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Differential Expression of miR-26b-5p, *EGR1*, and *STAT1* in Peripheral Blood of Schizophrenia Patients

Yilin Liu[1,†], Fuyi Qin[1,†], Lei Yu[2], Xinling Zhao[3], Qing Long[1], Xiao Ma[4], Xu You[5], Yunqiao Zhang[1], Yatang Chen[6,*], Yong Zeng[1,*]

[1]*The Second Affiliated Hospital of Kunming Medical University, Kunming, Yunnan, China*
[2]*Qujing Third People's Hospital, Qujing, Yunnan, China*
[3]*People's Hospital of Chongqing Liangjiang New Area, Chongqing, China*
[4]*Yunnan Land And Resources Vocational College, Kunming, Yunnan, China*
[5]*Honghe Second People's Hospital, Jianshui, Yunnan, China*
[6]*Chongqing University Central Hospital, Chongqing, China*

ABSTRACT

Background: This study aimed to investigate miRNAs and upstream regulatory transcription factors involved in schizophrenia (SZ) pathogenesis.

Methods: Differential expression of miRNAs and genes in SZ patients was investigated utilizing the gene expression omnibus dataset, gene ontology annotations, and Kyoto encyclopedia of genes and genomes (KEGG) pathway enrichment analysis. Real-time quantitative polymerase chain reaction experiments were conducted to validate the predictive screening of regulatory genes in peripheral blood samples from 20 SZ patients and 20 healthy controls. The diagnostic potential of these factors within these samples was assessed via receiver operating characteristic (ROC) curve analyses.

Results: Fifty-eight miRNAs were identified as differentially expressed in the peripheral blood of SZ patients. miR-26b-5p exhibited significantly reduced expression in SZ patients compared to healthy individuals. Additionally, 1422 mRNAs were differentially expressed, including 5 transcription factors potentially regulating miR-26b-5p expression. Among these, *EGR1* and *STAT1* displayed significantly lower expression levels in SZ patients. Receiver operating characteristic analysis revealed areas under the curve of 0.76 for miR-26b-5p, 0.74 for *EGR1*, 0.82 for *STAT1*, and 0.85 for the combined *STAT1*-miR-26b-5p diagnosis.

Conclusion: The reduced expression of miR-26b-5p, *EGR1*, and *STAT1* in the peripheral blood of SZ patients, compared to healthy controls, suggests a strong association with SZ. These molecules represent potential diagnostic biomarkers, with the combined marker *STAT1*-miR-26b-5p potentially offering enhanced diagnostic accuracy.

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INTRODUCTION

Schizophrenia (SZ) is a severe mental illness ranking among the top 10 causes of disability globally, affecting approximately 1% of the world's population.[1] Currently, SZ diagnosis primarily relies on subjective mental state assessments and clinical interviews conducted by clinicians, rather than objective pathophysiological indicators. Despite extensive research, the exact pathogenesis of SZ remains elusive, and the absence of reliable biomarkers has hampered accurate diagnosis and treatment.[2] Recently, a hypothesis has emerged suggesting that immune inflammation within the nervous system may underlie SZ pathogenesis. Supporting this hypothesis, studies have demonstrated differential expression of certain inflammatory factors in SZ patients compared to healthy controls.[3] Elevated levels of inflammatory factors are significantly associated with the manifestation of clinical symptoms and the underlying pathological mechanisms of SZ.[4] Evidence suggests that inflammatory factors can induce alterations in the neuroendocrine system of patients with early-stage SZ, such as elevated interleukin-6 concentrations that may be linked to severe cognitive dysfunction, particularly in patients who have undergone prolonged untreated illness or those with refractory SZ.[5] Numerous researchers posit that the onset of SZ arises from a complex interplay between genetic and environmental factors.[5] Furthermore, the dynamics and interactions of epigenetic mechanisms may contribute to the multifaceted etiology of mental disorders, including

[†]These authors contributed equally to this work.

***Corresponding author:** Yatang Chen and Yong Zeng, e-mail: yatangchen@foxmail.com or zengyong@kmmu.edu.cn

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SZ.[6] MicroRNAs (miRNAs) serve as crucial regulatory factors in cell development, differentiation, and other vital biological functions, encompassing nearly all life processes.[7] Notably, miRNAs can modulate the expression of approximately one-third of protein-coding genes,[8] thus functioning as both targets and modifiers of epigenetic modification.[9] Numerous studies have demonstrated that miRNAs in the blood hold potential as biomarkers for the diagnosis of SZ.[10] Xu et al[11] demonstrated that aberrations in the co-expression network, arising from interactions between transcription factors (TFs) and miRNAs, could be pivotal in the pathogenesis and clinical outcomes of SZ. In our current investigation, we employed bioinformatics techniques to mine large datasets and predict miRNAs that might regulate inflammatory factors in SZ. Furthermore, we integrated these predictions with molecular biological methods to clinically validate these miRNAs and assess their association with SZ, exploring their potential as diagnostic biomarkers. Additionally, we investigated the potential of TF-miRNA combinations as combined diagnostic markers, predicted their upstream regulatory TFs, and provided novel insights for the research, diagnosis, and treatment of SZ.

