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The Role of Peripheral MicroRNA-107 as Biomarker for Schizophrenia.

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Abstract.

The prevalence of schizophrenia is 1%, and it is a debilitating disorder that often results in a shortened lifespan. Peripheral blood samples are good candidates to investigate because they can be easily drawn. MicroRNAs are small non-coding RNA transcripts. They regulate the expression of genes by bindingto the 3'-untranslated region (UTR) of mRNAs and pointing them to degrade. The association between miR-107 gene expression and schizophrenia in a sample of Iraqi patients was investigated. Forty schizophrenia patients (20 males and 20 females) were enrolled and their age range was 25– 73 years. In addition to patients, 20 apparently healthy control subjects were also enrolled in the study. They matched patients for ethnicity (Iraqis), gender (20 males and 20 females),geographical variation (urban and rural) and age (25-35Y, 36-45Y and Y>46). The samples were analyzed by two steps Real Time – PCR techniques for detecting the expression of miR-107 in patients. Schizophrenia patients showed statistically significant up regulation of miR-107 in both male and female groups compared to healthy controls. There is no significant difference between males and females in control group (p=0.207), whereas; schizophrenia patients group showed significant difference between them (p=0.022). It was found that significant difference in expression between control and patients groups in both males and females with p-value (p=0.0094) and (p=0.0075) respectively. The results showed that no significant difference among the age groups in control (p=0.411) while, there was a significant difference in schizophrenia patients (p=0.0039). Moreover, all these groups (25-35Y, 36-45Y and Y>46) showed significant differences between control and schizophrenia patients with P-value (p=0.0074, p=0.0103 and p=0.0097) respectively. This study showed that no significant difference urban control and rural control (p=0.126) but significant difference between these two groups in schizophrenic patients (p=0.0108). In comparison between control and patients, there was significant difference in urban and rural groups with P-value (p=0.0063 and p=0.0091) respectively.

Keywords:*Schizophrenia, Peripheral blood, MicroRNA, Gene expression.*

INTRODUCTION

The prevalence of schizophrenia is 1%, and it is a debilitating disorder that often results in a shortened lifespan. Many studies, including neuroimaging, genetic, or biochemical parameters, have been performed in an attempt to identify the neurobiology of schizophrenia [1, 2]. Regarding its symptoms, SCZ is a long-term mental health condition that causes a range of different psychological positive symptoms, including hallucinations (hearing or seeing things that do not exist) and delusions (unusual beliefs not based on reality that often contradict the evidence). It has been found that schizophrenic patients experience negative symptoms such as depression and withdrawal [3].

The genetic contribution is important, since SCZ has been revealed to have a heritability risk of ~60% suggesting a much greater role of the environment SCZ development [4].Because of the mentioned burden of schizophrenia in patients' lives, researchers focused on identifying markers that represent disease status and prognosis. Peripheral tissues likeplasma, serum, and lymphocytes are easily accessible, and are thus convenient to investigate [3]. In this context, many studies have been done to find peripheral markers like serum interleukins, leptin, oxidative stress parameters, and antioxidant enzyme activity for psychiatric disorders[5, 6].MicroRNAs are approximately 22- nucleotide-long, small non-coding RNA transcripts. They bind to the 3'-untranslated region of mRNAs and induce degradation of pointed mRNA. The interaction between microRNAs and mRNAs usually results in decreased gene expression in a cell[7, 8]. Although microRNAs usually decrease the translation of mRNA, they rarely increase translation[9].With this mechanism, microRNAs have the potential to regulate more than 10,000 genes in the cell [10]. MiR-107 contributes to regulation of granulin/progranulin with implications for traumatic brain injury and neurodegenerative disease [11]. In schizophrenia increased levels of miR-107 contribute to the marked loss of cortical CHRM1 in schizophrenia which may be a differentiating pathophysiology [12].This studyaimed to investigate the expression of miR-107 in schizophrenia patients and healthy controls and highlighted the potential of miRNA levels to be used in the diagnosis of psychiatric disorders and offer novel targets for therapeutic development.

PATIENTS AND METHODS

Patients

In this descriptive study, blood samples were collected from 40 Iraqi patients affected by schizophrenia from Ibn Rushd Psychiatric Hospital, Baghdad, Iraq, were included 20 of them are males and their age ranged from (25-73) years old and the other 20 are females and their age ranged from (25-65) years old. As for a control 20 healthy individuals were control (healthy person)**.** After collection of the whole blood, the blood leaved to clot at room temperature for approximately 30 minutes. Then, the clot was removed by centrifuging at 1,000–2,000 x g for 10 minutes in a refrigerated centrifuge. The resulting supernatant is designated serum. Following centrifugation, the liquid component (serum) was transferred immediately into a clean polypropylene tube using a Pasteur pipette.

