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Original Article

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Genome-wide, integrative analysis implicates exosome-derived microRNA dysregulation in chronic insomnia

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Abstract

Study Objectives: Insomnia, characterized by difficulty in initiating and sustaining sleep or waking prematurely without the ability to return to sleep, affects approximately 30% of the population. The underlying mechanisms of insomnia remain unclear, and the objective diagnostic measures are scarce. It is an opportunity to explore the roles of peripheral blood exosomal miRNAs in insomnia patients.

Methods: Exosomal miRNAs were isolated from 20 insomnia patients and an equal number of healthy individuals. A comprehensive genome-wide miRNA expression analysis was conducted to identify differential miRNAs between the two groups. To evaluate the diagnostic potential of these miRNAs, receiver-operating characteristic (ROC) curves were employed. Furthermore, Gene Ontology enrichment analysis and Kyoto Encyclopedia of Genes and Genomes pathway analysis were performed on the target genes of differential exosome miRNA to explore their regulated signaling pathways and molecular functions.

Results: We identified 51 differentially expressed miRNAs. Among these, miR-182-5p and miR-451a were markedly downregulated in patients as evidenced by their area-under-the-curve values. The areas under the ROC curves and 95% confidence intervals (CIs) of miR-182-5p and miR-451a in predicting the possibility of chronic insomnia patients were 0.863 (95% CI = 0.75 to 0.97) and 0.813 (95% CI = 0.68 to 0.95). Coexpression network analysis and enrichment analysis of miRNA target genes shed light on the molecular pathophysiology of insomnia, including autophagy and mitophagy.

Conclusions: These findings suggest that peripheral blood exosomal miRNAs may serve as potential noninvasive biomarkers for insomnia diagnosis and provide new insights into the molecular mechanisms underlying this sleep disorder.

Key words: sleep disorders; exosomes; microRNAs; biomarkers

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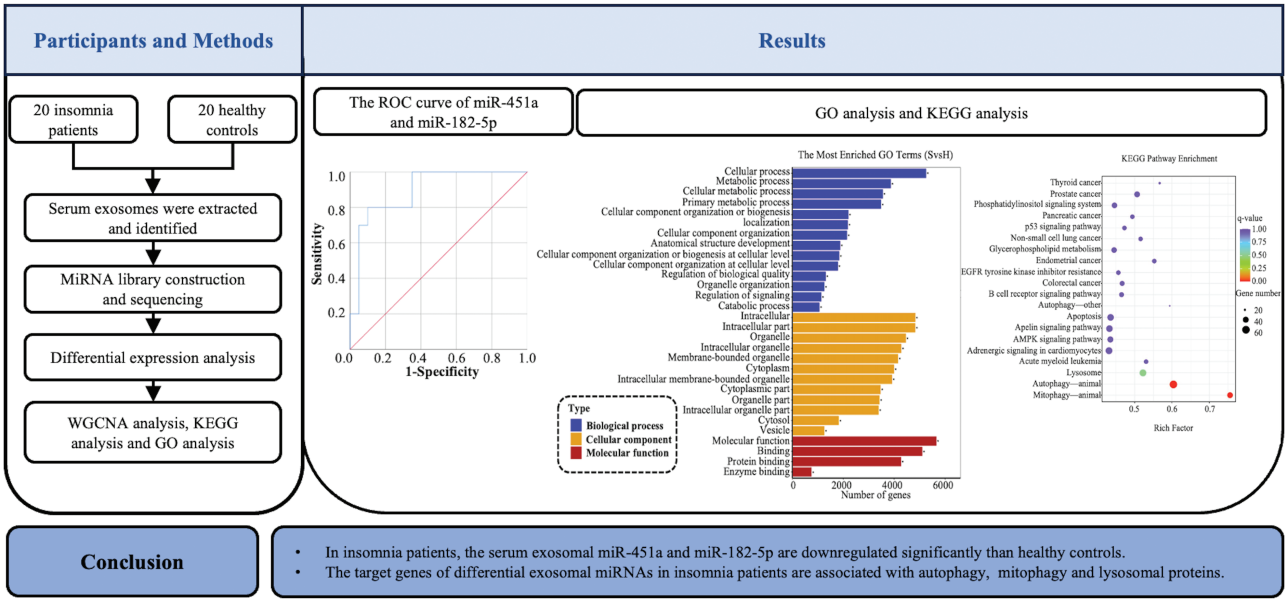
Graphical Abstract

Genome-Wide, Integrative Analysis Implicates Exosome-Derived MicroRNA Dysregulation in Chronic Insomnia



Statement of Significance

Inflammation serves as a crucial mechanism underlying insomnia. Exosomal miRNAs possess the ability to traverse the blood–brain barrier and exhibit correlations with inflammatory factors, thus emerging as diagnostic biomarkers in various neuropsychiatric disorders. This study pioneers the exploration of the role of serum exosomal miRNAs in insomnia patients, revealing significant downregulation of miR-182-5p and miR-451a in insomnia patients. Our study uncovered the changes in serum exosomal miRNA profiles in patients with insomnia through genome-wide analysis, providing further evidence to support the liquid biopsy technique for diagnosing insomnia in the future.

Introduction

Insomnia is a prevalent sleep disorder characterized by difficulty in initiating and maintaining sleep or waking prematurely without the ability to return to sleep. This disorder leads to considerable physical and mental health complications [1, 2]. The incidence of insomnia varies internationally, reported between 10% and 15% with trends suggesting a potential rise to 30% of the population affected [3]. Diagnoses of sleep disorders currently depend largely on subjective reporting rather than objective measures, highlighting a significant gap. Numerous models of chronic insomnia exist, yet a consensus on its initiation mechanism is absent.

Exosomes are small extracellular vesicles (40–160 nm) that facilitate intercellular substance and information exchange. Secreted by various cells, they transport proteins, lipids, and miRNAs, which are essential for stress response, communication, synaptic remodeling, and neuronal development. Their capacity to cross the blood–brain barrier renders them promising noninvasive biomarkers for neurological disorders, evidenced by their cargo correlating with inflammatory factors in blood and cerebrospinal fluid [4–8]. Numerous studies have demonstrated the potential of liquid biopsies (serum exosomes miRNA) as biomarkers in depression, anxiety, and other psychiatric disorders [6, 9–12]. However, there are few studies about exosomes in patients with insomnia.