MATERIAL AND METHODS

Clinical Samples

Blood samples were obtained from 20 patients diagnosed with SZ residing in the closed ward of a major psychiatric hospital in Yunnan Province, China, between November 2021 and February 2022. These patients, inclusive of those individually diagnosed by at least 2 psychiatrists, exhibited a concordant diagnosis of SZ. Individuals who had refrained from antipsychotic medication during their initial episode or had experienced a relapse and had not undergone a systematic treatment with antipsychotics for a minimum of 3 months preceding their admission were included. Patients with comorbid psychiatric and central nervous system disorders, rheumatic and immunologic conditions, a recent history of severe infections or brain trauma, pregnant and lactating women, as well as individuals with a history of blood transfusion within the preceding 3 months, were excluded from the study. Additionally, 20 healthy individuals, matched for age and gender, were recruited and served as the control group for the physical examination. All clinical experimental protocols and enrollment criteria utilized in this study adhered to the guidelines approved by the Ethics Committee (2021kmykdx6f111). Before participation, all subjects or their legal guardians provided written informed consent.

Screening of Differentially Expressed miRNAs

The information regarding differentially expressed genes in patients with SZ in comparison to healthy individuals was acquired from the gene expression omnibus (GEO) database. The database was searched using keywords including "Schizophrenia" (research keyword), "*Homo sapiens*" (organism), and "Expression profiling by array" (research type). The inclusion criteria for the dataset were as follows: (1) the data originated from studies investigating miRNA/mRNA expression differences in peripheral blood samples from patients with SZ and healthy controls, and (2) the data were sufficiently comprehensive to support subsequent analysis.

Gene Ontology and Kyoto Encyclopedia of Genes and Genomes Analysis

In this study, we employed database for annotation, visualization and integrated discovery (DAVID) (https://david.ncifcrf.gov/), a comprehensive visual data integration database capable of evaluating the biological functions of diverse genes or proteins, to analyze the associated functions and pathways of the miRNAs under investigation. The miRNAs identified from the GEO database were subsequently subjected to functional annotation and pathway enrichment analysis. For this analysis, the entire human genome was utilized as the background reference. During the screening of significant gene ontology (GO) and Kyoto encyclopedia of genes and genomes (KEGG) pathway terms, as well as the calculation of the number of distinct genes exhibiting notable enrichment, statistical significance was established at a threshold of $P < .05$.

Analysis and Prediction of miRNAs and Upstream TFs

To predict the molecules mediating the regulation of inflammatory factors by miRNAs, we employed 3 online databases: TargetScan,[12] miRDB,[13] and miRWalk,[14] to identify and screen miRNAs targeting inflammatory factors. The predicted results were subsequently overlaid and intersected with the differentially expressed miRNAs previously identified from the GEO database, exhibiting a negative correlation with the occurrence of SZ. Consequently, miRNAs differentially expressed in SZ patients and predicted to regulate inflammatory factors were determined. Additionally, we utilized the TransmiR v2.0 database to predict the upstream regulated TFs of miRNAs that were confirmed to be differentially expressed in our experiments.[15] Given that TFs regulate the promoter regions of miRNA precursors, the names of miRNA precursors were chosen for retrieval. Furthermore, a comprehensive dataset encompassing studies on differential mRNA expression in SZ patients and healthy controls was retrieved from the GEO database. Finally, the predicted TF results were cross-referenced with the obtained dataset to identify the target genes.

Real-Time Quantitative Polymerase Chain Reaction Experiment

Extraction of total ribonucleic acid (RNA) was conducted from 5 mL of peripheral whole blood employing TRIZOL

reagent, and the RNA concentration was subsequently determined using an ultra-micro spectrophotometer. The RNA reverse transcription and real-time quantitative polymerase chain reaction (RT-qPCR) kits were sourced from Takara Bio (Shiga, Japan), while the primers utilized in this study were synthesized by Sangon Biotech (Shanghai, China). Sequences of the primer pairs used were 5′-CTCAACTGGTGTCGTGGAGTCGGC AATTCAGTTGAGACCTATCC-3′ (stem-loop primer) and 5′-GGGGTTCAAGTAATTCAGG-3′ (forward primer) and 5′-CTCAACTGGTGTCGTGGA-3′ (reverse primer) for miR-26b-5p; 5′-CTCGCTTCGGCAGCACA-3′ (forward primer) and 5′-AACGCTTCACGAATTTGCGT-3′ (reverse primer) for U6; 5′-GGTCAGTGGCCTAGTGAGC-3′ (forward primer) and 5′-GTGCCGCTGAGTAAATGGGA-3′ (reverse primer) for EGR1; 5′-GTTATGGGACCGCACCTTCA-3′ (forward primer) and 5′-CAGTGAACTGGACCCCTGTC-3′ (reverse primer) for STAT1; and 5′-AGGATTCCTATGTGGGCGAC-3′ (forward primer) and 5′-GTAGAAGGTGTGGTGCCAGA-3′ (reverse primer) for ACTIN. Real-time polymerase chain reaction (PCR) was conducted on the Bio-Rad CFX96 Sequence Detection System. Real-time quantitative polymerase chain reaction reactions were performed in a total volume of 10 μL using Tsingke reagents. Each sample was assayed in triplicate wells, with triplicate measurements of the *U6*/*ACTIN* internal reference gene serving as a control. Relative quantification was achieved using the formula RQ=2^−ΔΔCt, which allowed for the determination of differences in cycle threshold values (Ct) between the target genes and the internal control group. Statistical significance was established at a threshold of $P < .05$.

