RESEARCH ARTICLE

Expression of micro-RNAs miR-31, miR-146a, miR-181c and miR-155 and their target gene IL-2 are altered in schizophrenia: a case-control study [version 1; peer review: 1 approved, 1 approved with reservations]

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Abstract

Background: Schizophrenia is a severe psychiatric disorder with a heterogeneous clinical phenotype. The association of interleukins and other cytokines and their receptors with schizophrenia has been previously reported. Additionally, a number of studies have reported altered micro-RNA (miRNA) expression in schizophrenia and other psychiatric disorders. The aim of our study was to explore the possible association of miR-31, miR-146a, miR-181c and miR-155 with schizophrenia pathogenesis, as well as their link to *IL2* gene expression in disease.

Methods: For this case-control study, 225 patients with paranoid schizophrenia and 225 sex- and age-matched controls with no family history of schizophrenia were recruited. The expression of studied miRNAs and the *IL2* gene was measured using qPCR. DNA samples of all patients and controls were genotyped for *IL2* rs2069778 single nucleotide polymorphism (SNP) using PCR with sequence specific primers (PCR-SSP). Statistical analyses include the Mann-Whitney U-test and Fischer's exact test.

Results: All studied miRNAs were over-expressed in schizophrenic patients *IL2* gene expression was down-regulated in schizophrenic patients. The *IL2* rs2069778 SNP is not associated with schizophrenia but regulates expression of the *IL2* gene.

Conclusions: Over-expression of studied miRNAs and down-regulation of *IL2* gene expression may be considered as genetic risk factors for chronic schizophrenia. Abnormalities in studied miRNA expressions result in the deregulation of the T-cell receptor signaling

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Any reports and responses or comments on the article can be found at the end of the article.

pathway in schizophrenia.

Keywords

schizophrenia, miRNA, interleukin-2, cytokines, SNP, expression, pathway

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Introduction

Schizophrenia (OMIM code: 181500) is a severe psychiatric disorder with a heterogeneous clinical phenotype¹. While the etiology of this disorder remains largely unknown, it has become evident that immune-inflammatory processes play an important multilevel role in disease development and progression²⁻⁴. Both cellular and humoral components of the innate and adaptive immune system were shown to be altered at different stages of disease development, both locally within the central nervous system as well as at a systemic level. Cytokines and chemokines are essential signal mediators of the immune system that modulate and guide immune/inflammatory responses as well as perform a wide range of other functions related to cell survival, proliferation, differentiation and migration⁵. Considering their biological importance, cytokines, especially interleukins (IL), have received considerable attention in the context of schizophrenia. The association of interleukins and other cytokines and their receptors with schizophrenia has been previously reported both on the level of genetic variants as well as the levels of gene expression and protein abundance, indicating their essential role in disease predisposition, development, progression and treatment response⁶⁻⁹.

IL-2 controls a wide range of biological activities, largely depending on the biological context. It is essential for T lymphocyte proliferation and differentiation, but it is also implicated in the generation and maintenance of regulatory T (Treg) cells^{10,11}. The role of the *IL2* gene in schizophrenia is still unclear and conflicting. However, previous studies, including our own findings, suggest the involvement of IL-2 in the pathogenesis of schizophrenia. Decreased lymphocyte production of IL-2 and increased IL-2 receptors have been reported previously¹²⁻¹⁶. Interleukin-2 receptor gamma (IL2RG) is an important signaling component of receptors for many cytokines, including IL-2, -4, -7, -9, -15 and -21¹³. Moreover, the *IL2RG* gene is over-expressed in the blood of schizophrenia patients¹³. On the other hand, other groups reported increased IL-2 serum levels in schizophrenia¹⁷⁻¹⁹. Some *IL2* genetic polymorphisms were also reported to be associated with schizophrenia^{20,21}. Expression of the *IL2* gene is controlled at multiple layers. The *IL2* gene contains at least two cis elements for transcript stability regulation, located in both the 3' and 5' untranslated regions (UTRs)^{22,23}. Single nucleotide polymorphisms (SNPs) in the promoter region of *IL2* influence the expression levels of this cytokine. Finally, expression of *IL2* is also regulated by micro-RNAs²⁴.