In our study, we performed genome-wide profiling of exosomal miRNA in patients with chronic insomnia and healthy controls. We identified distinct exosomal miRNA expressions and revealed significant downregulation of two specific exosomal miRNAs. Through bioinformatics and network analyses, we discovered several coexpression modules of exosomal miRNAs, enhancing our understanding of the disorder's molecular basis.

Methods

Participants and samples

Our investigation included 20 participants—chronic insomnia patients juxtaposed with healthy individuals. All subjects completed an extensive medical questionnaire before testing, which encompassed instruments such as the Pittsburgh Sleep Quality Index (PSQI), Morningness-Eveningness Questionnaire Self-Assessment Version (MEQ-SA), Clinical Global Impression Scale (CGI), and the Quality of Life Enjoyment and Satisfaction Questionnaire(Q-LES-Q-SF), among others.

The inclusion criteria of insomnia patients were (1) individuals diagnosed with chronic insomnia; (2) age range between 18 and 65 years; (3) no recent intake of sleep-affecting medication or ineffective drugs; (4) possession of complete clinical data; and (5) voluntary study participation with signed informed consent. Exclusion criteria encompassed (1) patients with concurrent psychiatric disorders such as schizophrenia, drug dependence, dementia, traumatic brain injury, etc; (2) recent history of

inflammation or fever; (3) pregnant or lactating women; (4) presence of tumors, infections, or severe organ failure; and (5) refusal to partake in the study. Our study was an exploratory research and there was a lack of reliable data available for sample size estimation. Based on previous relevant reports and the actual progress of patient enrollment, the sample size was set at 20 patients in the insomnia group. Twenty age- and gender-matched healthy controls were recruited. The principal characteristics of the participants are presented in Table 1.

Ethical compliance for this study was secured through approval by the Xuanwu Hospital Capital Medical University Ethics Review Board and the IRB approving number is [2022]095. Written informed consent was obtained from all subjects. Study methods and results are reported following the reporting recommendations for tumor marker prognostic studies (REMARK).

Serum samples were collected from our participants. Serum exosomes were extracted by using size-exclusion chromatography. Then we performed transmission electron microscopy, nanoparticle tracking analysis, and Western blot methods to identify exosomes. The methodologies for blood exosome isolation and validation, along with the miRNA library construction and sequencing, Western blots, and quantitative reverse transcription-polymerase chain reaction (RT-PCR), are detailed in the Supplementary File.

Differential expression analysis

We selected miRNAs for differential expression analysis between the insomnia and healthy cohorts using DESeq2, based on a mean expression threshold of ≥10 transcripts per million (TPM) across all samples. Significantly differentially expressed miRNAs (DE miRNAs) were determined using Benjamini–Hochberg corrected *p*-values (*q*-values, false discovery rate) of <.05. Additional bioinformatics analyses, including random forest classification, weighted gene coexpression network analysis (WGCNA), Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis, and Gene Ontology (GO) enrichment analysis, are elaborated upon in the Supplementary Methods.

Statistical analysis

Using SPSS version 29.0.1.0, we identified the potential biomarkers of blood exosomal miRNA through the estimation of receiver-operating characteristic (ROC) curves and conducted the descriptive analysis of the associated clinical information. Categorical variables were characterized using counts and percentages, with statistical significance being assessed through the application of the chi-squared (χ^2) test. For continuous variables, we first evaluated their normality. If the data followed a normal distribution, the mean and standard deviation (reported as mean ± *SD*) were used to describe central tendency and variability, and statistical comparisons were performed using the two independent-samples *t*-test. Alternatively, for continuous variables that did not conform to a normal distribution, we employed the median and interquartile range (IQR) to describe central location and dispersion, with statistical significance being evaluated using the Mann–Whitney U test. The Pearson correlation

Table 1. Clinical characteristics and medical questionnaire scores with participants

		Insomnia group	Healthy group	*p*-Values
Number, *n*		20	20	–
Age, mean ± *SD*		43.82 ± 15.87	41.50 ± 14.05	.341
Course, month, median (IQR)		51.5 (33, 114)	0	<.001
Female, *n*(%)		9 (45%)	12 (60%)	.342
Height, mean ± *SD*		168.05 ± 10.13	166.10 ± 9.68	.809
Weight, mean ± *SD*		65.11 ± 11.98	66.13 ± 9.94	.264
BMI, mean ± *SD*		22.96 ± 3.04	23.95 ± 2.73	.654
Medicine, *n* (%)		12 (60%)	0	<.001
The history of smoking, *n* (%)		1 (5%)	3 (15%)	.605
The history of drinking, *n* (%)		0	3 (15%)	.231
The scores of PSQI, mean ± *SD*		17.65 ± 5.83	2.6 ± 1.93	<.001
HAMD, median (IQR)		7.5 (4.5, 12.75)	0 (0, 1)	<.001
HAMA, median (IQR)		8 (3, 13)	0 (0, 1.75)	<.001
MEQ-SA, mean ± *SD*		54.95 ± 10.79	59.95 ± 10.73	.921
MEQ-SA	Definite evening, *n* (%)	0	0	–
	Moderate evening, *n* (%)	4 (20%)	1 (5%)	
	Intermediate, *n* (%)	8 (40%)	7 (35%)	
	Moderate morning, *n* (%)	7 (35%)	6 (30%)	
	Definite morning, *n* (%)	1 (5%)	6 (30%)	
CGI, median (IQR)		4 (3, 5.75)	0 (0, 0)	<.001
Q-LES-Q-SF, mean±SD		53.85 ± 11.58	64.75 ± 10.26	.771

Abbreviations: BMI, body mass index = weight(kg)/(height(m))^2; CGI, Clinical Global Impression Scale; HAMA, Hamilton Anxiety Scale; HAMD, Hamilton Depression Scale; IQR, interquartile range; MEQ-SA, Morningness-Eveningness Questionnaire Self-Assessment Version; PSQI, Pittsburgh Sleep Quality Index; Q-LES-Q-SF, Quality of Life Enjoyment and Satisfaction Questionnaire; *SD*, standard deviation.

coefficients were calculated using the statistical function in ggpubr within R version 4.3.1. *p*-Values in miRNAs were adjusted by age, gender, smokin,g and drinking as covariates using multivariable linear regression models.

Results

Differential expression of blood exosomal miRNAs in chronic insomnia

We harvested blood exosomes from patients with chronic insomnia and healthy controls (Figure 1, A–C). Utilizing the Illumina HiSeq 2500 platform, we conducted miRNA-seq to compare miRNA profiles within serum exosomes of both groups. Thousands of miRNAs were initially identified, from which we filtered out those with less than 10 reads (mean TPM <10), ultimately discerning 51 miRNAs with significant expression alterations in the blood exosomes between the two groups. Among these, 21 miRNAs were upregulated, and 30 were downregulated in the insomnia group relative to the controls (Figure 1, D and E; Tables 2 and 3).