Statistical Analysis

Statistical package for social sciences (SPSS) version 26 and GraphPad Prism version 7 were utilized for data analysis. For numerical variables such as age, expressed as mean ± standard deviation after the normality test. The chi-square test was used to compare the differences between gender and SZ family history between both groups. Age differences were analyzed with a *t*-test. The molecular expression data for the case and healthy control groups, which did not conform to a normal distribution, were described using the median and interquartile range. A non-parametric Mann-Whitney *U* test (2-sided) was subsequently performed to evaluate the statistical significance of the expression level differences between the 2 groups. Receiver operating characteristic (ROC) curves were employed to assess and compare the diagnostic potential of molecules for SZ. The area under the curve (AUC), specificity, and sensitivity of the respective molecules were calculated to assess their diagnostic utility. For AUC values ranging from 0.5 to 1.0, a higher AUC value indicated increased diagnostic accuracy.[16] The optimal cut-off point for ROC was determined using the Youden index, defined as the maximum perpendicular distance between the ROC curve and the diagonal or the line of opportunity, calculated as the maximum (sensitivity + specificity − 1).[16] Subsequently, we investigated the potential use of the TF-miRNA axis as a diagnostic biomarker. Logistic regression analyses were conducted in which the model used the enter method, utilizing the expression level of a particular molecule as the dependent variable and incorporating the expression of other differentially expressed molecules as covariates in a binary logistic framework. Following the acquisition of pertinent parameters for the co-diagnosis, an ROC curve analysis was subsequently executed on the co-diagnostic model. Statistical significance was set at $P < .05$ for all data analyses.

RESULTS

Gene Expression Omnibus Database Screening for SZ-Related miRNAs

In this study, the gene expression dataset GSE54914 was retrieved from the GEO database. The original data were subsequently analyzed using the GEO2R online tool, which is based on the limma package within the R software platform available on the NCBI website.[17] After analysis, the blanks within the downloaded data were removed, leading to the identification of 298 differentially expressed miRNAs. Ultimately, a total of 58 differentially expressed miRNAs were selected based on the inclusion criteria of "hsa-miR" and "$P < .05$." Among these, 7 miRNAs were up-regulated, while 51 were down-regulated (Figure 1).

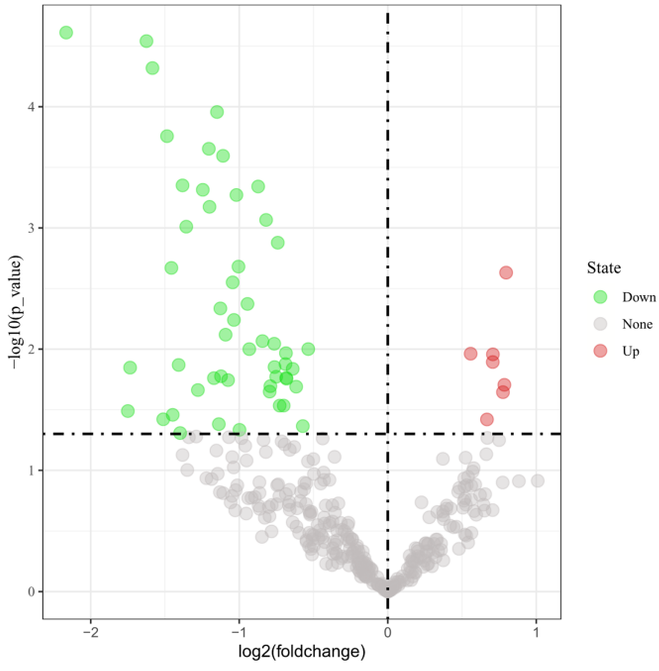

Figure 1. Differentially expressed miRNAs in the GSE54914 dataset. Green dots represent down-regulated genes, and red dots represent up-regulated genes.

Gene Ontology and Kyoto Encyclopedia of Genes and Genomes Pathway Enrichment Analysis of miRNAs

Database for annotation, visualization and integrated discovery was employed to conduct a comprehensive analysis of the GO and KEGG pathways associated with differentially expressed miRNAs. Our findings indicate that the biological processes identified in the GO analysis were predominantly enriched in biosynthetic processes, cellular nitrogen compound metabolic processes, cellular protein modification processes, gene expression, NeuroTrophin Receptor Kinase (NTRK) receptor signaling pathway, and Fc epsilon receptor signaling pathway. Furthermore, the cellular components were significantly enriched in organelles, protein complexes, nucleoplasmic bodies, cytoplasm, and postsynaptic membranes. Molecular functions, on the other hand, were primarily enriched in ion binding, enzyme binding, nucleic acid-binding transcription factor activity, protein binding transcription factor activity, and cytoskeletal protein binding. Additionally, the KEGG pathway analysis revealed enrichment in various pathways, including morphine addiction, nicotine addiction, GABAergic synapses, Ras signaling pathway, Mitogen-Activated Protein Kinases (MAPK) signaling pathway, signaling pathways regulating stem cell pluripotency, Rap1 signaling pathway, cancer-related pathways, thyroid hormone signaling pathway, phosphatidylinositol signaling system, renal cell carcinoma, Wnt signaling pathway, proteins involved in cancer polysaccharide metabolism, ubiquitin-mediated protein degradation, long-term depression, and other related pathways. For a clearer visualization of the results, Sangerbox Tools were utilized (http://vip.sangerbox.com/home.html). These findings are comprehensively presented in Figure 2.