Methods

The total RNA was extracted from the collected serum using the protocol of TRIzol™ Reagent Thermo Scientific (USA), then, it was subjected to Two Step RT-PCR (cDNA synthesis, Real Time PCR) quantification. Two primers were designed (Reverse transcript primer miR-107: 5`GTCGTATGCAGTGCAGGGTCCGAGGTAGTCGCACTGC ATACGACTGATAG-3` and Forward primer miR-107: 5`-GCCGCAGCAGCATTGTACAGGG-3`) and another primer for real time-PCR Reverse primer universal reverse miRNA-107: 5`-GTGCAGGGTCCGAGGTAT-3`, as well as,(Reverse transcript primer RNU: 5`GTTGGCTTCTGGTGCAGGGTCCGAGGTATTCGCACCAG AGCCAACAATCAG-3` and Forward primer RNU: 5`-GTGAACTTATTGACGGGCG-3`) Reverse primer universal reverse RNU: 5`-GTGCAGGGTCCGAGGT-3`, act as housekeeping gene for detection of gene expression.

First step (cDNA synthesis).

This step involved synthesis of cDNA that performed in a thermal cycler (BioRad, USA). Initially, with the use of the GoTaq® qPCR Master Mix Kit (Promega, USA), reverse transcription using stem loop primers was performed. This Kit is contain M-MLV Reverse Transcriptase, dNTPs, M-MLV Reaction Buffer,0.15 µl of RNasin (40 u/µl), 2 µl of RT primer (20 p-mole / µL), RNA template and Nuclease Free Water was added to

achieve total volume of 10 µl. The reactions are achieved under aseptic condition. Thermal cycler protocol for cDNA synthesis were annealing at 16°C for 30 minutes (1 cycle), followed by extension at 42°C (30 minutes), enzyme inactivation at 85°C (5 minutes) and hold at 4°C (10 minutes). The RT product (cDNA) concentration was detected using QuantusFlorometer, Promega (USA).

Second Step

Quantitative-Comparative CT (ΔΔCT) Real-time PCR ΔΔCT Real-time PCR was performed in a Mic qPCR Cycler from Bio Molecular System, Australia. The 10 µl PCR included 1 µl RT-PCR product, 5 µl of 2X GoTaq® qPCR Master Mix Promega(USA), 0.5 µl of each10 µM primer (Primer F and Primer R) and 0.25 µl of MgCl2. The reactions were incubated in a 48-well plate Mic qPCR Cycler Bio Molecular System (Australia) of preincubation at 50°C for 2 minutes and at 95°C for 10 minutes, followed by 40 cycles at 95°C for 15 seconds and at 60°C for 90 seconds. All reactions were run in triplicate.

Statistical analysis

Relative quantification determines the changes in steady-state levels of a gene across multiple samples and expresses it relative to the levels of an internal control RNA. This reference gene is often a housekeeping gene; therefore, relative quantification does not require standards with known concentrations and the reference can be any transcript, as long as its sequence is known. Relative quantification is based on the expression levels of a target gene versus a reference gene and in many experiments is adequate for investigating physiological changes in gene expression levels. To calculate the expression of a target gene in relation to an adequate reference gene various mathematical models are established. Calculations are based on the comparison of the distinct cycle determined by threshold values (Ct) at a constant level of fluorescence. Mathematical models that determine the relative expression ratio are illustrated in the following equations.

Folding $=2^{-\Delta\Delta CT}$

$\Delta\Delta CT = \Delta CT_{patient} - \Delta CT_{Control}$

$\Delta CT = CT_{gene} - CT_{House\ Keeping\ gene}$

The data were processed and analyzed using the SPSS software package for Windows. Normality assumption of $2^{-\Delta\Delta CT}$ values was checked to detect the expression of the target genes. Since the assumption of normality was not met, the comparisons between groups were performed. Descriptive statistics for $2^{-\Delta\Delta CT}$ values were expressed as mean, standard deviation and Significant differences (two-tailed *p*) less than 0.05 were regarded as significant. Parametric variables (age and gender) compare between groups.

