mental functions that are observed in autism patients with important its vital role in brain development (Penagarikano *et al*., 2011).
Genomic analysis of *CNTNAP2* showed that single nucleotide polymorphisms (SNPs) can be associated with tendency to these disorders and may provide possible explanations for the phenotypic variability in autism (Abrahams and Geschwind, 2008; Mefford *et al*., 2012) such as impaired language function, abnormal social behavior, intellectual deficiency, schizophrenia and epilepsy (Nascimento *et al.*, 2016). And can be consider SNPs in the *CNTNAP2* gene as genetic markers for tendency to autism (Li *et al*., 2010).

II. MATERIALS AND METHODS:

1. Patients and controls

One hundred and forty five children (95 autistic children and 50 healthy children), for both the sexes

during the period from July to November, 2016 were included in this study. The diagnosed children with an autism were submitted by pediatrician according to Diagnostic and Statistical Manual of Mental Disorders, 4th edition (DSM-IV) criteria, using available historical information from interviews and clinical records.

2. Genotyping

Two mL from blood samples were collected in a sterile K_2EDTA tube. Genomic DNA was extracted from whole blood by using kit (gSYNC™ DNA Extraction Kit 100Preps Cat.No.GS100) according to manufacturer's protocol.

A single nucleotide polymorphisms (SNP) in the *CNTNAP2*(rs7794745) was targeted and selected from the National Center for Biotechnology Information SNP database genotyped. The SNP was genotyped using polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP).PCR reaction to amplify a 311 bp fragment containing the target SNP was performed using primers described in table (1).

Gene (Marker)	Primer Sequences (5´- 3´)	Product	Reference
CNTNAP2 **(rs 7794745)**	F: CAACATTGATCCCTTCAGCCAT R: CTCACCAGTGTGCTTCAGACCA	T(78/223)bp A (311) bp	(Nascimento *et al.*, 2016)

Table (1): Primers sequences used for genes amplification

The final volume of reaction tubes was 25µl, consist of 5µl Tag PCR Pre Mix, 1µl of each forward and reverse of the primers specific for the this gene, 5µl of DNA template and 13 µl nuclease free water. The optimum condition of detection gene was mentioned in table (2).

Step	Temperature(°C)	Time	Cycle
Initial denaturation	95	3 min	1
Denaturation	95	45 sec	35
Annealing	61	45 sec	
Extension	72	45 sec	
Final extension	72	10 min	1

Table (2): Program of *CNTNAP2* gene(Li *et al.*, 2010)with modification

Finally the PCR products were separated on 2% agarose gel and stained in ethidium bromide and visualized under UV light.

Restriction enzyme digestion was carried out using Mluc1 (New England Biolabs) which cuts the wild-type sequence into 311-bp fragment. This A-to-T base pair substitution in the *CNTNAP2* gene creates a Mluc1 restriction site. The digested DNA fragments were subjected to electrophoresis on a 2% agarose gel with ethidium bromide.

Protocol	Volume
PCR Product	5 µl
Restriction enzyme	0.5 µl
Restriction enzyme buffer 10x	4.5 µl
Free nuclease water	3 µl
Temperature/Time	37°C /30 min

Table (3): Reaction condition of Restriction EnzymeMIUCI (Biolab/newengland).

3. Statistical analysis

Statistical analysis was performed using SPSS statistics program version 16. P value of less than 0.05 was considered statistically significant.

III. RESULTS

In the present study, there were 77 males (81%) and 18(19%) females, 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).

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

Table (-4): Distribution and percentages of autistic children and control according to gender.
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%) when compared with the other age groups, that record lower percentage shown in 12-14 years (4.21%) , 9-11 years (7.36%) and less than 3 years group (11.57%). The results showed significant differences (P≤ 0.05) between the age groups.

Age group (Years)	Patients No.(%)	Control No.(%)
Less than 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(26)
Total	95(100)	50(100)

Table (5): Distribution and percentages of autistic children and control according to age group.
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 (6/95) 6.3%.