Serum exosomal miRNAs coexpression modules in insomnia patients

Using WGCNA, we assigned serum exosomal miRNAs to 13 coexpression modules based on the similarity of their expression patterns (Figure 2A). The boxplot showed the differences in the expression of different gene modules in patients of insomnia (Figure 2C). The correlations between each module and different disease states were analyzed, as shown in the heatmap (Figure 2B). The connections between genes within the module are illustrated by the network diagram (Figure 2, D–F). In our results, three modules displayed significant correlations with insomnia: one was downregulated (turquoise) and two were upregulated (yellow-green and red) (Figure 2, B–F). The turquoise module's primary miRNAs, such as miR-517a-3p and miR-7-5p, were negatively associated with drug use in insomnia patients ($p = .04$). The yellow-green module demonstrated a positive association with the patient's smoking history ($p = .03$), featuring miRNAs such as miR-3127-5p and miR-4435. The red module, with miRNAs like miR-411-5p and miR-409-3p, exhibited a negative correlation with patients' age ($p = .03$).

Significant downregulation of three exosomal miRNAs in insomnia patients

To investigate the potential of exosomal miRNAs as biomarkers, we studied the expression differences of 51 miRNAs between insomnia patients and healthy subjects, identifying 3 miRNAs that exhibited significant differences between these two groups. We incorporated age, gender, smoking, and drinking into the regression models as covariates to control for their impact on miRNAs, which confirmed the significant downregulation of the three miRNAs in insomnia patients. We constructed ROC curves for these three miRNAs to evaluate their effectiveness as potential biomarkers (Figure 3). MiR-20a-5p emerged as the most promising single miRNA biomarker, with an area-under-the-curve (AUC) value of 0.888 (95% confidence interval [CI] = 0.79 to 0.98), and demonstrated diagnostic sensitivity and specificity of 0.75 and 0.95, respectively. The areas under the ROC curves and 95% CI of miR-182-5p and miR-451a in predicting the possibility of chronic insomnia patients were 0.863 (95% CI = 0.75 to 0.97) and 0.813 (95% CI = 0.68 to 0.95), with the sensitivity and specificity of 0.9 and 0.8 for miR-182-5p and 0.65 and 0.9 for miR-451a (Figure 3).

QRT-PCR validation of miRNA sequence data

To substantiate our sequencing results, we further exactly assessed the expression levels of the three miRNAs (hsa-miR-451a, hsa-miR-182-5p, and hsa-miR-20a-5p) using quantitative reverse transcription-polymerase chain reaction (qRT-PCR) in the same cohort of insomnia patients and healthy controls. Consistent with the miRNA-seq data, levels of hsa-miR-451a and hsa-miR-182-5p in blood exosomes were reduced in the insomnia group. However, contrary to the sequencing data, hsa-miR-20a-5p levels did not significantly differ between insomnia patients and controls (Figure 4). The AUC is 0.895 for combined analysis of hsa-miR-451a and hsa-miR-182-5p when predicting the possibility of chronic insomnia patients, with sensitivity rates of 0.8 and specificity rates of 0.9 (Figure 3).

Pathway enrichment analysis of differential exosomal miRNA-targeted genes

To elucidate the molecular underpinnings of insomnia, we undertook a multigroup analysis to identify potential target genes of differentially expressed exosomal miRNAs. The GO analysis, facilitated by ClusterProfiler, assessed shared functions among targets of insomnia-affected miRNA modules, revealing enrichment in genes involved in intracellular metabolic processing and molecular function linkage. KEGG pathway analysis further highlighted significant enrichment within biochemical metabolic pathways and signal transduction pathways, including those related to autophagy, mitophagy, and lysosomal functions (Figure 5).

Correlation between miRNA expression profiles and insomnia, depression, and anxiety

We evaluated the correlation between the exosomal miRNA expression levels and clinical scores related to insomnia, depression, and anxiety. The results of Pearson correlation coefficients are depicted in Figure 6. The expression levels of miR-451a and miR-182-5p all had a negative correlation with the scores of PSQI, HAMA, and HAMD. The correlation between miR-451a and the three scores was found to be significant (PSQI: $R = -0.56$, $p = .00015$; HAMD: $R = -0.47$, $p = .0021$; HAMA: $R = -0.37$, $p = .018$). However, the miR-182-5p exhibited stronger correlations with the scores of PSQI and HAMD than HAMA (PSQI: $R = -0.51$, $p = .00083$; HAMD: $R = -0.32$, $p = .043$; HAMA: $R = -0.25$, $p = .12$).

Discussion

In this study, we conducted a genome-wide analysis to identify serum exosomal miRNAs that are significantly associated with insomnia. Our findings revealed the different exosomal miRNA expression patterns between chronic insomnia patients and healthy volunteers and summarized miRNA coexpression modules in insomnia patients. There are 51 differentially expressed miRNAs with 21 miRNAs upregulated and 30 downregulated. Notably, our study indicated that the downregulation of miR-182-5p and miR-451a in serum exosomes is significant. To our knowledge, this study is the first to compare serum exosomal miRNAs in insomnia patients and healthy subjects.