Screening and Validation of miR-26b-5p

Utilizing the keyword "*IL-6*," we conducted a search and prediction analysis in the widely used miRNA target gene databases. By intersecting the predicted miRNAs targeting *IL-6* with the previously screened differentially expressed miRNAs in SZ from the GEO database GSE54914 dataset (Figure 3), we identified 6 miRNAs: hsa-miR-26b-5p, hsa-miR-4756-3p, hsa-miR-3924, hsa-miR-5002-5p, hsa-miR-3667-5p, and hsa-miR-1304-5p. To validate our findings, we employed the RT-qPCR assay to measure the expression levels of 6 miRNAs in peripheral blood samples from 20 SZ patients and 20 healthy controls. Gender and age did not significantly differ between the patients with SZ and healthy controls (Table. 1). Relative quantitative analysis was conducted using *U6* as an internal reference, and a comparison of miRNA expression levels between the disease and control groups was made. The results demonstrated a significantly lower relative expression of miR-26b-5p in the peripheral blood of SZ patients compared to the healthy control group ($P = .005$) (Figure 4). The expression levels of the remaining 5 miRNAs did not differ significantly between the 2 groups.

Screening and Validation of *EGR1* and *STAT1*

The gene expression dataset GSE46509 was retrieved from the GEO database and analyzed utilizing the GEO2R online tool. Data processing adhered to the stringent criteria of $P < .05$ and $|\log2FC| \geq 1$, resulting in the identification of 1422 differentially expressed genes. Of these, 788 genes were downregulated, while 634 genes were upregulated (Figure 5). Using TransmiR, potential upstream TFs for miR-26b-5p were predicted. A search employing the precursor miRNA name "miR-26b" yielded 67 putative TFs. Subsequently, a Venn diagram was constructed by intersecting the predicted TFs with the differentially expressed genes identified in patients with SZ (Figure 6). This analysis revealed 5 overlapping genes. Among these overlapping genes, early growth response 1 (*EGR1*), signal transducer and activator of transcription 1 (*STAT1*), and nuclear factor kappa B subunit 2 (*NFKB2*)

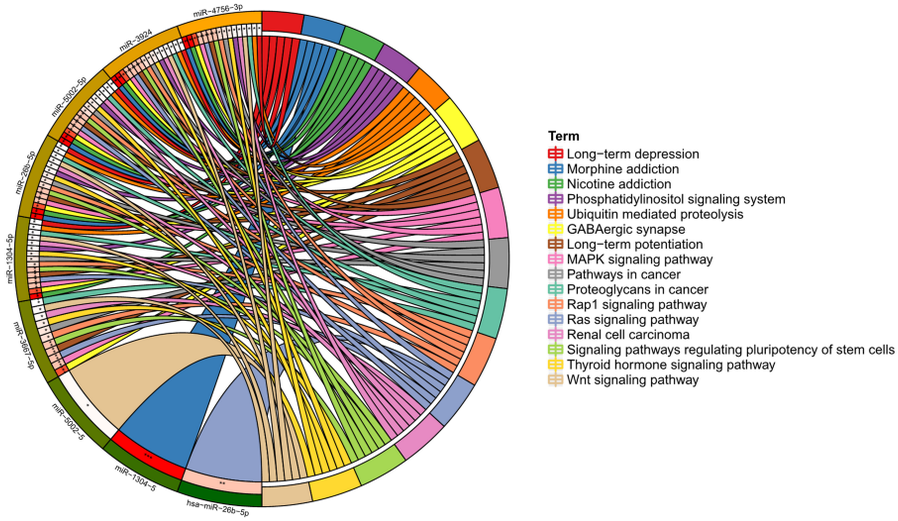

Figure 2. The results of the KEGG analysis.

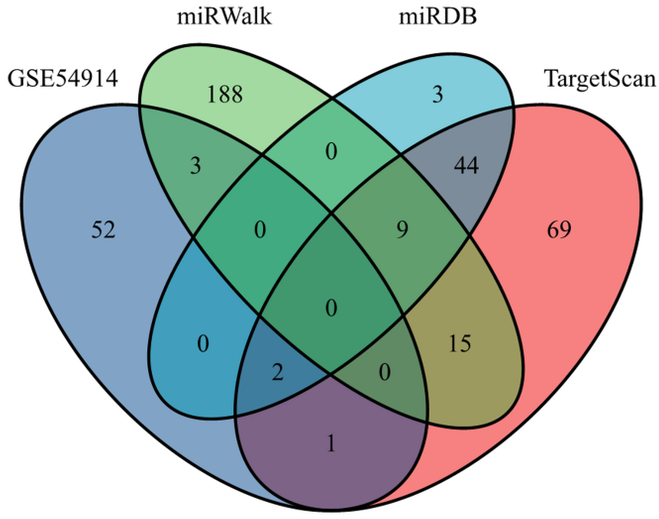

Figure 3. Venn diagram of predicted IL-6-targeted regulatory miRNAs versus SZ differentially expressed miRNAs.

exhibited reduced expression levels in patients with SZ. Conversely, hepatocyte nuclear factor 4 alpha (*HNF4A*), the broad complex tramtrack and bric a brac pox virus and zinc finger protein (*BTB/POZ*), and hook-containing zinc finger 1 (*PATZ1*) were upregulated in these patients. Using the *ACTIN* gene as an internal reference, RT-qPCR was conducted under identical conditions as the prior experiment to assess the expression levels of the predicted TFs *EGR1*, *STAT1*, and *NFKB2*. The findings revealed that the relative expressions of *EGR1* and *STAT1* were significantly downregulated in patients with SZ compared to the healthy control group (P = .023, P = .002) (Figure 7). Conversely, no statistically significant difference was observed in the expression of *NFKB2* between SZ patients and healthy controls (P = .111).