SNP (rs 779475) of *CNTNAP4* gene

PCR products were of the expected size were 311 bp, fig. (4-10). The results of digestion by restriction enzyme (MICUI) for patients and control samples, showed genotype (AA) do not have mutation (A/T) in the *CNTNAP2*, while, the heterozygous genotype (AT) their PCR products have three bands 78 , 223 and 311 bp. Also, patients and healthy with homozygous genotype (TT) that had a mutation (A/T), they PCR product have two segments 223 and 311bp, as showed in fig. (1).

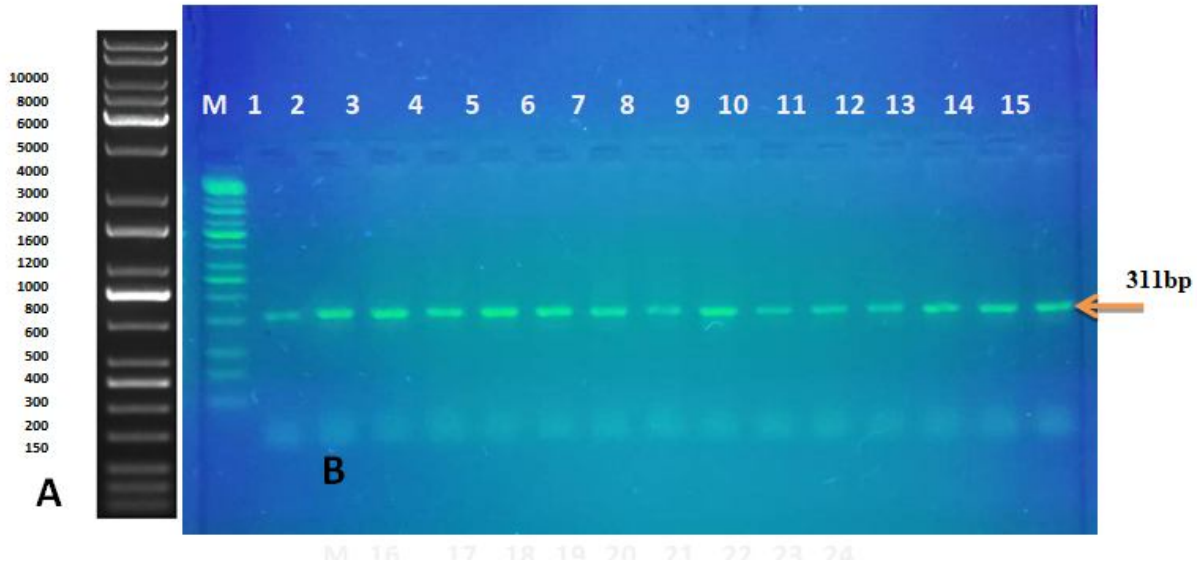

Fig.(1): A; DNA ladder (100bp). B;PCR product the band size 311 bp. The product was electrophoresis on 2% agarose at 70 voltages, 1x TBE buffer for 1:30 hours.