Micro-RNAs (miRNAs) are class of small, non-coding RNAs (comprised of about 22 nucleotides). MiRNAs are found in animals, plants and some viruses. They function in the regulation of gene expression at posttranscriptional level and RNA silencing²⁵⁻²⁷. As miRNAs are involved in the normal functioning of eukaryotic cells, deregulation of miRNAs has become associated with disease. There is a manually curated "miR2Disease" database, which aims to provide a comprehensive resource of microRNA deregulation in various human diseases²⁸. Also, a number of studies have reported altered miRNA expression in schizophrenia, bipolar disorder and major depression and anxiety disorders²⁹⁻³².

The aim of our study was to explore the possible association of miR-31, miR-146a, miR-181c and miR-155 with schizophrenia pathogenesis, as well as their link to *IL2* gene expression in disease. This study is the first to report genetic association between schizophrenia and mentioned above miRNAs; however, several studies have reported a role for the *IL2* gene in schizophrenia. All of these four micro-RNAs play a major role in regulating expression of the cytokine network. Particularly, miR-31, miR-146a and miR-181c are regulators of *IL2* gene expression³³⁻³⁵, while miR-155 expression is greatly enhanced following stimulation of macrophages and dendritic cells by Toll-like receptors³⁶. We also studied the possible association of the *IL2* rs2069778 SNP genotype with *IL2* and miRNA levels. This SNP was chosen due to its high minor allele frequency, clinical significance in autoimmune diseases³⁷, as well as its location near the regulatory elements of the *IL2* gene.

Methods

Ethical statement

Informed written consent was obtained from all study participants. The study has been approved by the Ethical Committee of the Institute of Molecular Biology of the National Academy of Sciences RA (IRB00004079, IORG0003427).

Study population

This case-control study was conducted from January 2016 to February 2017. A total of 225 patients with paranoid schizophrenia (SCZ) and 225 sex- and age-matched controls (CTRL) with no family history of schizophrenia were involved in this study (Table 1). This was the maximum available number of schizophrenia patients in Armenia who agreed to participate in this study. From these subjects, 61 patients and 60 controls were tested for micro-RNA expression and 66 patients and 99 controls for *IL2* gene expression. There was no specific criteria for dividing subsets in this study; subjects were divided into groups according to availability of biological material (DNA and RNA). All subjects were genotyped for this study.

Paranoid schizophrenia (OMIM code: 181500, ICD-10-CM code: F20.0, DSM-5 code: 295.90) was diagnosed by two independent psychiatrists. Schizophrenia patients were recruited from the clinics of the Psychiatric Medical Center of the Ministry of Health of the Republic of Armenia (MH RA). Healthy subjects with any psychiatric illness during their lifetime, any serious endocrine or neurological disorder, any treatment or medical condition known to affect the brain or meeting the DSM-5 criteria for intellectual disability³⁸ were excluded from this study. Exclusion criteria for all study subjects included any treatment with immune-modulating drugs and serious medical disorder.

Table 1. Demographic data of study participants.

	SCZ	CTRL
Age (mean ± SD)	46.34±13.78	46.16±15.01
Sex male/female	132/93	137/88
Age of first onset of schizophrenia	30.83±12.92	NA

Healthy control subjects were recruited among the blood donors of the Erebouni Medical Center (MH RA) and were interviewed by psychiatrists.

Blood sampling and isolation of genomic DNA and total RNA from peripheral blood mononuclear cells

A total of 10 ml of peripheral blood was collected in EDTA containing tubes (5ml for RNA and 5ml for DNA isolation) from each study participant.