Receiver Operating Characteristic Curve Analysis

The AUC for miR-26b-5p was determined to be 0.76 (95% CI: 0.585, 0.927), with a statistical significance of P = .006. The optimal cutoff value was found to be less than 0.053,

Table 1. General Characteristics of the Schizophrenic Patient Group and the Healthy Control Group

	Healthy Control (n = 20)	Schizophrenia Control (n = 20)	*P*
Gender			1.000
Male (%)	10 (50)	10 (50)	
Female (%)	10 (50)	10 (50)	
Age	37.45 ± 12.43	39.9 ± 13.26	.550
Family history of schizophrenia			.008**
Yes (%)	0 (0)	6 (30)	
No (%)	20 (100)	14 (70)	

**P < .01.

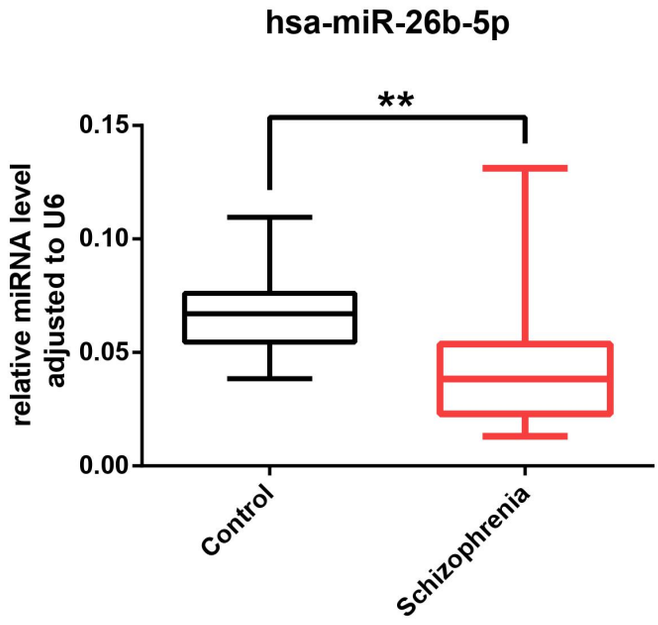

Figure 4. miR-26b-5p expression levels in the peripheral blood of SZ patients were significantly lower than in normal controls (**P < .01).

achieving a sensitivity of 75% and a specificity of 80%. These findings suggest the potential utilization of miR-26b-5p expression levels as a diagnostic biomarker for SZ. Maintaining the groups as state variables, we evaluated the relative expression levels of *EGR1* and *STAT1* as test variables. For *EGR1*, the AUC was calculated as 0.74 (95% CI: 0.556, 0.919), with a statistical significance of

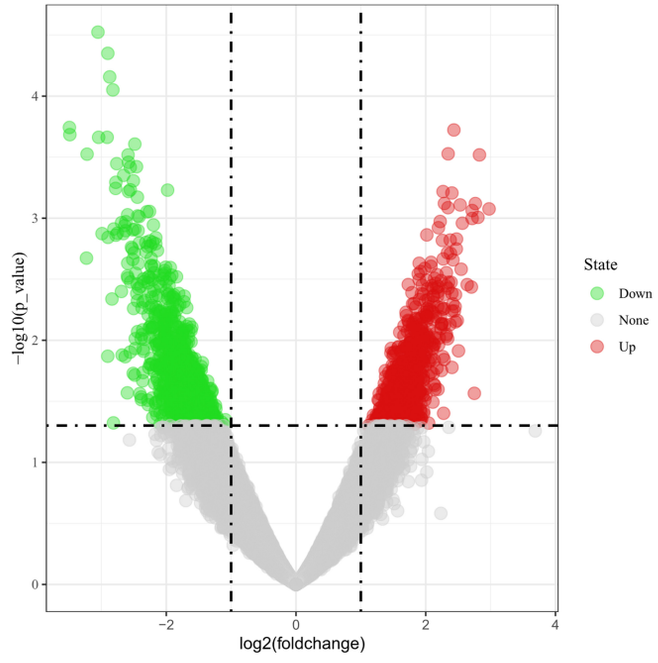

Figure 5. Differentially expressed mRNAs in the GSE46509 dataset. Green dots represent down-regulated genes, and red dots represent up-regulated genes.