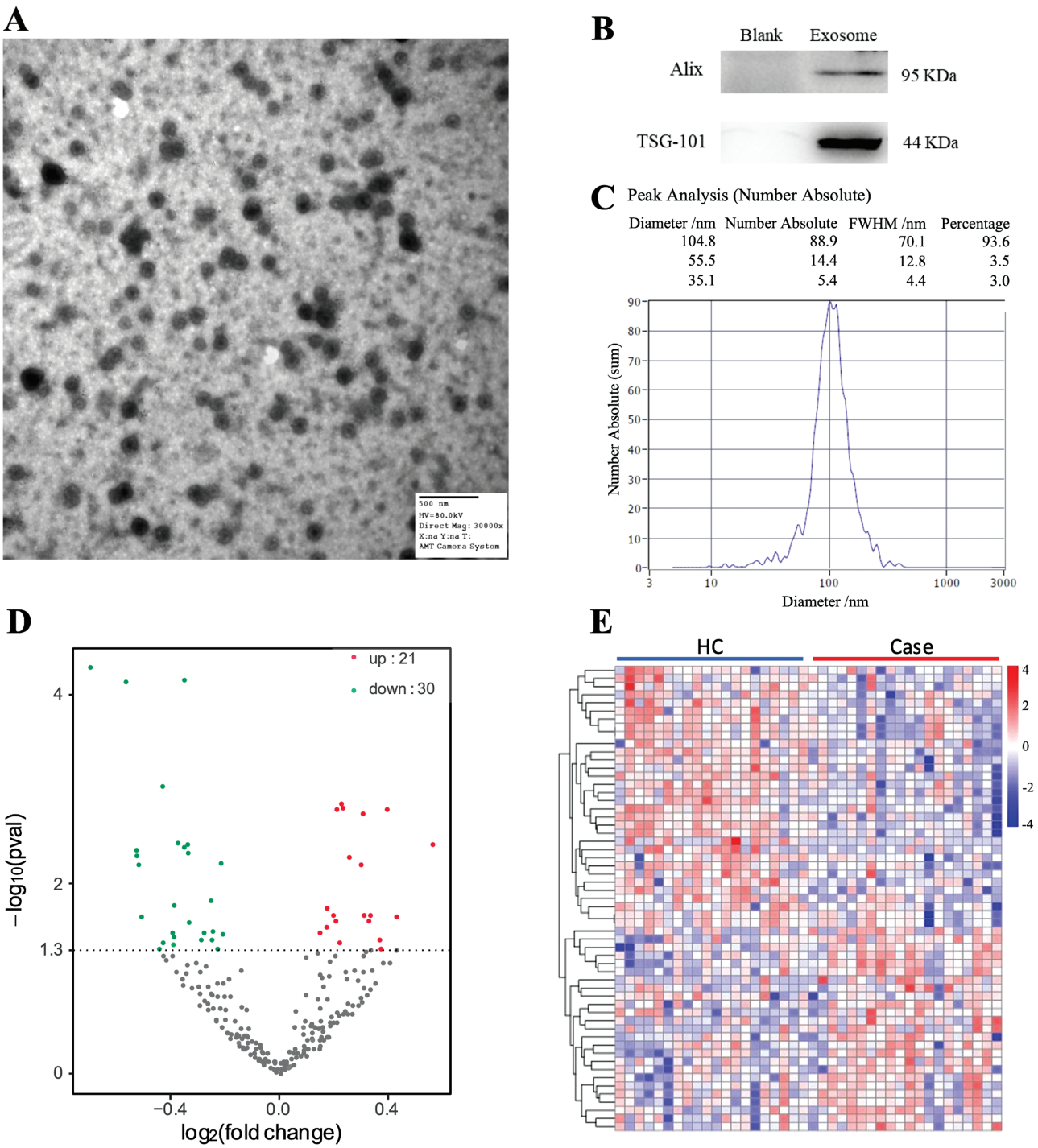


Figure 1. Blood exosomal miRNA changes in patients with insomnia. (A) Exosome morphology observed by electron microscopy at ×7000 magnification. (B) Western blots showed exosome-specific protein markers Alix and TSG-101 were present in the exosomes derived from peripheral blood. (C) The particles peak around at 100 nm verified by nanoparticle size tracking analysis—Zeta View. (D) Volcano plot of expressed exosomal miRNAs between insomnia patients and healthy control. The X-axis represents $\log_2$ foldchange differences between the compared samples. The Y-axis represents the statistical significance of the change in miRNA expression. (E) Hierarchical cluster plot of differentially expressed miRNAs for insomnia patients compared to healthy controls. The subjects are shown on the X-axis and the miRNAs are shown on the Y-axis. On the X-axis, the groups starting with "H" represent the healthy group, and the groups starting with "C" represent the sleeplessness group.

MicroRNAs in exosomes are endogenous, noncoding, single-stranded small RNAs approximately 20–25 nucleotides in length, playing a crucial role in post-transcriptional gene regulation [13]. MiRNAs are comparable in number to transcription factors or RNA-binding proteins, but they exhibit tissue-specific expression patterns, significantly contributing to the cell-specific protein expression profile. MiRNA genes are transcribed by RNA polymerase II into primary miRNAs (pri-miRNAs) [13]. The canonical miRNA biogenesis pathway involves two key steps, primarily catalyzed by the RNase III family enzymes Drosha and Dicer.

Table 2. Exosome miRNAs with significant differences and downregulation in patients

sRNA	log2FoldChange	*p*-Values	*q*-Values*
hsa-miR-451a	−0.69621	5.29E−05	0.0072203
hsa-miR-20a-5p	−0.3488	7.07E−05	0.0072203
hsa-miR-182-5p	−0.56486	7.42E−05	0.0072203
hsa-miR-92a-3p	−0.42904	0.00092952	0.059749
hsa-miR-181b-5p	−0.37256	0.0037747	0.08998
hsa-miR-361-5p	−0.31225	0.0038812	0.08998
hsa-miR-181a-5p	−0.34918	0.0043501	0.08998
hsa-miR-1180-3p	−0.52552	0.0046642	0.08998
hsa-miR-22-3p	−0.33434	0.005027	0.08998
hsa-miR-183-5p	−0.52431	0.0052287	0.08998
hsa-miR-185-5p	−0.21293	0.0061547	0.093326
hsa-miR-1287-5p	−0.51723	0.0063922	0.093326
hsa-miR-16-5p	−0.25029	0.015097	0.20992
hsa-miR-486-5p	−0.38832	0.016625	0.2178
hsa-miR-486-3p	−0.38618	0.017155	0.2178
hsa-miR-181d-5p	−0.50684	0.022363	0.23174
hsa-miR-598-3p	−0.33099	0.025836	0.23746
hsa-miR-15b-5p	−0.22419	0.031489	0.27044
hsa-miR-3184-5p	−0.39207	0.033035	0.27083
hsa-miR-107	−0.27576	0.033905	0.27083
hsa-miR-320a-3p	−0.18246	0.036525	0.27083
hsa-miR-150-5p	−0.38686	0.036612	0.27083
hsa-miR-423-3p	−0.3843	0.037101	0.27083
hsa-miR-320b	−0.24304	0.039708	0.27606
hsa-miR-15a-5p	−0.28649	0.041779	0.27733
hsa-miR-196a-5p	−0.42739	0.04356	0.27733
hsa-miR-200c-3p	−0.38532	0.044246	0.27733
hsa-miR-93-3p	−0.3898	0.044638	0.27733
hsa-miR-532-5p	−0.2256	0.048302	0.28004
hsa-miR-363-3p	−0.44091	0.049238	0.28004

Abbreviations: hsa, homo sapiens; miR, micro RNA; sRNA, small RNA.
* Corrected *p*-value.