Peripheral blood mononuclear cells were isolated from whole blood using the following protocol, as described in 13: 10 ml of Red Cell Lysis Buffer (RCLB) (containing 0.144M ammonium chloride, 1 mM sodium bicarbonate) was added to 5 ml of fresh blood. After 5 minutes, the mixture was centrifuged at 1000g for 10 minutes, the supernatant was discarded and the pellet was gently rinsed with RCLB. The pellet was re-suspended in 5 ml of the RCLB buffer and centrifuged at 1000g for 10 minutes. The final purified pellet was stored in RNeasy lysis buffer (Ambion, Austin, TX, USA) at -20°C for later use. Total RNA was extracted using High Pure miRNA Isolation Kit (Cat. No. 05080576001 Roche Applied Science, Penzberg, Germany) according to the manufacturer's instructions. The quantity and quality of RNA and DNA samples were assessed using a 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA) according to the manufacturer's protocol and stored at -80°C until further use. The average RNA yield was 25 mg per 5 ml of blood sample.

The method for genomic DNA isolation was a modification of Miller's salting-out procedure³⁹ where a chloroform extraction phase is added (2ml of chloroform added to each tube supernatant and centrifuged at 3000g for 10 minutes)⁴⁰.

cDNA synthesis

cDNA synthesis was performed by reverse transcription (0.5 µg total RNA, total volume of cDNA 20 µl) using Transcriptor First Strand cDNA Synthesis Kit (Cat. No. 04897030001, Roche Applied Science) with anchored dT primers (0.4 µg; ABgene, Waltham, MA, USA) at 47°C for 45 min. cDNA samples were stored at -20°C until further use.

Quantification of IL2 gene expression

mRNA levels of *IL2* (target) and *PSMB2* (housekeeping) genes and were measured by quantitative real-time polymerase

chain reaction with the Rotor Gene 3000 instrument (Corbett Research, USA). cDNA (5 µl, corresponding to 20 ng calculated on input total RNA) was added to 20 µl PCR-mix. The final reaction mix contained 900 nM of each sense and antisense primer (Roche Applied Science), 100 nM LNA probe (Roche Applied Science), 3.5 mM MgCl₂, 200 µM of each dNTP (ABgene), 0.2µl Thermo-Start TAQ polymerase with concentration 5 U/µl and 1x ThermoStart Buffer (ABgene).

qPCR was performed using following thermal cycling conditions: initial denaturation for 15 minutes at 95°C; 40 cycles of denaturation for 20 seconds at 95°C, annealing for 30 seconds at 60°C and extension for 60 seconds at 72°C; and a final extension for five minutes at 72°C.

The primers and fluorescently-labeled Locked Nucleic Acid (LNA) probe from the Universal Probe Library for the *IL2* gene were selected using the Probe Finder web-based software as follows:

***IL2* gene:**

Left primer: 5'-AAG TTT TAC ATG CCC AAG AAG G-3'

Right primer: 5'-AAG TGA AAG TTT TTG CTT TGA GCT A-3'

Probe: #65

***PSMB2* gene:**

Left primer 5'-GTG AGA GGG CAG TGG AAC TC-3'

Right primer 5'-GAA GGT TGG CAG ATT CAG GA-3'

Probe #50

Measurement of miRNA expression levels

Reverse transcription for selected micro-RNAs and measurement of micro-RNA expression by quantitative real-time polymerase chain reaction (RT-PCR) were performed using TaqMan Micro-RNA Assays (see Table 2) and TaqMan Universal PCR Master MIX II (no UNG) (Cat. No. 4440040, Thermo Fisher Scientific, Waltham, USA), according to the manufacturer's instructions. qPCR was performed using Realist-DX IAB real-time PCR system (GeneTICA, Czech Republic).

qPCR was performed using following thermal cycling conditions: polymerase activation for 10 minutes at 95°C; 40 cycles

Table 2. List of miRNA assays used in the study.

miRNA	miRBase Accession Number	Mature miRNA Sequence	TaqMan Assay cat number	Assay ID
miR-16	MI0000070	UAGCAGCACGUAAAUAUUGGCG	4427975	000391
miR-31	MI0002673	GGCAAGAUGCUGGCAUAGCUG	4427975	001100
miR-146a	MI0000477	UGAGAACUGAAUUCUAGGGUU	4427975	000468
miR-155	MI0000681	UUAUUGCUAAUCGUGAUAGGGGU	4427975	002623
miR-181c	MI0000271	AACAUUCAACCGUCGGUGAGU	4427975	000482

of denaturation for 15 seconds at 95°C and annealing/extension for 60 seconds at 60°C.