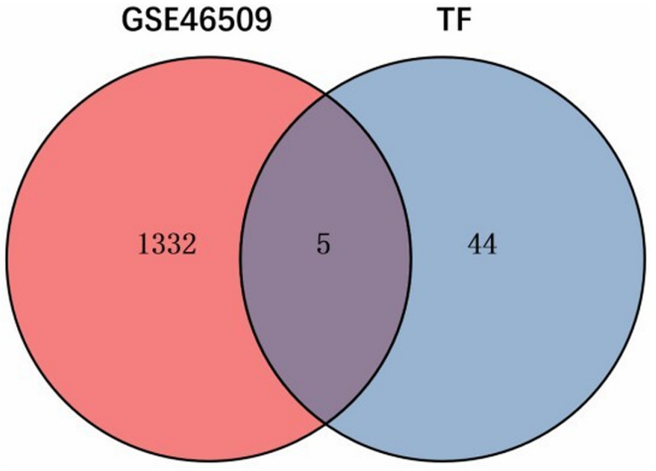

Figure 6. Venn diagram of predicted miR-26b-5p upstream TF versus SZ differentially expressed mRNAs.

P = .024. The optimal cutoff value was less than 0.0094, demonstrating a sensitivity of 56% and a specificity of 93%. Similarly, the AUC for *STAT1* was found to be 0.82 (95% CI: 0.644, 0.997), also with a statistical significance of *P* = .002. The cutoff value for *STAT1* was less than 0.065, exhibiting a sensitivity of 100% and a specificity of 73%. These results underscore the promising diagnostic potential of both *EGR1* and *STAT1* in SZ. To investigate the combined diagnostic impact of differentially expressed miRNAs and upstream TFs in SZ, the expression patterns of miR-26b-5p and 2 key TFs, *EGR1* and *STAT1*, were analyzed in the peripheral blood of SZ patients using logistic regression through SPSS 26. This analysis yielded regression equations for *STAT1* and miR-26b-5p (*P*=.697) and *EGR1* and miR-26b-5p (*P*=.121), as shown in Table 2. In the Hosmer-Lemeshow goodness-of-fit test, a *P*-value greater than .05 indicates a well-fitting model. Both models passed this test, demonstrating a good fit. However, due to the lack of statistical significance in the regression coefficients for *EGR1* and miR-26b-5p, this relationship was excluded from further ROC analysis. The combined analysis of *STAT1* and miR-26b-5p revealed an AUC of 0.85 (95% CI: 0.703, 1.006), with a statistical significance of *P*= .001. The optimal cut-off value was determined to be less than 0.63, demonstrating a sensitivity of 80% and a specificity of 94%. This combined diagnostic approach offers promise as a biomarker for SZ, potentially offering advantages over previously identified single diagnostic markers, as illustrated in Figure 8.

DISCUSSION

Schizophrenia remains a complex condition lacking a definitive etiology, characterized by a polygenic inheritance pattern involving numerous pleiotropic genes. It is now widely recognized that the pathogenesis of SZ is primarily attributed to the intricate interactions between predisposing genetic factors and environmental influences.[18] Within this context, our study employed an epigenetic perspective to explore the potential associations between miRNAs and their related genes in the etiology of SZ, as well as their potential utilization as diagnostic biomarkers. Our investigation comprised 20 SZ patients designated as the disease cohort and 20 healthy individuals serving as controls. Notably, no statistically significant differences were observed between the 2 groups in terms of age and sex demographics. Interestingly, 30% of the SZ patients enrolled in our study reported a family history of SZ, whereas none of the healthy controls had such a history. This significant familial aggregation of SZ aligns with recent epidemiological insights, further supporting the role of genetic factors in the etiology of this complex disorder.[19] Utilizing bioinformatics techniques, we screened for differential expression in 58 miRNAs within the peripheral blood of SZ patients. Subsequent GO and KEGG pathway analyses of these miRNAs demonstrated enrichment in neurotrophin TRK receptor signaling, synaptic transmission, and associations with morphine addiction, nicotine addiction, GABAergic synapses, MAPK signaling, long-term dementia, and long-term depression. These findings align with previous reports on their relevance to SZ progression.[20-27] Furthermore, we narrowed our focus to identify regulatory miRNAs specifically implicated in the

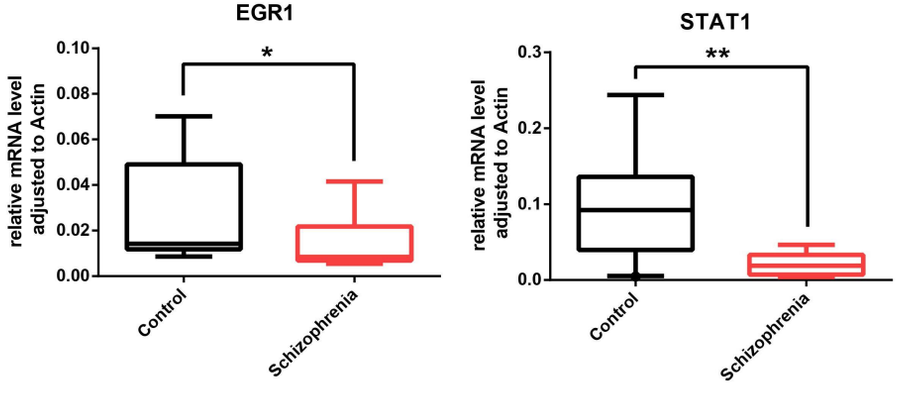

Figure 7. Comparison of EGR1 and STAT1 expression levels in peripheral blood of SZ patients versus normal controls (*P < .05, **P < .01).

