Molecular Genetics Study on Autistic Patients in Iraq

Basheer K. Karmeet [1]*Abdul Kareem A. Al-Kazaz [1] and Mahdi Saber [2]
[1] Department of Biotechnology, College of Science, Baghdad University, Baghdad, Iraq.
[2] Department of Biology, College of Science, Wasit University, Wasit, Iraq.

Abstract

This study reflected on the relationship between contactin associated protein-like 2 gene *(CNTNAP2*) and autism spectrum disorders. The study includes forty autistic patients and forty non autistic children as control groups (twenty unaffected sibling and twenty unrelated children). DNA was extracted from Blood samples for molecular detection of *CNTNAP2* mutations associated with ASDs by using Polymerase Chain Reaction (PCR) technique and sequencing analysis. PCR reaction was performed to amplify exon 20 of *CNTNAP2* gene. The PCR results revealed that identical bands related to exon 20 of *CNTNAP2* gene were present in all samples. Therefore, five samples (four from autistic patients and one from control sibling) were selected for genotype analysis of *CNTNAP2* (exon 20) by direct sequencing. Genotype analysis revealed that there were no any variants in *CNTNAP2* (exon 20), but it shows that two different mutations were identified in non coding region (introns) of the *CNTNAP2* gene (Single Nucleotide Polymorphisms (SNPs),ID SNP: rs3779032 A/G in 2118282 position and ID SNP: rs3779031 A/C in 2118436 position). rs3779031 A/C are located at intron 19 while SNP rs3779032 A/G are located at intron 21. These mutations were seen only in autistic patients but not present in control sample. The current study showed that two common SNPs (rs3779031 and rs3779032) in *CNTNAP2* were strongly associated with ASDs, where the frequencies of these SNPs were relatively high. SNP rs3779032 were identified in two autistic patients while rs3779031 were identified in three autistic patients from four unrelated families with ASDs.

Keywords: Autism spectrum disorder, CNTNAP2, PCR, Sequencing.

دراسة وراثية جزيئية على مرضى التوحد في العراق

بشير كاظم خرميط *[1] ، عبد الكريم القزاز [1] ، مهدي صبر [2]
[1] قسم التقنيات الاحيائية، كلية العلوم، جامعة بغداد، بغداد، العراق
[2] قسم علوم الحياة، كلية العلوم، جامعة واسط، واسط، العراق

الخلاصة:

اجريت هذه الدراسة بهدف التعرف على العلاقة بين جين *CNTNAP2* واضطراب طيف التوحد. شملت الدراسة اربعين طفل مصاب باضطراب التوحد و اربعون اخرون غير مصابين باضطراب التوحد كمجاميع سيطرة (عشرون طفل من اشقاء المرضى وعشرون اخرون غرباء). تراوح معدل العمر في جميع الاطفال المتوحدين وغير المتوحدين ما بين3- 10 سنة. استخلص الحامض النووي (DNA) وذلك للتحري عن الطفرات المتزامنة مع اضطراب التوحد في جين *CNTNAP2* من خلال استخدام تقنية تفاعل البلمرة المتسلسل ومتابعة تسلسل القواعد النتروجينية. تم اجراء تفاعل البلمرة المتسلسل لتضخيم

Email Basheerbio2014@yahoo.com

الاكسون 20 من جين *CNTNAP2*. اظهرت نتائج تفاعل البلمرة المتسلسل ان الحزم المتماثلة التي تعود لاكسون 20 في جين *CNTNAP2* كانت موجودة في كل العينات. لذلك تم اختيار خمس عينات (اربع عينات تعود لاطفال مصابين باضطراب التوحد وعينة واحدة لطفل غير مصاب) لمتابعة تسلسل القواعد النتروجينية في هذا اكسون . من خلال النتائج

تبين انه لا يوجد اي تغير في تسلسل القواعد النتروجينية في الاكسون 20، لكن هنالك طفرتان تقع في المنطقة غير المشفرة (Intron) توجد في مرضى التوحد فقط (SNPs rs3779031 C>A and rs3779032 G>A)اوضحت هذه الدراسة ان SNPs هي شائعة ومتزامنة بشكل قوي مع اضطراب التوحد حيث تملك تردد عالي نسبيا. حيث (SNP rs3779031C>A) ظهرت في ثلاث مرضى بينما (SNPrs3779032C>A) ظهرت في اثنين فقط .

Introduction:

Autism Spectrum Disorders (ASDs) are complex neurodevelopmental disorders characterized by severe deficits in socialization, impaired communication, and a limited range of interests and behavior. Autism was originally described by American psychiatrist Leo Kanner in 1943; his work is the basis for the modern definition and diagnostic criteria [1]. A diagnosis of autism can typically be made by 3 years of age [2].

The prevalence of diagnosed ASD in 2011–2012 was estimated to be 2 %. The male to female ratio is approximately 4:1, suggesting a possible imprinting effect and/or involvement of the sex chromosomes [3].