MiRNAs guide the RNA-induced silencing complex to downregulate gene expression post-transcriptionally, either by inhibiting translation or promoting mRNA decay.

Recent research works have highlighted the potential of miRNAs as biomarkers for sleep disorders. For instance, a study on isolated rapid eye movement sleep behavior disorder (IRBD), a risk factor for Lewy body dementia, identified specific serum miRNA profiles that could predict the progression to Parkinson's disease and dementia with Lewy bodies [14]. Another study by the University of Colorado-Boulder found that chronic sleep deprivation significantly reduced circulating levels of specific miRNAs, implicating their role in inflammation, immune function, and vascular health [15]. Furthermore, miRNA-mediated regulation has been proposed as a novel mechanism by which nocturnal breathing disorders exacerbate post-myocardial infarction remodeling [16]. Additionally, miR-137 has been implicated in the regulation of the sleep–wake cycle through its effect on the neuropeptide hypocretin/orexin, modulated by the immune system [17].

Although insomnia was affected by age, gender, smoking, and alcohol consumption, there were no statistically significant differences in these factors between insomnia group and healthy controls. The miR-182-5p and miR-451a remain promising biomarkers after adjustment for all relevant covariates and QRT-PCR validation. In our study, we found miR-451a had significantly negative correlations with the scores of PSQI, HAMA, and HAMD. However, miR-182-5p displayed significant negative correlations with scores of PSQI and HAMD, but not with HAMA. Overexpressing miR-451a can reverse the depression phenotype and the loss of dendritic spines by inhibiting chronic restraint stress-induced corticotropin-releasing factor receptor 1 expression via targeting transcription factor 2 [18]. The low CSF levels of miR-451a have been found to correlate with the comorbidity of cognitive impairment and depression [19]. These studies further demonstrate that changes in peripheral blood miR-451a can reflect central nervous system disorders. Besides, miR-451a was shown to regulate proliferation and increase apoptosis via IL-6R/JAK2/STAT3 pathway in oncological diseases [20]. Previous

Table 3. Exosome miRNAs with significant differences and upregulation in patients

sRNA	log2FoldChange	*p*-Values	*q*-Values*
hsa-let-7f-5p	0.23158	0.0014375	0.059749
hsa-miR-98-5p	0.23718	0.0016074	0.059749
hsa-miR-335-5p	0.40032	0.0016558	0.059749
hsa-miR-191-5p	0.21481	0.0016565	0.059749
hsa-miR-30e-3p	0.31104	0.0018416	0.059749
hsa-miR-889-3p	0.56885	0.0038105	0.08998
hsa-miR-28-3p	0.2609	0.0052386	0.08998
hsa-miR-27a-3p	0.3044	0.0063477	0.093326
hsa-miR-30e-5p	0.17898	0.018174	0.22112
hsa-miR-146a-5p	0.31563	0.021558	0.23174
hsa-miR-556-5p	0.43559	0.022377	0.23174
hsa-miR-1-3p	0.33828	0.022706	0.23174
hsa-miR-142-3p	0.20178	0.023015	0.23174
hsa-miR-143-3p	0.33289	0.025382	0.23746
hsa-miR-339-3p	0.21093	0.026023	0.23746
hsa-miR-151a-3p	0.1767	0.031427	0.27044
hsa-let-7a-5p	0.15265	0.034998	0.27083
hsa-miR-376a-3p	0.37328	0.038983	0.27606
hsa-miR-126-5p	0.22527	0.044056	0.27733
hsa-miR-411-5p	0.3771	0.047841	0.28004
hsa-miR-409-3p	0.3782	0.048971	0.28004

Abbreviations: hsa, homo sapiens; miR, micro RNA; sRNA, small RNA.
* Corrected *p*-value.

