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Association of the Intronic Polymorphism rs3773364 A>G in Synapsin-2 Gene with Epilepsy Patients in Iraq

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Abstract

Epilepsy is a central nervous system disease which is characterized by a recurrent seizure that distinguishes it from other similar diseases. Epilepsy may occur due to defects in genes that encode some receptors in the brain. For this reason, this study aimed to understand the association between Synapsin-2 (*SYN2*) gene and susceptibility to epilepsy. Blood samples were collected from 40 volunteers, including 30 patients suffering epilepsy with an age range of 26-49 years old and 10 healthy individuals with an age range of 25-53 years old. The study sample involved 16 males and 14 females with epilepsy along with 6 males and 4 females healthy subjects. DNA was isolated from the volunteers for PCR-RFLP assay. Genotyping of rs3773364 A>G *SYN2* was conducted and the results refer to a highly significant difference in the distribution of AG genotype ($P=0.0001$), while there is no significant difference in the distribution of AA and GG genotypes, with p values of 0.1702 and 1.00, respectively. The results also showed that gender did not significantly affect the results when comparing patients with the control ($p=0.0934$). Our findings indicates that *SYN2* rs3773364 A>G confers risk to epilepsy and may be implicated in epileptogenesis.

Keywords: Epilepsy, *SYN2* Gene Polymorphism, rs3773364 A>G, PCR-RFLP

ارتباط تعدد الاشكال في الانترون عند rs3773364 A>G لجين سينابسين 2- مع مرضى الصرع في العراق

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الخلاصة

يعتبر الصرع مرض النظام (الجهاز) العصبي المركزي و يتميز بنوبات متكررة تميزه عن أعراض الأمراض العصبية الأخرى. ينتج الصرع من خلل في الجينات المشفرة لبعض المستقبلات في الدماغ، لهذا السبب هدفت هذه الدراسة لبيان العلاقة بين جين *SYN2* وقابلية التأثر بالصرع. جمعت عينات الدم من 40 متطوع (30 منهم مصاب بالصرع تتراوح أعمارهم بين 26-49 عام و 10 سليمين من أي مرض وتتراوح أعمارهم بين 25-53 عام) متكونين من 16 ذكر و 14 أنثى يعانون من الصرع و 6 ذكور و 4 إناث سليمين، وتم عزل الحمض النووي من المتطوعين لفحص PCR-RFLP. اجري التتميط الوراثي لجين rs3773364 *SYN2* A>G وتشير النتائج إلى وجود فرق معنوي كبير في توزيع التركيب الوراثي AG حيث كانت قيمة P

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0.0001 =، بينما لا يوجد فرق معنوي في توزيع النمط الوراثي AA و GG الذي له قيمة $P = 0.1702$ و $P = 1.00$ على التوالي. كما أظهرت النتائج أيضا إن الجنس غير مؤثر عند مقارنة مجموعة المرضى مع مجموعة الأصحاء حيث كانت قيمة $P = 0.0934$. بينت الدراسة الحالية على إن SYN2 rs3773364 A>G يساهم في خطر الإصابة بالمرض وقد يكون سببا في العمليات التي تؤدي إلى حدوث الصرع.

1. Introduction

Epilepsy is one of the most known neurological disorders with the major characteristic being seizures [1]. This disease is considered a neurological disorder with symptoms like recurrent seizures. Being a genetic disorder, its onset can be associated with several genes having their roles at different levels of metabolism or cell signaling [2]. However, with adequate care and treatment, most of the patients lead a yet normal lifespan [3]. In Iraq, some studies attempted to design more efficient methods to detect epileptic seizures. One study used Electroencephalogram records (EEG) data to design an algorithm to detect epileptic seizures in patients suffering epilepsy. This gives an easier and faster way to detect and diagnose epilepsy, where it is very difficult for physicians to detect it visually [4]. However, on the molecular level, the PCR-RFLP technique is used widely in the molecular assays in Iraq for diagnosing and analyzing some of the genetic diseases, including epilepsy, diabetes, and many others. For example, in Basra city (south of Iraq) PCR-RFLP was used to study the association between the IL-10 gene and diabetes mellitus [5].