There is an evidence from family studies for the importance of genetic factors in the development of autism spectrum disorders (ASDs). Compelling evidence for a genetic basis for autism has been provided by twin studies, demonstrating a significantly higher concordance rate for monozygous versus dizygous twins, with an overall heritability of 80%–90% [4]. Although ASD is highly heritable, the identification of susceptibility genes has been hindered by the heterogeneity of the syndrome. The hypothesis implicating are genetic heterogeneity, in which rare highly penetrant mutations (some of which may be de novo) in different genes specific to single families have ASD. Several dozens of candidate genes have been tested for association with ASD. This can be attributed to the fact that over one third of all human genes are expressed in the adult or developing brain [5]. ASD are synaptic disorder, reduced dendritic spines have been observed in neurons of autistic patients and nearly all the genes associated with ASD are involved in the formation, regulation, or normal function of the synapse [6].

CNTNAP2 gene is encoding protein involved in various synaptic processes. A rare, common and de novo mutations in the *CNTNAP2* synaptic gene cause ASD in a small number of families [7]. A linkage and association studies demonstrated an increased familial risk for autism with a SNP in intron 2 of the *CNTNAP2* gene (rs7794745) [8]. Independent studies have highlighted significant association between other SNPs and language endophenotypes of ASD, including age at first word (rs2710102) [9] and age at first phrase (rs1718101) [10].

Materials and methods

Forty patients (31 males and 9 females) with Autism Spectrum Disorder were selected for this study. The ages were ranged between 3-10 years. The samples were collected from specialized institutes to care autistic children (Rahman Specialist Centre for the care and service autistic children in Baghdad). Control groups included forty healthy children, twenty unaffected children (without ASDs) of patients' brothers and twenty unrelated healthy children.

Three milliliters of blood were collected by vein puncture in EDTA tubes from all patients and control groups and stirred gently for few seconds to avoid blood's clotting. The genomic DNA isolation from the whole frozen blood collected in EDTA anticoagulant tubes was applied using Geneaid genomic DNA purification kits (Geneaid, USA). After genomic DNA extraction, agarose gel electrophoresis was adopted to confirm the presence and integrity of the extracted DNA [11]. The PCR reaction and specific primers (**Forward**/ 5`GACATCAAGGGAGGGAGTAAAG3`, **Reverse**/5`CTATCCCCTCAAAA CAAAACCA 3`) were used to amplify exon 20 of *CNTNAP2* gene [12]. Components of PCR reaction and mixing amounts were shown in table 1.

Table 1- PCR component for amplification of exon 20 of *CNTNAP2* gene

Component	Concentration	Component of one sample (µl)	Component of 10 sample (µl)
Deionized water	---	6.5	65
Green master mix	1 X	12.5	125
Forward primer	10 picomols/ µl	1.5	15
Reverse primer	10 picomols/ µl	1.5	15
DNA Sample	0.05-0.15µg/ µl	3	*
Total Volume	---	25	220

* DNA template 3 µl for each sample.

Optimization of PCR reaction was accomplished after several trials; thus the following program was adopted. The PCR reaction was carried out as shown in table 2.

Table 2- PCR Program for *CNTNAP2* gene (Exon 20)

Steps	Temperature (C°)	Time	No. Of cycles
Initial denaturation	95	5 min	1
Denaturation	95	30 sec	35
Annealing	56.5	30 sec	
Extension	72	45 sec	
Final extension	72	7 min	1

Sequencing of *CNTNAP2* gene (Exon 20)

PCR product was purified for sequencing by using EZ-10 spin column DNA cleanup minipreps Kit (Bio Basic Inc., Canada). The PCR products (5 samples) of the *CNTNAP2* (exon 20) and primers were sending to Source Bioscience Company (Nottingham, UK) for sequencing.

Results and Discussion

Description of the study sample

The results presented in this study were based on analyses of data from a total of 80 cases: 40 Autistic children, 20 non autistic (siblings) and 20 non-relatives healthy children. The results of this study pointed to the higher risk of autism in males 31 (77.5 %) than in females 9 (22.5%) with a ratio of 4:1 table 3.

Table 3- Frequency rates for cases and controls according to gender group

Case-control	Gender				Total
	Male	%	Female	%	
Autism	31	77.5	9	22.5	40
Non autistic (siblings)	9	45	11	55	20
Control (non relatives)	10	50	10	50	20
Total	50		30		80

The high risk of autistic males may be due to several X-linked genes that are found to be ASD risk genes. The current results agreed with other studies, Jamain *et al*. [13] who observed that Mutations of the X-linked genes were associated with autism. Werling and Geschwind, [14] observed that there is sex differences in autism spectrum disorders.

Polymerase Chain Reaction (PCR) analysis

The PCR results revealed that identical bands related to the *CNTNAP2* exons were present in all samples. Exon 20 PCR amplified regions showed a molecular weight of 667 bp figures 1.