Table 2. Logistic Regression Results of miR-26b-5p with STAT1

Variables in the Equation								95% Confidence Interval for EXP (B)	
Step 1[a]		*B*	*S.E.*	*Wald*	*df*	*Sig*	*Exp (B)*	Lower Bound	Upper Bound
	miR-26b-5p	−0.378	0.474	−0.797	1	0.425	0.685	0.251	1.737
	STAT1	−3.410	1.272	−2.682	1	0.007	0.033	0.001	0.244
	Constant	−0.570	0.672	−0.848	1	0.396	0.566	0.108	1.810

Dependent variable: presence of schizophrenia.
[a]Variable(s) entered on step 1: miR-26b-5p, STAT1. df, degrees of freedom; STAT1, signal transducer and activator of transcription 1.

IL-6 mediated pathogenesis of SZ, namely hsa-miR-26b-5p, hsa-miR-5002-5p, hsa-miR-3667-5p, hsa-miR-4756-3p, hsa-miR-1304-5p, and hsa-miR-3924. Accumulating evidence suggests that *IL-6* expression is upregulated in SZ.[4,5] Given that miRNAs exert suppressive effects on gene silencing, we chose to further investigate miRNAs that are expressed at low levels in SZ. Radiation-related studies have demonstrated that miR-26b-5p expression is upregulated in patients with breast cancer, indicating its potential as a radiation marker in conjunction with other molecules.[28] In neuropsychiatric-related studies, hsa-miR-26b-5p is upregulated in Alzheimer's disease.[29] Additionally, hsa-miR-3924 plays a crucial role in inhibiting the invasive process of pancreatic cancer.[30] hsa-miR-4756-3p serves as a regulator in triple-negative breast cancer.[31] Our literature review revealed that the miRNAs screened in our study are primarily associated with oncological diseases. Notably, previous studies have primarily employed bioinformatics techniques for analysis,[32] lacking clinical sample validation. These miRNAs have not been extensively investigated in the context of SZ. We validated the RT-qPCR results for miR-26b-5p, miR-4756-3p, and miR-3924. Real-time quantitative polymerase chain reaction is widely recognized as the gold standard for sensitive, high-throughput, and accurate quantification, surpassing traditional RNA detection methods.[33] Our PCR analysis revealed that only miR-26b-5p exhibited specific amplification, achieving statistical significance. Conversely, the hsa-miR-4756-3p and hsa-miR-3924 samples were not reproducible and exhibited low Ct values. This inconsistency may stem from the fact that the miRNA data were derived from bioinformatics predictions. As different algorithms employed across various databases and platforms can introduce false positives, the reproducibility and accuracy of our findings underscore the need for further validation in clinical samples. A comprehensive analysis of prior studies indicates that limited research has been conducted on the aforementioned miRNAs in SZ, necessitating their validation in a larger cohort of clinical samples.

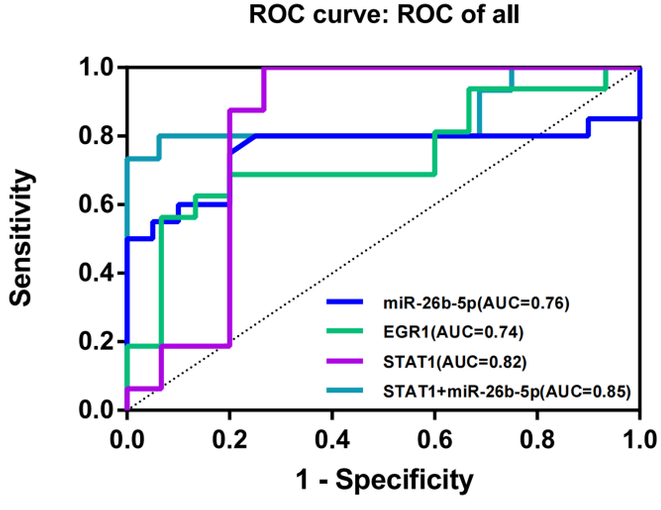

Figure 8. Summary of ROC curves for single indicator and combined diagnostics.

Furthermore, accumulating evidence suggests that the phenotypic manifestations of psychiatric disorders may mediate dynamic gene-environment interactions at the molecular level through TFs that regulate epigenetic processes.[6] miRNAs are primarily transcribed by polymerase II promoters and are regulated by TFs and chromatin structures similarly to mRNAs.[34] The role of TFs in disease development through the regulation of miRNAs has been demonstrated in studies about rheumatoid arthritis,[35] and cardiovascular disease,[36] highlighting their potential significance in SZ as well. Given these findings, we hypothesized the existence of a regulatory axis involving TFs, miR-26b-5p, and *IL-6*. Using bioinformatics, we predicted the TFs regulating miR-26b-5p. Subsequently, a secondary screening was conducted, incorporating experimentally validated differentially expressed genes in SZ, resulting in the identification of 5 TFs: *EGR1*, *STAT1*, *NFKB2*, *HNF4A*, and *PATZ1*. Literature review revealed that TFs involved in most TF-miRNA regulatory axes often exhibit similar expression trends as the miRNAs they regulate.[11] Therefore, we hypothesized that the predicted TFs and the miRNAs targeted in this study would exhibit comparable expression patterns in patients with SZ. Furthermore, we postulated a mechanistic framework where the downregulation of miR-26b-5p in SZ patients results in the upregulation of *IL-6* expression, thereby contributing to the pathogenesis of SZ. Consequently, we prioritized TFs *EGR1*, *STAT1*, and *NFKB2* for RT-qPCR validation, given their similarly low expression levels in