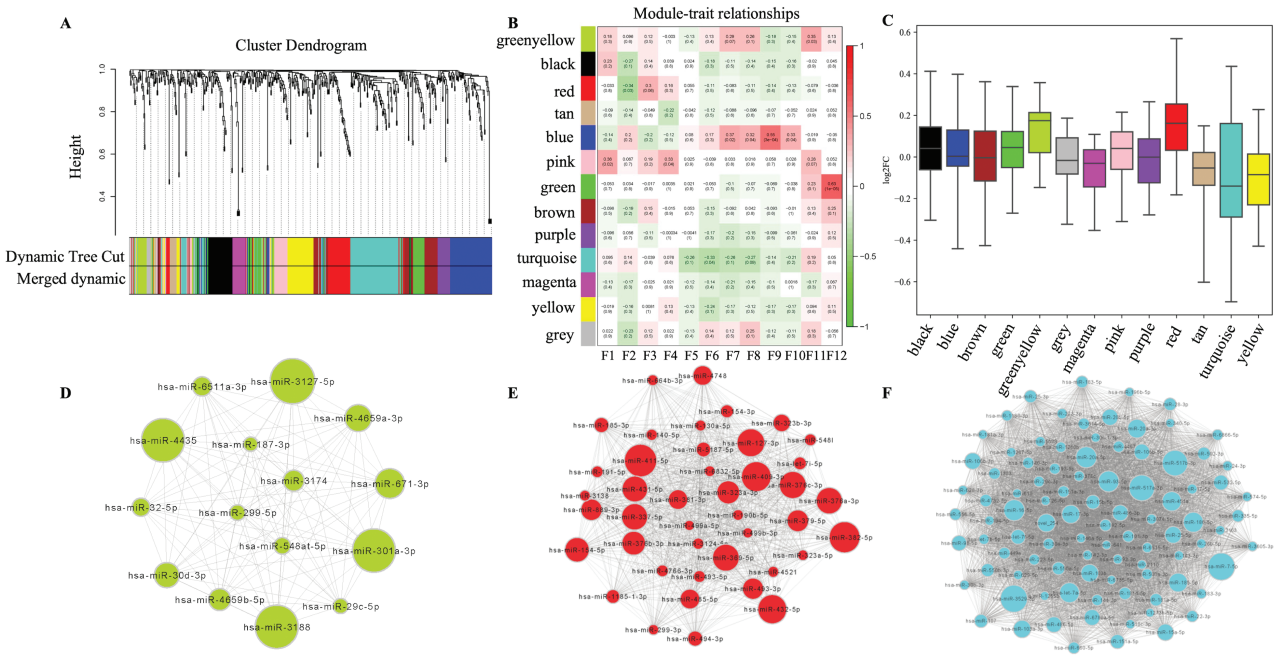


Figure 2. miRNA coexpression modules dysregulated in blood exosomes of insomnia patients. (A) Dendrogram showing miRNA coexpression modules defined in 20 samples for insomnia. miRNAs were clustered based on a dissimilarity measure. Correlated miRNAs were grouped into modules identified through the Dynamic Tree Cut function. Color bars indicate dynamic module assignments (Dynamic Tree Cut) and merged module assignments (Merged dynamic). (B) Pearson's correlation coefficient (and *p*-value in parentheses) between disease status and gene module. (The abscissa is different clinical traits, F1: gender F2: age F3: height F4: weight F5: course of disease F6: whether there is drug treatment F7: number of traditional Chinese medicine types F8: the use of traditional Chinese medicine F9: number of western medicine types F10: the use of western medicine F11: smoking F12: alcohol consumption [drug use is divided into: never used drugs; used drugs but not within 3 months; used drugs within 3 months].) (C) Log_2 transformed the fold-change distribution of microRNA in different color modules. Log_2(Fold-Change) greater than 0 represents upregulated expression in disease samples, and vice versa, downregulated expression in disease samples. (D–F) Coexpression network plots for yellow-green, red, and turquoise-colored modules. Node size is proportional to node connectivity, and edge indicates coexpression of connected nodes with intramodular connectivity greater than 0.5.

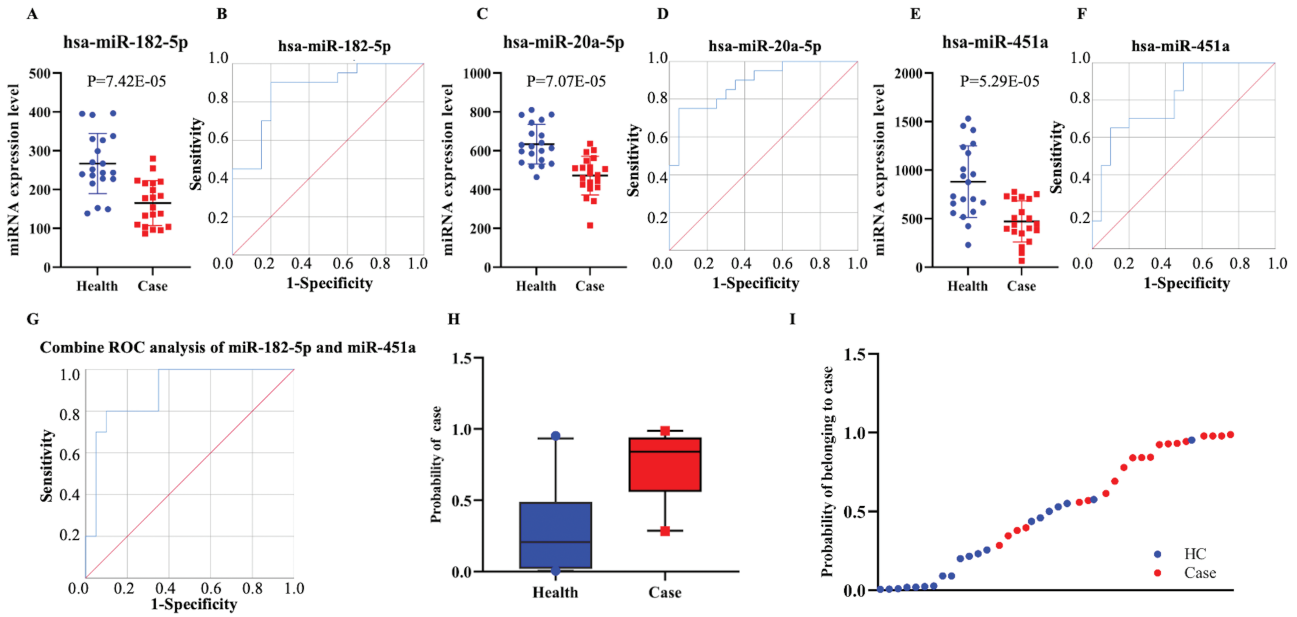


Figure 3. Blood exosome miRNAs as potential biomarkers to distinguish insomnia patients from healthy people. (A) Scatter box plot of median values for the expression of differential miR-182-5p between insomnia group and healthy group. (B) ROC curves and area under the ROC curve of miR-182-5p for the diagnosis of insomnia patients. (C) Scatter box plot of median values for the expression of differential miR-20a-5p between insomnia group and healthy group. (D) ROC curve and area under the ROC curve of miR-20a-5p for the diagnosis of insomnia patients. (E) Scatter box plot of median values for the expression of differential miR-451a between insomnia group and healthy group. (F) ROC curves and area under the ROC curve of miR-451a for the diagnosis of insomnia patients. (G) ROC curves and area under the ROC curve of a cluster of miR-182-5p and miR-451a for the diagnosis of insomnia patients. (H) Boxplot of the probability of participants belonging to insomnia group in the testing set by the combination of miR-182-5p and miR-451a. (I) Scatterplot of the probability of participants belonging to insomnia group in the testing set by the combination of miR-182-5p and miR-451a.

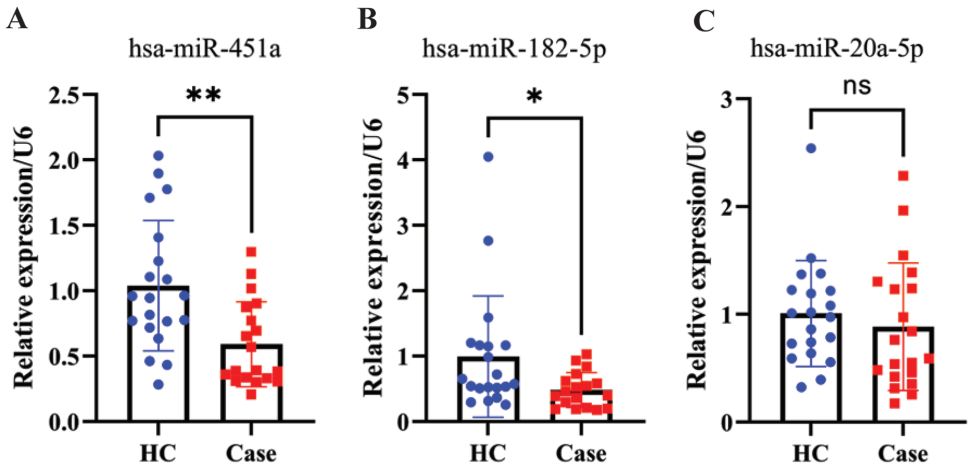


Figure 4. QRT-PCR validation of miRNA sequence results of three differential exosomal miRNAs. The Y-axis represents the relative expression level based on qRT-PCR. *p-value <.05, **p-value <.01.

