ISSN: 0067-2904

Genetic Polymorphisms rs643627 in Serotonin Receptor Gene *(5-HTR2A)* with Schizophrenia

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Received: 28/6/ 2019 Accepted: 31/ 7/2019

Abstract
Schizophrenia(SCZ) is oneof the most destructive and complicated chronic diseases of the human nervous system. Serotonin receptors have been involved in the pathophysiology of psychiatric disorders including schizophrenia. Fortyschizophrenia subjects (14females and 26 males) with an age range of 23– 57 years were enrolled, in addition to twenty healthy control subjects (10female and 10 male) with an age range of 19-44 years.
This study aimed to evaluate the frequency of one single nucleotide polymorphism (SNP), namelyrs643627 in *HTR2A*gene,inIraqi patients with schizophrenia in comparison with controls, along with the association between this SNP and the incidence of schizophrenia.
The genetic variantrs643627 within the intron region of 5-*HTR2A*gene was genotyped by Real Time –PCR.
The results showed that differences in the demographic data of gender and age between schizophrenia subjects and controls were statically non-significant. Also, the genotype frequencies distribution of rs643627 polymorphism showed no deviation from Hardy–Weinbergequilibrium in both groups (patients and controls). In addition,differences in the genotypes(AA, AG, and GG) and allele frequencies of *5-HTR2A* were statically non-significant between SCZ patients and controls. However,the present study results demonstrated an association between rs643627 polymorphism of *5-HT2A*geneand age and gender in schizophrenia patients group.

Keywords: Schizophrenia, serotonin,*5-HTR2A,* Polymorphism, RT-PCR.

تعددالأشكال الوراثيrs643627 في مستقبلات السيروتونين لجين(*5-HTR2A*) عند مرضى انفصام الشخصية

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الخلاصة
يعتبر مرض الفصام (SCZ) واحد من اكثر الامراض تدميرا وتعقيدا لكونه يصيب الجهاز العصبي. وتعد مستقبلات السيروتونين احدى الاسباب الفيزيولوجيا المؤدية للاضطرابات النفسية بما في ذلك الفصام (SCZ). وقد اجريت هذه التجربة على أربعين مريض بالفصام(14 اناث و 26ذكور) وكانت أعمارهم تتراوح بين23 و57 سنة. بالإضافة إلى ذلك تمت الاستعانة بعشرين متطوع من الاصحاء (10اناث و 10ذكور) وتتراوح أعمارهم بين 19 و 44 عامًا. وتهدف هذه الدراسة لتقييم تعدد الاشكال الوراثية ل rs643627 في

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جين *5-HTR2A* عند مرضى عراقين مصابين بالفصام من خلال مقارنتهم مع اخرين اصحاء وايضا دراسه العلاقه بين هذا التعدد للاشكال الوراثيه وحدوث المرض .

تم تحديد النمط الوراثي ل rs643627 الذي يقع في المنطقه غير المشفرة لجين 5-HTR2A باستخدام تقنية RT-PCR.

اظهرات النتائج ان البيانات الديموغرافيه للجنس والعمر عند مرض الفصام والاحصاء قيم غيرذات دلالة احصائية في حين ان توزيع ترددات الانماط الاليلية ل rs643627 لم يظهر اي انحراف عن توازن هاردي وينبرج في كلتا المجموعتين (المرضى والاصحاء) بالاضافة الى ذلك فان الانماط الوراثية (AA,AG,GG) وترددات الاليل كانتلقيم غير ذات دلالة احصائيه عن مرضى الفصام والاصحاء ومع ذلك فان النتائج الحالية تظهر وجود علاقة بين تعدد الاشكال ل rs643627 في جين *5-HTR2A* مع العمر والجنس عند مجموعه مرضى الفصام .