RESULT AND DISCUSSION

The study results were constructed by analyzing the information and data obtained from a 60 blood samples: 40 patients of schizophrenia (26 schizophrenia patients reside in urban and 16 schizophrenia patients reside in rural) and 20 apparently healthy as controls, all subjects are in ages in the range of 25-73 years as shown in table1

It was found that most of patients were located within 25 – 35 years in urban group with a percentage of 48% whereas most patients are located within 36-45 and Y>46 years with a percentage of 44% to both in rural group. The 36-45years constitutes the least percentage of 24% in the urban group and 25-35 years of 11% in the rural group. The results showed that males are more than females in urban group but in rural group females are more than males. The present results were almost similar to those obtained by Kathryn, Richard and Jill, [13] showed thatschizophrenia is diagnosed in more men than women, with a male to female ratio of 1.4:1.

Estimation of mir-107 expression by two steps RT-PCR.

Table2 show some of concentration results of cDNA after reverse transcription detected byQuantusFlorometer, Promega (USA).

RT-PCR result calculations are based on the comparison of the distinct cycle determined by threshold values (Ct) at a constant level of fluorescence as in figure1.a and b

The results of RNU (housekeeping gene) were ranged from (6.7-29) for both control and patients samples. MiR-107 cDNA showed different results for control and patient samples that ranged from (8.1-27). Estimation of cDNA concentration is important for subsequent RT-PCR.

Analysis of miRNA expression in peripheral tissues has also revealed associations with schizophrenia. We compared the microRNA expression levels of the schizophrenia group with controls.

Table.1 Distribution of patient's schizophrenia according to the age and gender and geographic area.

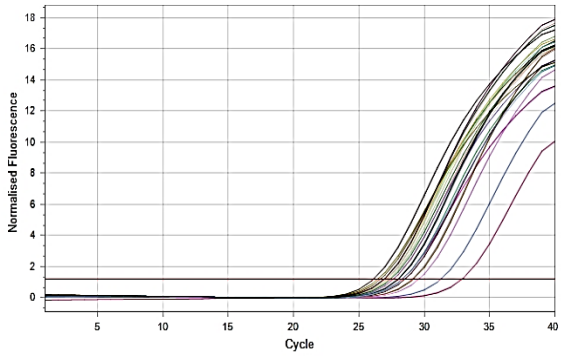
Age Years	Urban				Rural			
	Gender				Gender			
	M	F	T	%	M	F	T	%
25-35	6	6	12	48	0	2	2	11
36-45	3	3	6	24	3	5	8	44
Y>46	4	3	7	28	5	3	8	44
Total	13 (52%)	12(48%)	25	100%	8(44%)	10(55%)	18	100%

Table2: Concentration results of cDNA after reverse transcription for miR-107 gene and RNU.

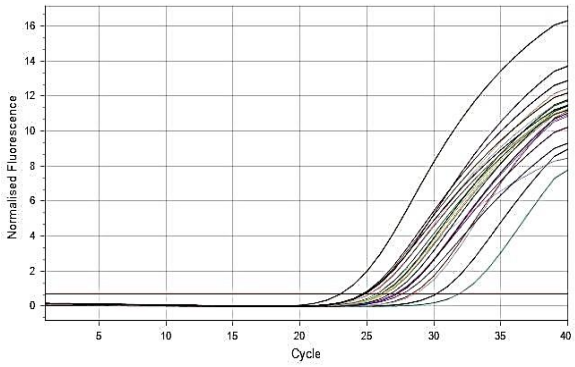
Sample	Conc. RNU (ng/µl)	Sample	Conc. Mir- 107 (ng/µl)
A	6.7	A	8.1
13	7.7	13	8.8
15	7.4	15	8.5
16	7.5	16	9
B	25	B	24
J	24	J	27
8	29	8	24
21	26	21	19
31	26	31	23

The study results shown significant difference between the groups regarding gender and age, as shown I;n Table 2.Table 3 and4.Schizophrenia patients showed statistically significant upregulation of miR-107 in both male and female groups compared to healthy controls. There was no significant difference between males and females in control group (p=0.207), whereas; schizophrenia patients group showed significant difference between them (p=0.022). The results were in agreement with the study of Kathryn *et al*. [13] who found thatschizophrenia is diagnosed in more men than women, with a male to female ratio of 1.4:1. It was found that significant difference in expression between control and patients groups in both males and females with *p*-value (p=0.0094) and (p=0.0075) respectively.