studies have found that miR-451a, which can promote the expression of IL-6-related genes, was differentially expressed in the peripheral blood of patients with IRBD, potentially explaining the elevated IL-6 levels observed in sleep disorder patients [14, 21]. Many observational studies have demonstrated that IL-6 is associated with insomnia [22–24]. IL-6 plays a key role in the transition between acute response and chronic inflammation, which may alter brain function by chronic stress, hypothalamic–pituitary–adrenal hyperactivity, sympathetic nervous system activity in insomnia disease, and psychiatric disorders [25–27]. Therefore, we provide the hypothesis that the downregulation of exosomal miR-451a in peripheral blood may lead to insomnia by increasing the level of IL-6. However, we did not analyze the level of serum IL-6 in insomnia patients. Because the elevated inflammation cytokines are also related to depression and suicide, the different roles of IL-6 in these disorders need to be further explored [28, 29]. miR-182-5p has been linked to oxidative stress and apoptosis, as well as various cancers. Its downregulation in plasma has been associated with adipocyte proliferation and metabolism under intermittent hypoxia via PI3K/AKT pathway [30]. The decreased exosomal miR-182-5p from sleep deprivation in mice or humans can lead to the activation of inflammatory pathways and contribute to atherosclerosis [31]. Furthermore, a mutation in the miR-182 precursor

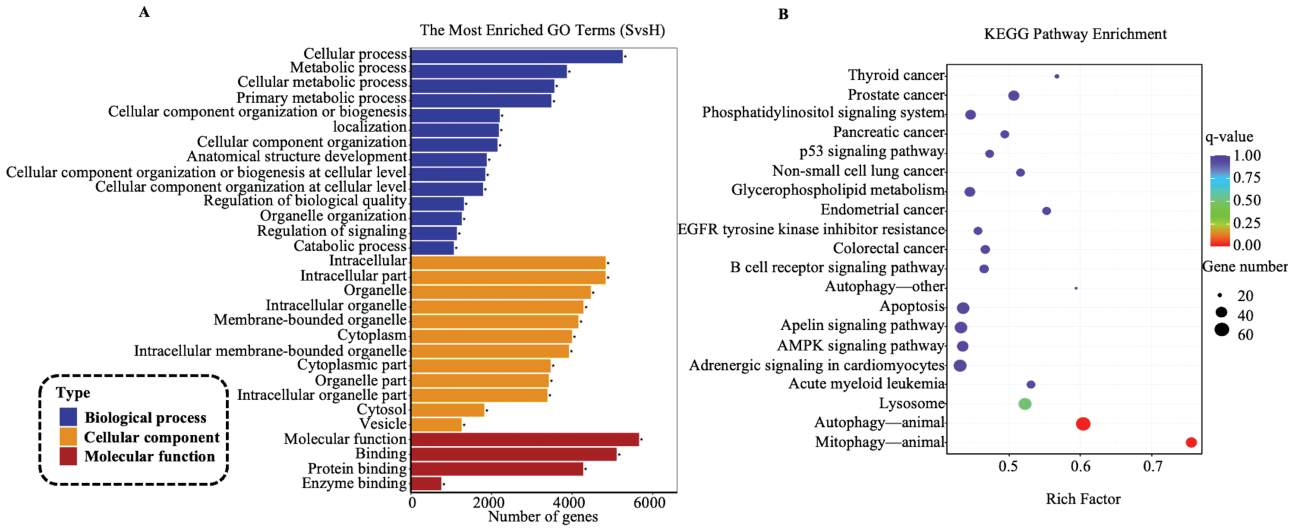


Figure 5. Enrichment analysis of miRNA target genes. (A) GO enrichment analysis for top 30 enrichment pathways. In the graph, the X-axis represents the number of genes, while the Y-axis indicates the distinct GO pathways. The height of each bar depicts the number of genes enriched in a special GO term. Additionally, the color of the bars serves to differentiate the types of GO pathways. (B) KEGG pathway enrichment analysis of multigroup analysis integrating miRNA data. The rich factor is presented as the enrichment degree of genes in various KEGG pathways. The Y-axis shows the names of the enriched pathways. The size of each bubble represents the number of enriched genes. The color of each node represents the statistical significance of the enrichment.