We used miR-16 as a housekeeping micro-RNA. Data were expressed as arbitrary units (miR-X/miR-16 ratio). miR-16 is currently one of generally accepted housekeeping micro-RNAs for normalizing micro-RNA expression in blood cells by qRT PCR⁴¹. In addition, there are no reports on miR-16 alterations in any psychiatric disease including schizophrenia.

PCR-SSP analyses

DNA samples of all patients and controls were genotyped for *IL2* rs2069778 SNP using a polymerase chain reaction with sequence-specific primers (PCR-SSP)⁴². The sequences of specific primers were designed based on relevant DNA sequences available in the NCBI [GenBank database](#) (RefSeq Accession: NG_016779.1). Primer sequences for the selected SNP were as follows:

Reverse standard: 5'-CAC CAC TAC AAA TTC TAC AAA TTC G-3'

Reverse mutant: 5'-CAC CAC TAC AAA TTC TAC AAA TTC A-3'

Forward constant: 5'-CTG GTG CCA GAA AGA GCT TG-3'

The presence/absence of allele-specific amplicons were visualized by electrophoresis using 2% agarose gel in 0.5x Tris-Borate-EDTA (TBE) buffer stained with ethidium bromide fluorescent dye. To check the reproducibility of results, randomly selected DNA samples of study subjects (10% of total) were genotyped twice.

Genotyping was carried out at Laboratory of Human Genomics and Immunomics, Institute of Molecular Biology NAS RA (Yerevan, Armenia).

Statistical analyses

The Shapiro–Wilk test for normality revealed non-parametric distribution of the obtained data. Therefore, the significance of difference in gene expression levels between each study group was analyzed by the Mann–Whitney U test. *p*-values less than 0.05 were considered as significant. Statistical analysis was performed using GraphPad Prism (version 5) software. Allele and genotype frequencies were checked for Hardy–Weinberg equilibrium and were in equilibrium. No investigation of potential sources of bias was undertaken.

Pathway analysis

For characterization of enriched functions and biological pathways of the studied miRNAs targets, we used miRsystem⁴³, which performs enrichment analyses, accounting both for target genes as well as the levels of individual miRNA expression.

Results

Expression of *IL2* in schizophrenia

In total, 66 SCZ patients (male/female: 33/33, mean age±S.D.: 51±11.2 years) and 99 healthy controls (male/female: 45/44, mean

age±S.D.: 50±13.9 years) participated in *IL2* gene expression step⁴⁴.

We studied mRNA expression levels of the *IL2* gene in schizophrenia and its possible association with the *IL2* rs2069778 C/T SNP. The median mRNA expression levels of *IL2* in the patient group were significantly lower than in healthy control subjects (patients vs. controls, median [interquartile range]: 0.06889 [0.6499–0.007519] vs. 1.469 [3.858–0.000], *p*=0.0095) (Figure 1).

Furthermore, analysis revealed a significant difference in *IL2* expression between those with and without the *IL2* rs2069778 C/T SNP in both the control and schizophrenia groups. Particularly, in schizophrenic patients, *IL2* mRNA expression levels were significantly higher in rs2069778*T minor allele carriers (CT+TT) than in CC homozygotes (CC vs. CT+TT, median [interquartile range]: 0.033711 [0.5433–0.00453] vs. 0.2178 [2.618–0.03726], *p*=0.0003) (Figure 2). The same difference was found in control groups (CC vs. CT+TT, median [interquartile range]: 1.083 [2.840–0.000] vs. 2.625 [4.966–0.000], *p*=0.0495) (Figure 2). It is worth noting that expression of *IL2* in schizophrenic patients carrying the CC genotype was lower than in corresponding controls (*p*=0.0216), while the difference in expression between carriers of the T minor allele was not significant (*p*=0.22).