Introduction

Schizophrenia is a serious and chronic mental disorder influencing more than twenty one million people around the world. It is characterized by errors in thinking, deformations in perception, emotions, feelings, sense of self and behaviour [1, 2]. The mutual experiences include delusions, hearing voices and seeing things (WHO, 2016). The etiology of schizophrenia is complicated, and many genetic studies have had a guiding influence on schizophrenia research. A genome-wide association study (GWAS) proposed that schizophrenia is a complex and polygenetic disease with heritability that reach to over 80% [3, 4]. Large numbers of studies have also focused on the neurotransmitters associated withthe pathogenesis of schizophrenia, including serotoninsystem. Serotonin plays an import and main role in different brain activities, including learning, pain, emotions and memory [5]. The neuromodulator action of serotoninsystem on brain depends largely on the actions of serotonin receptors (5-hydroxytryptamine, or 5-HT), which consist of at least fourteen different classes and subtypes [6]. The serotoninreceptor type twois a G protein coupled receptor (GPCR) thatworks as a primary target for serotonin signalling andis expressed on numerous cell types in the brain and periphery regions and [7]. The gene*5-HT2A* has a great scientific interest due toits multiples roles in normal biological functions, such as cerebral cortex excitability [8], platelet aggregation,dilation,vasoconstriction, smooth muscle contraction, inflammatory processes [9], and hormone signalling [10]. Among many tissues expressing *5-HT2A*, it is particularly prevalent in the cerebral cortex where it is enriched at the apical dendrites of pyramidal neurons [11]. Proportionate with its broad biological influences, studies examining single nucleotide polymorphisms in the gene encoding 5-HT2A *(HTR2A)* have identified more than hundred genotypic associations with a wide range of phenotypes, especially brain-related disorders [12,13]. Drugs that directly or indirectly adjust serotonergic signalling through *5-HT2A* receptors are used to treat neurologic, neuropsychiatric, and cardiovascular conditions, and 5-HT2A is an emerging drug target for a variety of other conditions[14,15,16].

Materials and Methods

Subjects

The study sample was composed offortySCZpatients (26 male and 14 female) and twenty genetically unrelated healthy volunteers (10 men and 10 women).Collection of blood samples extended through the period fromOctober 2018 to February 2019.Patients' blood samples were collected from IbnRushdPsychiatry, Baghdad, Iraq. All patients providedtheir informed written approval to participate in the study. Diagnosis was confirmed by psychiatric observations performed by experienced psychiatrists. The entire participants were of unrelated Iraqi origin, and had similar geographic and socio demographic data.

DNA Extraction

DNA was extracted from blood leucocytes usingReliaPrep^{TM} Blood g DNA Miniprepkit (promega / USA) according to the manufactures instructions. Polymorphism rs643627was assessed by real time polymerase chain reaction (RT-PCR). Primer and probe sequences were designed by Macrogene Company (Korea).

Genotyping

The genetic polymorphism rs643627 within the 5-HTR2A gene was the chosen variant to be genotyped by PCR technique. RT-PCRwas performed with the senseprimer HTR2A:

CCCAAGTCTGAAATGAAC andanti-sense primer HTR2A: CAGCGATGTATCTAATAAGC. In addition, two probes were used; sensewild probe CATGAGCTCTATTATGTGCCCCTCTT and sense mutant probe CATGAGCTCTATTGTGTGCCCCTCTT.

Real-time PCR was performed in a MicqPCR Cycler from Bio Molecular System, Australia, using Go Taq® Probe qPCR Master Mix kit. The 10 µl PCR contained 5µl of GoTaq® qPCR 2x Master Mix Promega (USA), 0.5 µl of each10 µM primer (Primer F and Primer R), 0 .5 µl of each probe 10 µM (Probe F and Probe R), 1.5 Nuclease free water and 1.5 µl of DNA.The reactions were performed in a 48-well plate with MicqPCR Cycler (Bio Molecular System/ Australia). Optimization for gradient annealing temperatureprogram was used to all probes and the most ideal annealing temperature was 60°C.

Initial incubation started at 95°C for 5 minutes followed by 40 cycles at 95°C for 15 seconds (Denaturation step), 60°C for 30 seconds (Annealing step), and 72°C for 30 seconds (Extension step).

Statistical analysis

Data analysis was performedby using SPSS for Windows, version 22(SPSS Inc. Chicago, Illinois, United States). Independent samples t-test was used to compare between means of the studied groups. Additionally, Hardy-Weinberg equilibrium was calculated using a web tool [17]. Odds ratios (ORs) with a 95% confidence interval (CI) were also calculated. A two-tailed *p* value ($p < 0.05$) was considered significant [18].

Categorical variables were analysed by Chi-square test. Bonfferoni Post Hoc test for multiple comparisons wasapplied after ANOVA test.

Results and Discussion

Characteristics of study subjects

The clinical and demographic characteristics of the sixty participants are shown in Table-1.In this study, the mean ages of controls and patients were37.20±7.58 and 39.90±9.02,respectively.There were no significant differences in age (P=0.67)and gender (P=0.264) between controls and patients groups .

Table 1-General characteristics of studied groups

Characteristics	C group (N=20)	P group (N=40)	P value
Age	37.20±7.58	39.90±9.02	0.67
Gender			0.264
Male	10 (50%)	26 (65%)	
Female	10 (50%)	14 (35%)	
Total	20 (100%)	40 (100%)	

5-HTR2Agenotyping and allele frequency

The genotype frequencies distribution of *5-HTR2A* polymorphism that were observed in both groups (Schizophrenia patients and controls) were consistent with those predicted by Hardy-Weinberg equilibrium (P>0.05) shown in Table-2.