The SYN2 gene is a family member of the synapsin genes. These genes encode neuronal phosphoproteins, the phosphorylation of which being involved in the regulation of all nervous system processes. The cytoplasmic surface of the synaptic vesicles is correlated with this kind of proteins. Family members are described by common protein domains, which are involved in synaptogenesis and neurotransmitter release regulation, indicating a possible role in many neurological disorders. This gene encodes a neuron-specific phosphoprotein that specifically attaches to the presynaptic nerve terminal's tiny synaptic vesicles. In this gene, polymorphisms and mutations are associated with presynaptic functional defects and related neuronal disorders, including epilepsy, schizophrenia, bipolar disorder, and autism [6]. These proteins are assumed to selectively control neurotransmitter release where they are bound to small synaptic vesicles in the presynaptic nerve terminal involved in synaptogenesis [7]. SYN2 is considered one of the genes of the synapsin family, which stretches to more than 140 kb regions on chromosome 3 in humans, where there are 14 exons. Early published information on genetic variants of SYN2 refers to a variation in this gene that is involved in susceptibility to schizophrenia and febrile seizures [8, 9]. Single nucleotide polymorphism of rs3773364 A>G was found to be associated with epilepsy Indian and Caucasian patients. SYN2 is encoded by three distinct genes in mammals, which are SYN1, SYN2, and SYN3 [10].

Synapsin II refers to two identical phosphoproteins, namely synapsin IIa and synapsin IIb, which are encoded by the SYN2 gene in humans. Synapsins associate to the surfaces of synaptic vesicles as endogenous substrates and act as regulators of the release of neurotransmitters in the nervous system through the presynaptic membrane of axonal neurons [11, 12]. Recent studies have shown that SYN2 also plays a role in the post-docking stages of release and their disturbance leads to imponderables between the activities of inhibitory and excitatory neurons [13, 14]. The aim of the current study was to look for a potential correlation between the SYN2 gene and epilepsy patients in Iraq by using the restriction fragment length polymorphism technique (PCR-RFLP) for rs3773364 A>G region.

2. Materials and Methods

Patients and Controls

A blood sample was taken from each person of 40 volunteers, including 30 patients (16 males and 14 females) with an age range of 26-49 years and 10 controls (6 males and 4 females) with an age range of 25-53. All samples of epilepsy patients were taken from the Hospital of Neurological Science, Baghdad, Iraq. The patients were diagnosed and classified by experienced neurologists. Samples collection period extended from November 2018 to April 2019. The entire participants were of unrelated Iraqi origin and had similar geographic and socio-demographic data. All patients provided their informed written approval to participate in the study and allow their blood samples to be taken for molecular assay.

Exclusion criteria

Exclusion criteria involved patients who have another neurological disease accompanied by epilepsy

or affects *SYN2* gene, which may lead to misinterpretation of the results.

DNA Extraction

Genomic DNA was isolated from 5mL of blood samples (isolated blood samples were stored in EDTA tubes and readily transported to the lab for DNA extraction) in compliance with the protocol of ReliaPrep™ Blood gDNA Miniprep System kit (Promega / USA). Polymorphism rs3773364 A>G was assessed by PCR restriction-fragment length polymorphism (PCR-RFLP). The primers were designed based on Lakhan *et al*, 2010 study (Table 1).

Table 1- Primers included in the present research[10]

SNP	Primer sequence	Restriction enzyme/ Products bp
SYN2 A>G (rs3773364)	5'-TCTGCTAGTTCATGGGAGTGG-3' 5'-AGAGCCTTTTCCAACCCAATC-3'	DdeI 146; 81,65

Genotyping of *SYN2* (rs3773364)

The PCR-RFLP technique is used in this research to carry out genotyping. In this technique, the *SYN2* intron-5 region was amplified using the site-specific primer found in Table 1. The reaction was conducted under the optimal conditions in PCR, with initial denaturation at 94 °C for 5 minutes through one cycle and 45 cycles of denaturation, annealing, and extension. Each denaturation cycle continued for 30 seconds at 94 °C, and each annealing cycle continued for 45 seconds at 55 °C, while the extension cycles lasted for 30 seconds at 72 °C. The final extension product was achieved through one cycle at 72 °C for 7 minutes. PCR reaction mixture included 4 µL of DNA sample, 12.5µL of OneTaq (NEB®) master mix, 4.5µL of free-nuclease water, and 2µL 10 pmol/µL of each primer. Single nucleotide polymorphism of rs3773364 A>G in intron 5 generated the restriction fragment at the target nucleotide site. The product size of 146 bp was yielded. The G allele yielded two fragments with product sizes of 84 and 62 base pairs, respectively, after the restriction digestion with DdeI. Polyacrylamide gel electrophoresis (15%) was used to separate the restriction endonuclease-digested products, and genotyping patterns were reported as indicated in Figure- 1.

Statistical Analysis

The Statistical Analysis System- SAS (2012) program was used for detecting the effects of different factors in the study parameters[15]. SNP genotype frequencies were calculated according to Hardy-Weinberg equilibrium. Chi-square test was used for significantly comparing between percentages (0.05 and 0.01 probability). The estimates of Odd ratio and CI were applied in this study. A two-tailed p-value of $p < 0.05$ was considered significant.