Levels of miRNAs in schizophrenia

A total of 61 SCZ patients (male/female: 37/24, mean age±S.D.: 45.4±13.9 years) and 60 healthy controls (male/female: 37/23, mean age±S.D.: 44.5±13.6 years) were tested for micro-RNA expression.

Median expression levels of all studied miRNAs were significantly higher in schizophrenic patients as compared to healthy controls (Table 3 and Figure 3)⁴⁵.

Further analysis indicated that in T allele carriers, miR-181c had significantly lower expression compared to CC homozygous variants, both in schizophrenia patients (TT+CT vs. CC, median [interquartile range]: 1.99 [2.92–1.39] vs. 3.46 [4.84–2.14], *p*=0.0045) and controls (TT+CT vs. CC, median [interquartile range]: 0.41 [2.41–0.10] vs. 1.90 [5.31–0.59], *p*=0.011) (Figure 4).

Interestingly, in T allele carriers, miR-31 also had significantly lower expression compared to CC homozygous variants in controls (TT+CT vs. CC, median [interquartile range]: 0.09 [1.61–0.03] vs. 1.84 [3.35–0.48], *p*=0.0015) but in schizophrenia patients there was no significant difference (TT+CT vs. CC, median [interquartile range]: 3.93 [7.74–2.91] vs. 5.03 [8.12–3.16], *p*=0.36) (Figure 4). We have not found any association between *IL2* rs2069778 variants and expressions of miR-155 and miR-146a.

Functional annotation of biological pathways containing targets for studied miRNAs

Our analysis demonstrated significant up-regulation of *IL2* expression-modulating miRNAs in schizophrenia. However,

Figure 1. Interleukin-2 (*IL2*) mRNA expression levels in peripheral blood mononuclear cells from schizophrenia patients (SCZ) and healthy control subjects (CTRL).

it is known that miRNAs can affect multiple targets. For characterization of enriched functions and biological pathways of the studied miRNAs targets, we used a freely available online integrated system called miRsystem⁴³. The analysis resulted in 30 pathways from 4 databases (KEGG⁴⁶, Biocarta, Reactome, Pathway Interaction Database) significantly enriched with miRNA targets. The majority of these were related to immune/inflammatory system pathways where *IL2* is either an effector or a target gene (Table 4). Figure 5 is an example of miR target enriched pathway⁴⁶.

Discussion

In this study, we observed decreased levels of *IL2* expression in peripheral blood mononuclear cells of schizophrenic patients, paralleled with increased expression of *IL2*-regulating miRNAs (miR-31, miR-146a, miR-155 and miR-181c). In addition, we demonstrated that carriage of the minor allele for *IL2* rs2069778 is associated with increased *IL2* expression levels which might suggest either a regulatory role for this SNP or a linkage with other SNPs that can modulate gene expression.

Figure 2. Interleukin-2 (*IL2*) mRNA expression levels in peripheral blood mononuclear cells from schizophrenic (SCZ) and healthy control (CTRL) rs2069778 CC homozygotes (CC) and rs2069778*T allele carriers (CT+TT).

Table 3. Levels of miR-31, miR-146a, miR-155 and miR-181c expression in schizophrenia patients and controls.

miRNA	SCZ patients (median [interquartile range])	Controls (median [interquartile range])	P value
miR-31	4.57 [8.12–3.38]	1.39 [2.998–0.1288]	$p < 0.0001$
miR-146a	0.802 [1.26–0.5205]	0.487 [1.318–0.1223]	$p = 0.0254$
miR-155	1.155 [2.1–0.8223]	0.04065 [0.1037–0.02585]	$p < 0.0001$
miR-181c	3.086 [4.637–1.97]	1.442 [3.454–0.3059]	$p = 0.0008$

Figure 3. Levels (miR-X/miR-16 comparative expression) of miR-31, miR-146a, miR-155 and miR-181c expression in schizophrenic patients (SCZ) and controls (CTRL).