SZ patients. Our results demonstrated that both *EGR1* and *STAT1* exhibited significantly lower expression levels in the peripheral blood of SZ patients compared to healthy controls. Conversely, no statistically significant difference was observed in the expression of *NFKB2*. *EGR1*, a member of the *EGR* family of cys2-his2 type zinc finger proteins, plays a crucial role in cell growth and proliferation,[37] neuronal plasticity,[38] immune responses,[39] and memory formation.[40] Previous studies have demonstrated that the transcription factor *EGR1* is significantly downregulated in postmortem brain samples from patients with SZ.[41] Furthermore, research has shown that *EGR1* expression is significantly altered in the peripheral blood cells of patients with major depression and bipolar disorder compared to healthy controls, suggesting its potential as a biomarker for the differential diagnosis of SZ.[41,42] This is consistent with the results of our experiments. Current research indicates that 2 key intracellular signaling systems primarily orchestrate the immune response *in vivo*: the *NF-κB* and *JAK-STAT1* pathways.[43] Activation of *NF-κB* occurs in response to pathogens and injury-sensing receptors such as *TLR4*, triggering the secretion of inflammatory cytokines, including *IL-6*. Conversely, the *JAK-STAT1* pathway is stimulated by interferons and subsequently upregulates the expression of genes, among them *CXCL10*, *STAT1*, and *TLR4*.[44] Investigations exploring childhood experiences and immune factors in the pathogenesis of SZ have revealed elevated *IL-6* expression and reduced *STAT1* expression in the peripheral blood of SZ patients.[45] Although there is a partial overlap in the genetic and biological processes triggered by these 2 pathways, some studies have reported discordant findings. Specifically, in peripheral blood mononuclear cells from a subset of SZ patients, increased *IL-6* gene expression and decreased *CXCL10*, *STAT1*, and *TLR4* gene expression have been observed. These observations suggest that there are distinct differences between these 2 signaling pathways,[46] and these findings are consistent with the results obtained from our predictions and experiments. Based on our findings, we propose that *IL-6* serves as a key inflammatory factor mediating the pathogenesis of SZ, with its upstream regulatory genes, miR-26b-5p and *EGR1*, exhibiting differential expression patterns. Notably, the low expression levels of *STAT1* observed in our experiments suggest that the inflammatory mechanism underlying SZ is primarily associated with the *NF-κB* pathway, rather than the *JAK-STAT1* pathway, which may play a less significant role. Schizophrenia is a complex central nervous system disorder characterized by the disruption of the blood-brain barrier during inflammatory processes.[47] It has been demonstrated that the expression levels of certain genes in the peripheral blood of SZ patients are identical to their expression levels in the central nervous system tissues.[48] In the realm of psychiatric disorder diagnosis, peripheral blood stands out as a paramount sample source, offering not only unparalleled convenience but also a significantly lower risk profile. Its utilization underscores the critical importance of this approach in facilitating accurate assessments while ensuring minimal harm to patients.[49] This suggests that peripheral blood may serve as a valuable surrogate for studying gene expression changes in the central nervous system of SZ patients, providing insights into the underlying pathophysiology of this disorder.

The results of our study demonstrated that the AUC values for miR-26b-5p, *EGR1*, and *STAT1* were all statistically significant in the ROC analysis. Based on these findings, we propose that miR-26b-5p, *EGR1*, and *STAT1* in peripheral blood have the potential to serve as biological markers for SZ. Furthermore, previous investigations have explored the utilization of the TF-miRNA-target gene axis as a biomarker for diagnosing and therapeutically monitoring SZ. One study reported significant downregulation of *EGR1* and target miRNAs in peripheral blood mononuclear cells of psychotic patients, accompanied by upregulated expression of their target genes. Conversely, the opposite trend was observed following antipsychotic drug treatment, indicating that the TF-miRNA-target gene axis possesses a significantly higher diagnostic value compared to individual genes. Employing statistical methods, we established a combined TF-miRNA diagnosis. Analysis of the ROC curve for the combined diagnostic marker *STAT1*-miR-26b-5p revealed an AUC, although not significantly higher than the AUCs of individual diagnostic markers, suggesting its potential utility. Therefore, *STAT1*-miR-26b-5p can be considered a combined diagnostic marker. Consistent with the results confirmed by existing studies, markers for co-diagnosis have the possible advantage of high accuracy and predictive value compared to single diagnostic markers.[50] Regarding the limitations of this paper and aspects that require further research, we believe that sampling challenges stemming from the scarcity of clinical samples collected precluded the inclusion of all first-episode patients who were not receiving medication in the disease group. Patients' current treatments and clinical responses were not included in the study. Consequently, future studies with enlarged sample sizes and more stringent inclusion criteria are imperative for analyzing and discussing different clinical presentations and treatments. Furthermore, our next consideration is to validate the regulatory function of *STAT1*-miR-26b-5p in the pathogenesis of inflammatory factor-induced SZ, employing cellular and animal models. This validation step is pivotal in further substantiating our findings and elucidating the diagnostic importance of these biomarkers in SZ.

Data Availability Statement: The data supporting the findings of the article are available in the NCBI at Gene Expression Omnibus, reference number [GSE54914 and GSE46509].

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