3. Results and Discussion

In the present study, blood samples were taken from forty volunteers (30 patients with epilepsy and 10 healthy people). There were 16 males and 14 females who suffered epilepsy, while in the control group, there were 6 males and 4 females. Statistical analysis for gender shows that is no significant difference, where the p-value for gender distribution was 0.0934 (Table- 2). In the present study, the mean age was 20.32 ± 1.76 in the patients and 47.00 ± 2.05 in the control, with a highly significant difference ($p=0.0001$).

Table 2- Results and distribution of gender and age in epilepsy patients and control

Factors	Patients group=30 No. (%)	Control group=10 No. (%)	P-value
Gender			
Male	16 (53.85%)	6 (60.00%)	0.0934
Female	14 (46.15%)	4 (40.00%)	
Mean ± SE of Age (year)	20.32 ± 1.76	47.00 ± 2.05	0.0001

The Polymorphism of rs3773364 of *SYN2* Gene

Genotype and allele frequency were determined in the 40 samples by PCR-RFLP assay. The current results found that the frequency of rs3773364 AG of SYN2 polymorphism is highly significant in patients groups compared with healthy controls, as shown in Figure- 1 and Table- 3 ($p=0.0001$, OR=0.684, C.I=0.353-1.327).

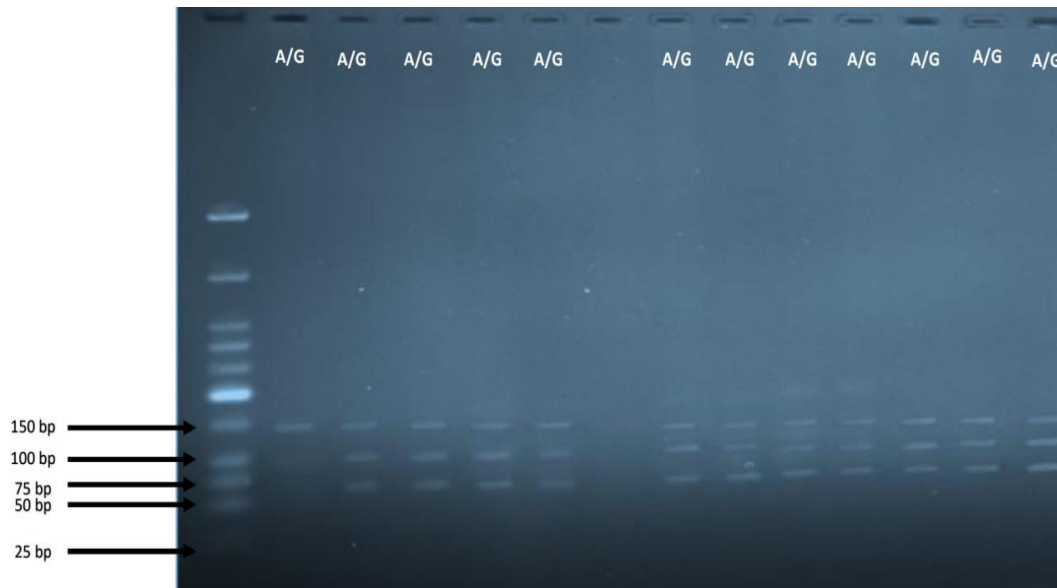


Figure1- Representative gel picture on 3% agarose gel for SYN2 A>G (rs3773364) genotyping, the voltage was mostly fixed for 80 minutes between 70-80V.

We also observed no significant difference in the distribution of the AA and GG genotypes, where the GG genotype was not present in any of the samples while AA genotype had results of $p=0.1702$, OR=1.857, and C.I=0.766-4.505 (Table- 3). The allele frequency for A allele was 0.63 in the patients and 0.75 in the control group, while that for the G allele was 0.37 in the patients and 0.25 in the control.

Table 3-Distribution of gene polymorphism and allele frequency in patients and control groups

Genotype	Patient(N=30) No. (%)	Control (N=10) No. (%)	P-value	Odd Ratio (C.I)
AA	9 (26.92%)	5 (50.00%)	0.1702 NS	1.857 (0.766-4.505)
AG	21 (73.08%)	5 (50.00%)	0.0001	0.684 (0.353-1.327)
GG	0 (0.00%)	0 (0.00%)	1.00	---
Allele frequency				
A	0.63	0.75		---
G	0.37	0.25		---

These results come in accordance with a study conducted in India that found a highly significant difference in the genotypes of rs3773364 AG of SYN2 polymorphism. The study observed a high distribution of AG genotype of rs3773364 in patients versus control. Furthermore, the study did not observe a significant difference in GG genotype between Indian control and patient individuals[10] (Lakhan *et al*, 2010). Another study on four Caucasian populations was conducted who analyzed SYN2 SNP, where they found a relationship between SYN2 and febrile convulsions only, but not with other seizure syndromes, which indicates the importance of SYN2 SNP in epilepsy[9].