Figure 4. IL2 rs2069778 minor allele carriage-dependent expression (miR-X/miR-16 comparative expression) of miRNAs in patients (SCZ) and controls (CTRL). CC – homozygous for rs2069778 SNP, TT+CT – carrier for rs2069778 SNP.

Table 4. Top pathways enriched with target genes for miR-31, miR-146a, miR-155, miR181c.

CATEGORY	TERM	Targets In The Term	% Of Targets In Genes	Raw p-value	Empirical p-value
KEGG	TOLL-LIKE RECEPTOR SIGNALING PATHWAY	10	9.8	3.08E-07	0.000167
KEGG	T CELL RECEPTOR SIGNALING PATHWAY	8	7.41	3.58E-05	0.00123
KEGG	CHEMOKINE SIGNALING PATHWAY	9	4.76	0.000325	0.003505
KEGG	B CELL RECEPTOR SIGNALING PATHWAY	5	6.67	0.001634	0.003546
KEGG	FCGAMMAR-MEDIATED PHAGOCYTOSIS	5	5.32	0.004168	0.025695
KEGG	NEUROTROPHIN SIGNALING PATHWAY	6	4.72	0.003129	0.047234
BIOCARTA	BIOCARTA BCR PATHWAY	3	8.57	0.007175	0.027159
BIOCARTA	BIOCARTA MAPK PATHWAY	5	5.75	0.003043	0.030354
PATHWAY INTERACTION DATABASE	IL2 SIGNALING EVENTS MEDIATED BY STAT5	4	13.33	0.000374	0.000314
PATHWAY INTERACTION DATABASE	CALCINEURIN-REGULATED NFAT-DEPENDENT TRANSCRIPTION IN LYMPHOCYTES	5	10.0	0.000268	0.001485
PATHWAY INTERACTION DATABASE	BCR SIGNALING PATHWAY	6	8.82	0.000131	0.001518
PATHWAY INTERACTION DATABASE	IGF1 PATHWAY	4	13.79	0.000328	0.002937
PATHWAY INTERACTION DATABASE	PDGFR-BETA SIGNALING PATHWAY	7	5.56	0.000602	0.005438
REACTOME	INTERFERON GAMMA SIGNALING	5	6.85	0.001455	0.000803
REACTOME	CYTOKINE SIGNALING IN IMMUNE SYSTEM	9	4.09	0.000917	0.001294
REACTOME	INTERFERON SIGNALING	5	4.54	0.007717	0.002176
REACTOME	CD28 CO-STIMULATION	3	9.68	0.005163	0.009527
REACTOME	INTERFERON ALPHA BETA SIGNALING	3	4.69	0.032552	0.01096
REACTOME	ADAPTIVE IMMUNE SYSTEM	13	2.70	0.002974	0.011809

Conflicting results on the levels of *IL2* have been reported for this disease both in medicated and undedicated patients. The data published by Singh *et al.*⁴⁷ and Theodoropoulou *et al.*⁴⁸ are in line with our findings. However, Ebrinc *et al.*⁴⁹ and Zhang *et al.*^{50,51} have found elevated levels of *IL2* in their subjects. Furthermore, there are studies reporting no difference in the *IL2* levels of schizophrenic patients as compared to controls⁵². Because expression was studied in different populations, we can speculate that the observed discrepancies could be partially explained by different genetic backgrounds. Moreover, the validity of our results is supported by the detected increase of *IL2* expression-modulating miRNAs measured using independent assay techniques. Finally, consistent with *IL2*'s role in maintenance of Treg cells, their low levels were also reported in schizophrenia⁵³.