Table 2-Number and percentage frequencies of (HTR2A) gene genotypes and their Hardy-Weinberg equilibrium (HWE) in C group and P group

Genotype	C group (n=20)		P value	P group (n=40)		P value
	Observed N (%)	Expected N (%)		Observed N (%)	Expected N (%)	
GG	6 (30)	7.2 (36)	P>0.05	13 (32.5)	12.1 (30.25)	P>0.05
AG	12 (60)	9.6 (48)		18 (45)	19.8 (49.5)	
AA	2 (10)	3.2 (16)		9 (22.5)	8.1 (20.25)	

In the present study, the results showed that the frequency of wild GG genotype was higher in patients(13; 32.5 %) than in the apparently healthy subjects (6; 30%) ,(OR =0.89. 95 %CI =0.28-2.85. P=0.84). The frequency of heterozygous AG genotype was higher in patients than in healthy subjects (18 (45%) versus 12 (60%), OR =1.83, 95 %CI = 0.62 -5.45, P=0.27), and the frequency of the mutant AA genotype was higher in patients than in healthy subjects (9 (22.5 %) versus 2 (10 %), OR= 0.38, 95 %CI =0.07-1.97, P=0.24).

In the analysis of allele distribution, there were no significant differences between the carriers of G allele in schizophrenia patients and controls (44(55%) versus 24 (60%), OR=1.23, 95 %CI= 0.57-2.65, P=0.60), and between the carriers of A allele in schizophrenia patients and controls (36 (45%) versus 16 (40%), OR=0.82, 95 % CI =0.38-1.76, P= 0.60). Therefore, the genotypes and allele frequencies showed non-significant difference between the patients and controls, and rs643627 within *5-HTR2A* was not associated to schizophrenia. Genotypes and allele frequencies of the SNP (rs643627) are summarized in Table-3.

Table 3-Genotype and allele frequencies of (5-HTR2A) gene in C and P group

Genotype / Allele	C group (N=20)	P group (N=40)	OR	95% CI	*P* value
	No. (%)	No. (%)			
GG	6 (30)	13 (32.5)	0.89	(0.28-2.85)	0.84
AG	12 (60)	18 (45)	1.83	(0.62-5.45)	0.27
AA	2 (10)	9 (22.5)	0.38	(0.07-1.97)	0.24
G	24 (60)	44 (55)	1.23	(0.57-2.65)	0.60
A	16 (40)	36 (45)	0.82	(0.38-1.76)	0.60

The association between HTR2A rs643627 polymorphism and gender

The schizophrenia samples consisted of 24 males and 14 females. The results showed that the frequency of AG and AA genotypes were statistically higher in males than females (AG: 14(53.8%) versus 4(57.1%); AA7:7 (26.9%) versus 2(14.3%)), respectively).The frequency of GG genotype was lower in males than females (5(19.2%) versus 8 (57.1%)). Statistically significant differences werefound between gender and the distribution of genotypes (P=0.04).It was found that the variation rs643627 within the 5-HTR2A gene is associated with the socio- demographic features (gender) in schizophrenia patients,as shown in Table-4.

Table 4-Distribution of patients (5-HTR2A) genotypes by gender

Genotype	Gender		P value
	Male group (N= 26)	Female group (N= 14)	
AG	14 (53.8%)	4 (28.6%)	0.04
AA	7 (26.9%)	2 (14.3%)	
GG	5 (19.2%)	8 (57.1%)	

Significant value (*P*<0.05)

The association between HTR2A rs643627polymorphism and age

In the present study, the lowest and highest ages of schizophrenia patientswere 23 and 57 years, respectively. The genotypes of schizophrenia patientswereclassified into two groups: above forty years group (N=19), and under forty years group (N=21).The frequency of AG genotype was equal in both ages group (9(42.9%) versus 9(42.9%)), and the frequenciesof AA and GG genotypes were slightly higher in the less than 40 years old than the above 40 years old group (AA: 5(23.8%) versus 4(21.0%); GG: 7(33.3%) versus 6(31.6%)). Statistically non-significant differences found between the two age groups for genotypes distribution were at p- value of 0.0957.Average ages for each genotype category are reported in Table-5.

Table 5-Distribution of patients (5-HTR2A) genotypes by age groups

Genotype	Age (y)		P value†
	less than 40 (N= 21)	more than 40 (N= 19)	
AG	9 (42.9%)	9 (47.4%)	0.957
AA	5 (23.8%)	4 (21.0%)	
GG	7 (33.3%)	6 (31.6%)	

The second distribution of patient genotypes was established without dividing patients into age groups. The number of carriers of GG genotype was 13, number of carriers of AG genotype was 18, and the number of carriers of AA genotype was 9. The number of patients with AG genotype was higher than those with AA orGG genotypes.Statistically significant differencesin genotype distribution were observed between two of the three age groups,specifically between the carriers of AG genotype and the carriers of AA genotype (p=0.03) as in Table-6.