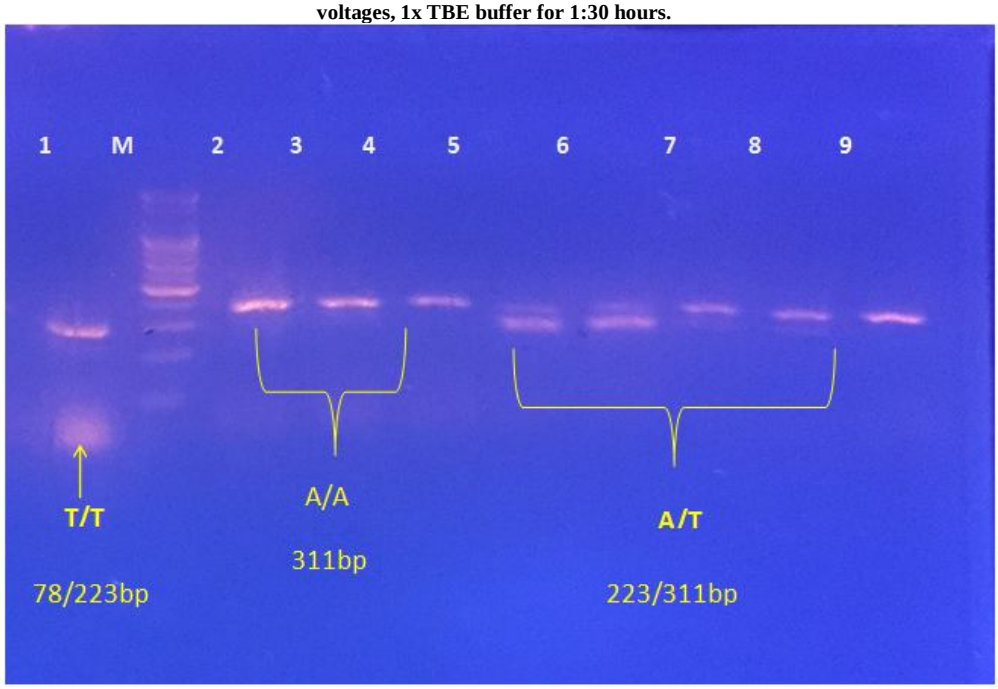

Fig. (3): Agarose gel electrophoresis of *CNTNAP2* gene after incubation with restriction enzyme (MIUCI), amplification on 2% agarose gel at 70 voltages for one hour, where M:DNA ladder (50-1000 bp); 1:T/T genotype, 2,3,4,9: A/A genotype, 5,6,7,8: A/T genotype.

The frequencies of the AA, AT and TT genotypes of rs7794745 were 25.58%, 60.46% and 13.96% in patients and 38.90%, 44.44% and 16.66% in controls, respectively. Thus, the significant association was observed in genotypes distributions of rs7794745 *CNTNAP2* gene polymorphism between autism patients and controls (P≤0.05).

The frequencies of A and T allele patients were 55.81%, 44.19% and the control group were 61.11% and 38.89% respectively (P≤0.05). There were no significant differences in allele frequencies between the two groups, table (6).

CNTNAP2 (rs7794745)	Autistic children(%)	Control (%)
Genotype frequency		
AA	25.58	38.9
AT	60.46	44.44
TT	13.96	16.66
Allele frequency		
A	55.81	61.11
T	44.19	38.89

Table (6): Genotype and allele frequencies of rs7794745 in ASD patients and control.

DISCUSSION

Distributing of the autistic children according to gender, age and sibling

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 (Giarelli *et al.*, 2010; Rose'meyer, 2013; Mezzelani *et al*., 2016), 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 (Betancur, 2011). Other studies have shown the role of hormonal influences in utero as a stimulated factor (Baron-Cohen *et al*., 2011).

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 (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 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 (Christensen *et al.*, 2015) this agree with the present study result that explains age group (3-5years) the highest percentage (41.05%). Following by, the age groups of (6-8years) with a percent (35.78%) this agree with Christensen *et al*.,(2016) study which explains approximately one in 68 children aged 8 years.

In current study, distribution of autistic children according to sibling explain six children were brothers with percentage 6.31%.

Many studies have estimated the recurrence risk of the sibling for autism disorder were 6 to 8% (Veenstra-VanderWeele *et al*.,2004). 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% (Lichtenstein *et al*., 2010 ; Constantino *et al*., 2012) indicates that autism is among the most genetic of neuropsychiatric diseases (Freitag, 2008). It is now well known that the same genetic lesion can lead to different behavioral and mental phenotypes within the same family (Blackwood *et al.*, 2001). And families with more than one autistic child would have increased risk to have an affected infant because the presence of more than one older affected sibling causes a two-fold increase in the risk of autism in the next children(Karimi *et al*., 2017).

Relationship between SNP of*CNTNAPA2*gene and autism:

Genetic and environmental factors had believed making epigeneticchanges in DNA as etiological causes of autism disorder(Millan,2013).The role of some genes and their single nucleotide polymorphisms (SNPs) as genetic funders of diseasesare involved (Nascimento*et al*., 2016).Many studies have proved role polymorphisms of *CNTNAP2* on brain function and some of autistic phenotypes such as abnormal social behavior, intellectual deficit and impaired language functionhave been associated with this gene (Newbury *et al*., 2010; Rodenas-Cuadrado *et al*., 2014).)