Though the studies of miRNA involvement in schizophrenia are a relatively new direction, there are already results implicating miRNA deregulation in the pathogenesis of schizophrenia. miR-137 is the micro-RNA best known for its role in schizophrenia pathogenesis^{54–56}. This micro-RNA is also well known due to a genetic polymorphism (SNP variant) in its gene, which was described as a genetic risk factor for the development of schizophrenia in a European population^{57–59}. There are other recent studies which confirmed the role of distinct micro-RNAs such as miR-195, miR-181b, miR-301a, miR-19, miR-206, miR-30a and miR-219 in the pathogenesis

of schizophrenia^{60–64}. In this study, we reported four miRNAs (miR-31, miR-146a, miR-155, miR-181c) that were up-regulated in schizophrenic patients. Besides targeting *IL2* expression, these molecules have many other targets that are involved in immune/inflammatory pathways, confirming the essential role of immune system disturbances in disease development and progression.

The limitation of the present study is the inability to recruit medication-free patients for assessment of the effect of treatment on *IL2* and miRNA expression. However, in many studies cited in this paper^{50,51}, regardless the direction of difference in *IL2* levels, no differences were observed between treated and untreated patient groups. It should be noted that we measured *IL2* gene and miRNA expression in two different patient groups with little overlap, which prevented us from performing direct correlation analysis between the levels of *IL2* and miRNAs.

Overall, our findings further strengthen the role of immune system deregulation in the development and progression of schizophrenia and necessitate further research towards understanding the changes of the Th1/Th2/T-reg response in this disease and in response to antipsychotic treatment.

Conclusions

All studied miRNAs (miR-31, miR-146a, miR-155, miR-181c) were over-expressed in schizophrenic patients, suggesting

Figure 5. Regulation of T cell signaling pathway by miR-31, miR-146a, miR-155 and miR-181c. Red boxes indicate target genes for studied miRNAs. This figure has been reproduced with permission from the KEGG PATHWAY database⁴⁶.

a role for them in disease pathogenesis. *IL2* gene expression was down-regulated in schizophrenic patients. The *IL2* rs2069778 C/T SNP is not associated with schizophrenia but regulates expression of the *IL2* gene. Abnormalities in studied miRNA expressions result in the deregulation of the T-Cell receptor signaling pathway in schizophrenia.

Data availability

Underlying data

Figshare: F1000 1990 Ghazaryan *et al.*.xlsx. <https://doi.org/10.6084/m9.figshare.9816860.v1>⁴⁴.

Figshare: F1000 1990 Ghazaryan *et al.* Raw Data miRNA.xlsx. <https://doi.org/10.6084/m9.figshare.10012442.v3>⁴⁵.

Data are available under the terms of the [Creative Commons Attribution 4.0 International license](#) (CC-BY 4.0).

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Ghanshyam N. Pandey

Department of Psychiatry, University of Illinois at Chicago, Chicago, Illinois, USA

In this study the authors determined several micro-RNAs and mRNA expression of IL-2 in the PBMC obtained from 225 paranoid schizophrenic patients and 225 healthy normal control subjects. They found that all studied micro-RNAs, for example miR-16, miR31, miR-146a, miR-155 and miR-181c were overexpressed in the peripheral blood mononuclear cells (PBMC) of schizophrenic subjects compared with normal control subjects. They also found that the IL-2 mRNA expression was down regulated in schizophrenic patients compared with normal control subjects.

This is an important study of miRNA and IL-2 in schizophrenia. The results are significant.

There are many strong points of the study. The number of study subjects is very large -- 225 patients with paranoid schizophrenia and 225 sex- and age-matched normal control subjects. They determined relevant miRNAs in the schizophrenic patients. They also determined the mRNA expression for IL-2, which has been studied in schizophrenia and was found down-regulated in this study. A procedure for isolating the RNA from PBMC is well described and the methods used for mRNA determinations and miRNA determination are also sound. The Introduction is to the point and relevant. The Discussion is pertinent and the Methods are described clearly. Overall, this is a very important and strong study of miRNA and IL-2 in schizophrenia.