The age of schizophrenia patient ranged from (25-75) years old, the control and patients are divided in to three groups according to the age (25-35Y, 36-45Y and Y>46). The results showed that no significant difference among these groups in control (p=0.411) while, there was a significant difference in schizophrenia patients (p=0.0039).Moreover, all these groups (25-35Y, 36-45Y and Y>46) showed significant differences between control and schizophrenia patients with P-value (p=0.0074, p=0.0103 and p=0.0097) respectively.



a. Typical Real Time PCR for miR-107 gene.



b. Typical Real Time PCR for RNU gene.

Fig.1a and b: Showing the typical Real Time growth curve with its phases in which any fluorescence detected above a certain level threshold as the reaction cycles.

Table 3: Expression results of males and females miR-107 in control and schizophrenia patients.

Sex	Male Mean+SD	Female Mean+SD	P-value	Sign.
Control	A,a 0.943+0.0841	A,a 1.180+0.0582	0.207	Non Sign.
Patient	B,a 2.963+0.318	B,b 3.772+0.941	0.022	Sign.
P-value	0.0094	0.0075		
Sign.	Sign.	Sign.		

Table 4: Expression results of miR-107 in control and schizophrenia patients regarding to the age.

Age	25-35Y Mean+SD	36-45Y Mean+SD	Y>46	LSD	P-value	Sign.
Control	A,a 0.969 +0.086	A,a 0.933 +0.0671	A,a 0.941 +0.0588	0.726	0.411	Non Sign.
Patient	B,a 4.175 +1.184	B,a 3.966 +0.918	B,b 2.858 +0.471	0.835	0.0039	Sign.
P-value	0.0074	0.0103	0.0097			
Sign.	Sign.	Sign.	Sign.			

Geographical variation

A number of studies have since investigated temporality and dose-response gradient, predominantly focusing on the association of urbanicity with schizophrenia. There is good evidence from studies investigating temporality of this association to suggest that the risk of schizophrenia and other non-affective psychosis increases as degree of urbanization at birth increases.Analysis thegeographical variation in orderto study the quality of life of schizophrenic patients who are living in both rural and urban areasin Baghdad.This study showed that no significant difference urban control and rural control (p=0.126) but significant difference between these two groups in schizophrenic patients (p=0.0108). In comparison between control and patients, there was significant difference in urban and rural groups with *P*-value (p=0.0063 and p=0.0091) respectively. The study results showed that miR-107 expression in a higher rate in urban than in rural as in table 5, this mean that schizophrenic patients living in rural areas, had relatively better quality of life than those living in urban areas. The results were agreed with the study of Jassam*et al*. [14]who showed that schizophrenic patients living in rural area had relatively better quality of life than those living in urban area, exploring different domains of quality of life like physical health, psychological health, level of independence,social relationship, environmental and spiritual domains

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Table 5:Expression results of miR-107 for geographical variation in incidence of psychosis.

Residence	Rural Mean+SD	Urban Mean+SD	P-value	Sign.
Control	A,a 1.291+0.073	A,a 1.022+0.043	0.126	NonSign.
Patient	B,a 3.952+1.055	B,b 2.528+0.163	0.0108	**Sign.**
P-value	0.0063	0.0091		
Sign.	Sign.	Sign.		

The genetic evidence has displayed that mutations that interrupt miRNA transcription were found in schizophrenia. Precise function of specific miRNA has been revealed tobe associated with schizophrenia. Furthermore, the manipulation of miRNAs expression could change the phenotypes in SCZ using animal model.

These model systems not only provide the basis for understanding the phenotype of miRNA dysfunction, they provide a platform for the exploring therapeutics. MiRNA and he fundamental miRNA biogenesis machinery are potentially both novel drug targets and new drug entities in the fight to control neuropsychiatric conditions.

Beyond sex differences, differential expression of non-coding RNA in schizophrenia has in one instance been shown to differentiate between molecular subpopulations with the illness. Schizophrenia has been proposed to be a syndrome of molecularly distinct disorders [15]. Deficits in cortical muscarinic M1 receptor (CHRM1) protein have been identified in a sub-group of over a

quarter ofindividuals with schizophrenia [16]. However, levels of CHRM1 mRNA were not different between schizophrenia cases with CHRM1 deficits compared to those with normal levels of CHRM1 protein [17]. Post-mortem studies have reported that the expression of miR-107, a miRNA that targets the CHRM1 sequence, is increased in schizophrenia [18].

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