Different results were observed in a study in Malaysia, where no association was found between the polymorphism of SYN2 rs3773364 A>G and epilepsy susceptibility. Therefore, this SNP of SYN2 was not considered as a risk factor for epilepsy susceptibility. Such findings are contrary to those of

our study as well as the Indian and Caucasian studies, where we found that there is a relationship between *SYN2* AG genotype distribution and epilepsy [16].

The distribution of the AA genotype of *SYN2* gene for the patients younger than 20 years old was 42.86%, with the same result for those between 20 to 30 years, whereas it was 14.29% for the patients with an age older than 30 years (Table 4). The AG genotype distributions were 57.89% for younger than 20 years, 36.84% for the age between 20-30 years, and 5.26% for older than 30 years. Thus, the difference in the distribution of the *SYN2* gene polymorphism with age was highly significant $p=0.0001$. On the other hand, the distribution of the AA genotype according to gender demonstrated 42.86% of affected males and 57.14% of affected females, while the AG genotype was present in 57.89% of affected males and 42.11% of affected females, reflecting a highly significant difference ($p=0.0001$). The results showed that the AG genotype distribution is higher in males and in the age of lower than 20 years, while AA genotype proportion is slightly higher in females.

Table 4- Distribution of gene polymorphism with age and gender

Factors	Levels	Genotype			P-value
		AA = 9 No. (%)	AG =21 No. (%)	GG = 0 No. (%)	
Age	Least than 20 yr.	4 (42.86%)	12 (57.89 %)	0 (0.00%)	0.0001
	20-30 yr.	4 (42.86%)	8 (36.84%)	0 (0.00%)	
	More than 30 yr.	1 (14.29%)	1 (5.26%)	0 (0.00%)	
Gender	Male	4 (42.86%)	12 (57.89%)	0 (0.00%)	0.0001
	Female	5 (57.14%)	9 (42.11%)	0 (0.00%)	

The study found a correspondence between *SYN2* (AG genotype) and idiopathic epilepsy, where the patients displayed a high ratio of AG genotype [10]. This result agrees with our findings. Many types of idiopathic epilepsy in humans and animals are a result from a deficiency of synapsin and phosphoprotein modulation, which have roles in regulating the release of neurotransmitters. Synapsin I and synapsin II, that are encoded by *SYN1* and *SYN2*, respectively, are naturally expressed in the central nervous system, where they have a relationship with synaptic vesicles, both GABA-containing vesicles at the inhibitory synapses and glutamate-containing vesicles at the excitatory synapses. Additional evidence for the implication of these genes in epilepsy in animals comes from mice experiments. It was found that the deletion in either *SYN1* or *SYN2* is associated with aggressive epileptic phenotypes, so that both genes are considered to be the most important epilepsy related genes [17]. Despite brain anatomy did not exhibit acute defect, *SYN1* and *SYN2* show spontaneous seizures, while *SYN3* did not show any epileptic seizure. However, cognitive impairment in the brain that results from knockout of both *SYN2* and *SYN3* may suggest the important role of *SYN2* in the control of high brain functions [18].

There is a strong underlying genetic basis for individuals with idiopathic generalized epilepsy (IGE) disorder. Patients with an IGE are typically otherwise normal and they have no abnormalities in brain structure. Individuals with IGE usually have a family history with this disorder, where those individuals show an increase in genetic predisposition to have a seizure. The genetic reasons for many IGE forms are known, although this disease does not follow the monogenic mechanism of inheritance. Seizure in IGE can be generalized and cover both halves of the brain or it can be a focal seizure which begins and remains on one side of the brain. This disorder is usually emerging in childhood and adolescence, although it is diagnosed later [19]. A mutation in this gene was also found to increase predisposition to autism spectrum disorder (ASD), which is a heterogeneous disorder that is described by rigid, abnormal language development, impaired social relationships, and repetitive behavior. In many cases, it was found that patients with ASD have epileptic seizures (approximately one-third patients had an epileptic seizures) [20-21].

4. Conclusions

Our study demonstrates the importance of *SYN2* rs3773364 A>G in increasing predisposition to epilepsy, where AG genotype may be conferring a risk factor to epilepsy in the Iraqi population. The results show that the different distribution of *SYN2* genotype with age and gender is highly significant, where AA genotype distribution is slightly higher in females, while AG genotype distribution is higher

in males and in those with age less than 20 years. The study also demonstrates no significant difference in gender when comparing patients with the control group $P=0.0934$.

Conflict of Interest The authors declare that they have no conflict of interest.

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