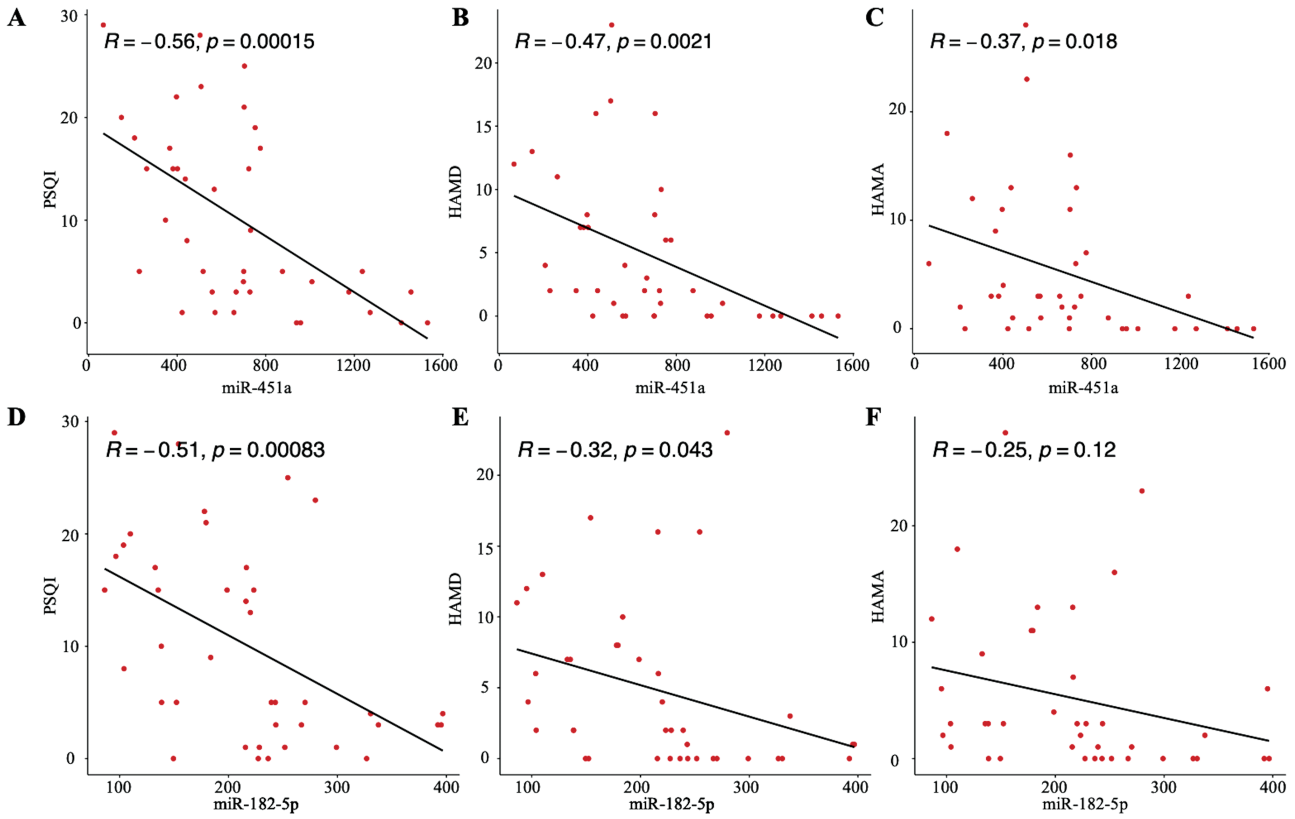


Figure 6. Pearson correlation coefficients between exosomal miRNAs and insomnia, depression, and anxiety in different groups. (A) Correlation between exosomal miR-451a and PSQI scores in insomnia patients and healthy controls. (B) Correlation between exosomal miR-451a and HAMD scores in insomnia patients and healthy controls. (C) Correlation between exosomal miR-451a and HAMA scores in insomnia patients and healthy controls. (D) Correlation between exosomal miR-182-5p and PSQI scores in insomnia patients and healthy controls. (E) Correlation between exosomal miR-182-5p and HAMD scores in insomnia patients and healthy controls. (F) Correlation between exosomal miR-182-5p and HAMA scores in insomnia patients and healthy controls.

has been linked to advanced insomnia in major depression patients, with overexpression leading to downregulation of the CLOCK gene, which regulates the sleep–wake cycle [32]. miR-182 upregulation was correlated with decreased brain-derived neurotrophic factor (BDNF) in depression-like behaviors [33]. In insomnia cohort, there were no reports presenting the correlation between miR-182 and BDNF level. A systematic review and meta-analysis showed the level of BDNF in subjects with insomnia is lower than controls [34]. In older insomnia patients, decreased BDNF concentration was observed [35]. It has been verified that inhibition of miR-182-5p can increase the level of BDNF [36, 37], which indicates miR-182 may play an important role in BDNF concentration. We did not test the level of BDNF in our patients, pitifully. However, we found a correlation between insomnia and downregulation of miR-182-5p, which may be a compensatory protection mechanism for insomnia patients. Depression comorbidity in insomnia patients suggests a complex interplay between these conditions, the specific role of exosomal miRNA still requires further investigation through mechanism research.

Our GO and KEGG enrichment analyses of target genes highlighted our differential exosomal miRNAs involved in key metabolic pathways and signal transduction, with autophagy playing a central role. Abnormal autophagy can lead to cellular damage and has been linked to metabolic stress [38–40]. Our findings suggest that differential exosomal miRNAs like miR-451a and miR-182-5p may also regulate autophagy, affecting cell metabolism and stress responses, thus contributing to insomnia [41]. The underlying mechanisms include (1) Insomnia may be associated with the increased level of orexin miRNA in the hypothalamus, increased mTOR-PI3K/Akt pathways, and the suppressed autophagy function [42, 43]; (2) autophagic activity can be regulated by lysosomal protein [44]. The disruption of lysosome membrane integrity and lysosomal cathepsin leakage involves autophagy dysregulation [45]; (3) mitochondrial autophagy damage is related to the Pink1/Parkin pathway in depression and insomnia [46]. However, there is a lack of research on which pathway miR-451a and miR-182-5p lead to deficiencies in autophagy and lysosome functions among insomnia patients. And coexpression analysis of exosomal miRNA and disease status revealed upregulated gene modules associated with miRNAs like hsa-miR-3127-5p, hsa-miR-4435, and hsa-miR-310a-3p, that are implicated in apoptosis and cancer [47–49]. These findings underscore the importance of apoptosis in mitigating chronic insomnia.

Our study has limitations, including a small sample size that may overlook other significant differential miRNAs. The effects of comorbidities on miRNA were not discharged due to the small sample size and the diversity of comorbidity and should be included in future studies. The insomnia patients also experience anxiety or depression, and the effects of differential exosomal miRNAs are unclear on the three different conditions. Moreover, the impact of medications on exosomal miRNA expression in the insomnia group warrants further investigation. In future research, insomnia patients with a wider age range and longer disease course need to be recruited to clarify whether there is a compensatory protection mechanism in insomnia patients. The identified differential peripheral blood exosomal miRNAs would require clinical replication in another independent cohort to confirm their specificity in diagnosing chronic insomnia. Future research works include more extensive animal and cell experiments that are necessary to elucidate the role of peripheral blood exosomal miRNAs in central nervous system diseases and their pathways related to chronic insomnia.

Conclusions

Peripheral blood exosomal miRNAs have emerged as pivotal regulators of insomnia and potential biomarkers. Our investigation identified key exosomal miRNAs, notably miR-182-5p and miR-451a, whose altered expression profiles delineate them as potential biomarkers for chronic insomnia. Our findings contribute to the understanding of miRNAs in insomnia regulation, supporting their role as serum biomarkers for diagnostic and therapeutic purposes. Future studies should validate these miRNAs' clinical relevance and explore their mechanistic roles in larger cohorts to establish their clinical utility and to explore the broader impact of miRNA dysregulation in sleep-related pathologies.

Supplementary material

Supplementary material is available at *SLEEP* online.

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Data availability

The datasets generated and/or analysed during the current study are not publicly available due to privacy concerns for patients, but are available from the corresponding author on reasonable request.

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