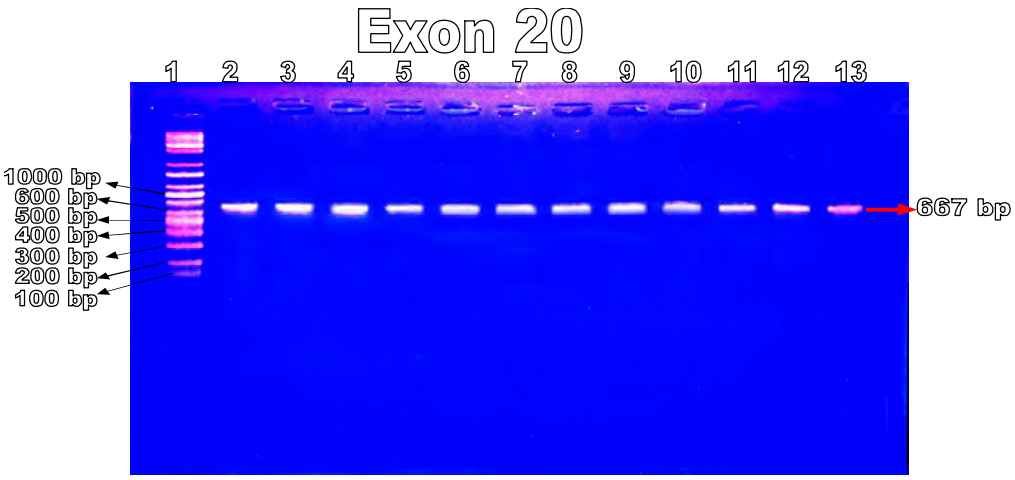

Figure 1-Gel electrophoresis of CNTNAP2 gene -exon 20 on 2% agarose gel at 70 voltages for one hour Lane 1: DNA ladder (100-2000 bp).
Lane (2-7): PCR products of the exon 20 from Autistic children.
Lane 8, 9 and 10: PCR products of the exon 20 from non autistic sibling.
Lane 11, 12 and 13: PCR products of the exon 20 from healthy children.

***CNTNAP2* gene sequencing**

Genotype analysis of *CNTNAP2* gene by direct sequencing revealed that there was no genetic alteration in exon 20. Moreover, the current study failed to replicate positive results of the *CNTNAP2* gene variants (exon 20) associated previously with autism and described by Bakkaloglu *et al*. [12], suggesting that further research is required to elucidate the role of this exon in ASD. Sequence analysis of *CNTNAP2* gene, summarized in table 4 showed that there were two mutations (SNPs) located in non coding region (introns), (rs3779031 A/C in 2118437 position are located in intron 21, rs3779032 A in 2118281 position are located in intron 19).

Table 4- Analysis of *CNTNAP2* gene (Subjects 1, 2 and 3, 4 represent autistic children while subject 5 represent non autistic child (sibling))

Intron	Subjects	Sequence (5`-`3)
21	1 2 3 4 5	ATACATGTACCTATAAATTAC ATACATGTACCTATAAATTAC ATACATGTACCTATAAATTAC ATACATGTACATATAAATTAC ATACATGTACATATAAATTAC ↓ SNP A/C: rs3779031
19	1 2 3 4 5	TTTTTCTTCTATAGGAAGCTT TTTTTCTTCTGTAGGAAGCTT TTTTTCTTCTGTAGGAAGCTT TTTTTCTTCTATAGGAAGCTT TTTTTCTTCTATAGGAAGCTT ↓ SNP A/G: rs3779032

The current study showed that two common SNPs (rs3779031 and rs3779032) in *CNTNAP2* are strongly associated with ASD where the frequencies of these SNPs were relatively high. SNPs (rs3779031 and rs3779032) are predicted to be deleterious (functional variants), where rs3779031 were identified in three affected individuals while rs3779032 were identified in two affected individuals from four unrelated families with ASD but was not present in control.

The current study agrees with other studies reported that common genetic risk variants in *CNTNAP2* associated with abnormal functional brain connectivity in humans [15] and specific-language impairment [16]. Two association studies suggest that common noncoding variants of *CNTNAP2* may be involved in autism susceptibility: single nucleotide polymorphism (SNP) rs2710102 in intron 13 showed an association with the 'age at first word' trait in autistic patients [9] and rs7794745 in intron 2 was associated with autism in an independent sample [8]. The multiple lines of evidence associating with the SNP rs2710102 provide strong support for contribution of this region to language endophenotypes [17, 18]. Other studies found that a common single nucleotide polymorphism (SNP) of *CNTNAP2* (rs7794745), known to be involved in autism [19, 20]. The results of these studies converge on *CNTNAP2*, suggesting that common variants in this gene may be involved in autism and influence language traits.

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