I have only a few minor comments:

1. What was the drug-status of the schizophrenic patients at the time of this study? Were all of them drug-free or were on treatment with any drugs?
2. They studied the mRNA expression of IL-2 in schizophrenic patients. The rationale for studying IL-2 has been described. However, it is not clear why they did not study the mRNA expression of the cytokines IL-1 β , IL-6 and TNF- α , the abnormalities of which have been implicated in schizophrenia.
3. They determined the mRNA expression of IL-2 and were comparing the results with the

protein expression determined by other investigators in the plasma of schizophrenic patients. These comparisons may be fine but may not be relevant. Can they describe if there are other studies of mRNA in schizophrenic patients and maybe they should cite those. For example, Pandey et al. (2015, 2018^{1,2}).

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Is the work clearly and accurately presented and does it cite the current literature?

Yes

Is the study design appropriate and is the work technically sound?

Yes

Are sufficient details of methods and analysis provided to allow replication by others?

Yes

If applicable, is the statistical analysis and its interpretation appropriate?

I cannot comment. A qualified statistician is required.

Are all the source data underlying the results available to ensure full reproducibility?

Yes

Are the conclusions drawn adequately supported by the results?

Yes

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Neurology of mood disorders, schizophrenia and suicide.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Reviewer Report 20 May 2020

<https://doi.org/10.5256/f1000research.21836.r62639>

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Mirko Grubor

Independent Researcher, Zagreb, Croatia

In this article, authors investigated association of miR-31, miR-146a, miR-181c and miR-155 with schizophrenia pathogenesis, as well as their link to IL2 gene expression in disease. It was noted that 225 patients with paranoid schizophrenia and 225 sex- and age-matched controls were recruited in study. Peripheral blood samples of each patient was used. Results have shown a higher expression of studied mi-RNAs in schizophrenic patients vs control group. As well as lower expression of IL2 gene in schizophrenic patients vs control group. IL2 rs2069778 SNP was not associated with schizophrenia.

It is kindly requested from authors to address following comments:

1. In Introduction section, it is indicated that interleukins and other cytokines and their receptors play an essential role schizophrenia predisposition, development, progression and treatment response. Most of the cited literature reported non-conclusive results in order to support such strong statement. Consideration to rephrase wording "essential" is suggested.
2. It is stated that 225 patients with paranoid schizophrenia and 225 sex- and age-matched controls were recruited in study yet only 61 and 66 patients were tested for micro-RNA expression and IL2 gene expression respectively. It is not clear which inclusion and exclusion criteria were utilized to have only 127 patients tested in total. Providing more information/explanation on these criteria is suggested. It is also stated that all subjects were genotyped for this study. Does this refer to total number of patients or only to ones which were tested or micro-RNA expression and IL2 gene expression?
3. No information on disease stage, type of medications, relevant medical history, concomitant medications and especially comorbidity was provided/discussed for tested patients. MiRNA and IL2 can effectively participate in the pathogenesis of several pathological conditions, such as cancer and metabolic, infectious, autoimmune and inflammatory diseases, Possibility of such comorbidity and influence on the study results should be considered and discussed.
4. Also, observed results of differences in miR-181c and miR-31 expression in T allele carriers should be discussed in more detail.

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Is the work clearly and accurately presented and does it cite the current literature?

Partly

Is the study design appropriate and is the work technically sound?

Yes

Are sufficient details of methods and analysis provided to allow replication by others?

Yes

If applicable, is the statistical analysis and its interpretation appropriate?

I cannot comment. A qualified statistician is required.

Are all the source data underlying the results available to ensure full reproducibility?

Yes

Are the conclusions drawn adequately supported by the results?

Partly

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Pharmacogenomics

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

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