Latterly, there are uniting evidence suggests that the *CNTNAP2* gene is a strong candidate gene for predisposition to autism (Stein *et al*., 2011; Penagarikano and Geschwind, 2012; Poot, 2015).

In current study, the results showed there was significant difference in the overall distribution of genotype frequencies between patients and control (P≤ 0.05). However, AT heterozygous was more frequent than TT and AA homozygous. Although, A allele had higher frequency compared with the T allele in both groups, belong to fact that T is the alternative allele while, the A allele is the major allele As well as, recent study found significantly higher frequency of the TT genotype of SNP rs7794745 in autistic children than in healthy control. The T allele of the *CNTNAP2* SNP rs779475 in autistic patients

was (0.43), with significant deference in ASD than healthy control. The presence of the risk allele T in its homozygous form has been associated with altered activation of brain areas responsible for language in non-autistic individuals (Whalley *et al*., 2011). This result alike to Nascimento *et al*., (2016) study, who has been found the frequency of the T allele in autistic patients (0.43) was similar to the frequency of 0.47 described in the database SNP polymorphisms/MAF (Minor Allele Count) of the National Center for Biotechnology Information (NCBI).

Several lines of evidence support that the SNP rs7794745 is associated with autistic disorder (Arking *et al*., 2008). Some studies suggested that T/T in rs7794745 of *CNTNAP2*, was related to autism disorders and has protective effects in healthy subjects. The risk allele of *CNTNAP2* is closely associated with reduced white matter and gray matter volume in ASD (Tan *et al*., 2010), and with a reduction of fractal anisotropy in the cerebellum and frontotemporal cortex (Tan *et al*., 2010). A positive association between the TT genotype and predisposition for autism has been observed by other researchers (Arking *et al*., 2008; Tan *et al*., 2010; Poot, 2015).

Another study has shown that A/T in rs7794745 of *CNTNAP2* gene is a risk genotype in autism compared with A/A (Li *et al*., 2010). These findings indicate that genotype more severely affects the reduction of cerebral response to human voice perception (Koeda *et al*., 2015).

Genetic variations and abnormal gene expression of *CNTNAP2* may increase the risks for specific language impairments by altering brain function during linguistic processing,

Therefore, based on the present study, the results suggested that a common variant of *CNTNAP2* (genotype TT for rs7794745) can contribute to susceptibility to autism, which is in agreement with previous results, that explain it is play a major role in the neural development, any impaired in function of this gene significantly increase the risk of some category of neural dysfunction (Alarcon *et al*., 2008). It was shown that *CNTNAP2* deficiency can causes social deficits linked with autism, hyperactivity, epilepsy (Penagarikano *et al*., 2012) and impairment of brain activity to speak (Ocklenburg *et al*., 2013).

CNTNAP2 is a very large gene spanning more than 2.5 Mb and maps to a region of chromosomal delicacy (Smith *et al*.,2006), So, it is possible that additional variants in this gene, including genomic copy number alterations, could also contribute to autism (Arking *et al*., 2008). Common genomic variants of *CNTNAP2* have been associated with autism as well as related phenotypes such as impaired language function, abnormal social behavior, intellectual deficiency, epilepsy and schizophrenia (Friedman *et al*., 2008; Miles, 2011; Angelidou *et al*., 2012). It was also showed that *CNTNAP2* rare variants may also contribute to the pathophysiology of ASD (Bakkaloglu *et al*., 2008). Genotype analysis showed two different mutations were identified in non-coding region (introns) of the *CNTNAP2* gene, belong to two common SNPs (rs3779031 and rs3779032) were strongly associated with ASDs (Karmeet *et al*., 2015).

CONCLUSION

This study suggest that *CNTNAP2* polymorphisms could be one of the important factors in the neural development related to verbal communication and language processing (Koeda *et al*., 2015). So, there are relationship between of rs7794745 *CNTNAP2* gene polymorphism and autism (Zare *et al*., 2017). Also, other study demonstrated with no significant association was seen between autism traits and this SNP (Jonsson *et al*., 2014; Kourtian *et al*., (2017).

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