The present result revealsthat thevariation rs643627 within 5-HTR2A gene is associated with the socio- demographic features (gender and age) in schizophrenia patients.

Table 6-Age of patients with each genotype of gene (5-HTR2A)

Genotypes	N	Age (mean± SD)	P -value
GG	13	41.03±7.5	0 .03
AG	18	42.40±8.0 [a]	
AA	9	33.70± 7.1	

In the present study, we investigated 5-HTR2A polymorphism rs643627 in sixty individuals of Iraqi origin, including forty schizophrenic patients and twenty healthy controls. There wasno evidenceregarding a relationship between HTR2A and schizophrenia found in any allele or genotype for rs643627. However, their association with SCZ and other psychiatric disorders were studied in other countries.

A case control comparison in Korea showed that rs643627 within HTR2A gene was significantly associated with the bipolar risk [1]. A recent study showed that rs463627 was non-significantly associated with SCZ in Chinese population [20].

In the current study, patients were classified by gender, where male patients were observed to have significantly higher frequenciesof genotypes GG, AG and AA than female patients (p=0.04).
These resultsare consistent with those obtainedby Kathryn, Richard and Jill (2010). Schizophrenia is diagnosed in more males than females, with a ratio of 1.4:1 [21]. It is known that sex differences occur in brain function as well as in the vulnerability, incidence,manifestation, and treatment of numerous psychiatric diseaseswhich are determined by inherent biological differences between females and males. For example, males show a higher tendencyfor Parkinson's disease, addiction, attention deficit hyperactivity disorder (ADHD),and autism. Females also tend to show higher susceptibility to anxiety/depression and Alzheimer's disease [22]. Large numbers of studies found that the onset age of schizophrenia isearlier in men than in women, in spite of no sex difference in total cases of schizophrenia [23]. Males with schizophrenia show more cognitive disturbances and greater reductions in temporal lobe volume than females with schizophrenia (1).

Moreover, evidence also supports sex differences in serotonin neurotransmission and psychiatric disorders caused by disruptions in the serotonin system. These kinds of differences are not only because of hormonal regulation, but are also due to genetic effects [24].In addition,the effects of sex hormones on serotonin regulation were also reported. It was shown that estradiol plays a protective role against the positive, negative, and cognitive symptom domains of schizophrenia [25]. Our results also indicated that rs643627is associated with age in schizophreniapatients. A previous study on humans showed a linear loss of receptor 5-HT2A of about 16 and 18% per decade of life in the hippocampus and prefrontal cortex, respectively [26]. The expression of the 5-HT2A receptor decreases dramatically in many brain regions after the age of 50[27]. Currently, little is known about genetic polymorphisms associated to mean life span. However, polymorphisms in the serotonergic system alter neurometabolic routes, causing neuropsychiatric diseases and behaviours that potentially lead to death or to conditions that shorten life expectancy [27].

Many factors may contribute to these inconsistent results.SCZ has been linked to genetic and environment factors.HT2A gene was reported to beassociated with more than one mental disorder (obsessive-compulsive disorder, schizophrenia, bipolar disorder and major depressive disorders) [28].The rs643627 within 5-HTR2Agene was also reported to be associated with psychiatric disorder, bipolar disorder and major depressive disorders [19].

In addition to what is mentioned, microRNAs have the potential to regulate more than ten thousand genes in the cell, including candidate genes for schizophrenia [29].MicroRNAs contribute in the regulation of post transcription of gene expression. They are responsible forregulating the translation of approximately sixty percent of genes that are translated to proteins [30]. Changes in the nucleotide sequence of certain microRNAs may lead to differences in theregulation of gene expression, andmay

lead to a mental disorder [31].MicroRNAs play crucial roles in brain propagation and have the capacity to target multiple genes [32]. Many of these miRNAs could modify the expression of genes associated with SCZ [33, 34].

On the other hand, the limited sample size (60 samples) in the present study could raise concerns as to whether negative findings observed in this study could reflect the lack of power to detect small differences that are possibly associated with SNPs.

Conclusion,

In summary, it was found that thers643627 within 5HTR2A intron region had no associationwith SCZ. However, the study of gender and age of SCZ patients showed significant association with thesevariants..The disease appeared more in malesthan in females,was not age-specific, and couldaffect people of all ages. Taking into account the limitations of the present study, further studieswith larger sample sizes are needed.

Acknowledgments

This work was supported by Ibn –Rushd Psychiatric, Baghdad, Iraq, and we appreciate the support of University of Baghdad, Collage of Sciences and the Biotechnology Department. In addition, we appreciate the contribution of ASCO learning Center.

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