Alterations in the Expression Levels of miR-9-5p and miR-4467 in Peripheral Blood of Patients with First-Episode Schizophrenia

Mengdi Jin
Jilin University School of Public Health
Xiaojing Zhu
Shandong University Qilu Hospital
Yaoyao Sun
Jilin University School of Public Health
Zhijun Li
Jilin University School of Public Health
Xinwei Li
Jilin University School of Public Health
Yang He
Jilin University School of Public Health
Yane Liu
Jilin University School of Public Health
Ningning Jia
Jilin University School of Public Health
Guoyan Hu
Jilin University School of Public Health
Xingyao Cui
Jilin University School of Public Health
Qiong Yu (yuqiong@jlu.edu.cn)
Jilin University School of Public Health https://orcid.org/0000-0003-1143-1749

Research Article

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Abstract

The diagnosis of schizophrenia (SCZ) patients largely rely on abnormal behavior and mental state examination, which is inevitably subjective and susceptible. Finding more stable and objective biomarkers has always been the vision of scholars. In this study, we aim to validate peripheral blood miRNA as potential biomarkers for patients with first-episode schizophrenia (FES). We performed high-throughput sequencing analysis and screened 138 differentially expressed miRNAs (DEmiRNAs) in the peripheral blood in a test cohort (15 FES patients and 15 healthy controls), followed by quantitative reverse transcription-polymerase chain reaction (qRT-PCR) in miR-9-5p, miR-144-3p, miR-328-3p, and miR-4467 in a validation cohort (35 FES patients and 60 healthy controls), compared with healthy controls, miR-9-5p of FES patients was significantly down-regulated, and miR-4467 was up-regulated. The receiver operating characteristic curve (ROC) demonstrated that miR-4467 had a better diagnostic value than miR-9-5p. Bioinformatics analysis predicted the potential target genes of miR-9-5p and miR-4467 mainly involved in the regulation of body behavior, neuronal differentiation, and nervous system development, and enriched in Neurotrophin, MAPK, and phosphatidylinositol signaling systems. The protein interaction assay identified NEDD4, EIF4G1, FBXL16, FBXL3, etc. as the hub target genes. Summarily, our data suggested the expression of miR-9-5p and miR-4467 in peripheral blood might serve as promising biomarkers for FES patients, and might involve in the occurrence and development of schizophrenia by regulating gene modules related to neurodevelopment and neuroprotective.

1. Introduction

Schizophrenia affects approximately 1% of the population in the world and is closely related to human disability, mortality, and economic burden on individuals and society (Baron 2002). Currently, the diagnosis of schizophrenia is mainly based on abnormal behavior and mental state examination by psychiatrists, which is inevitably subjective and susceptible. In view of the fact that early diagnosis and intervention of schizophrenia have a significantly positive impact on the prognosis, there is still a great need for detectable biomarkers to assist in the diagnosis of SCZ.

MicroRNAs (miRNA, miR) are a class of non-coding small RNA, which can regulate up to one-third of human genes by incomplete base pairing with the target 3'-UTR sequence at the post-transcription level (Krol et al. 2010). And over 60% of human protein-coding genes are regulated by miRNA (Sayed and Abdellatif 2011). Alteration in miRNA expression has been confirmed in a variety of human diseases, including cancer (Lee and Dutta 2009), cardiovascular diseases (Zhou et al. 2018), and metabolic diseases (Rottiers and Näär 2012), while dysregulation of miRNAs is closely related to disease progression (Paul et al. 2018). The changes in miRNA level can also reflect the genetic and biological changes in neuropsychiatric disorders, like schizophrenia (Miller and Wahlestedt 2010; Sun and Shi 2015). Actually, abnormal expression levels of miRNAs have been found in the cerebrospinal fluid and postmortem brain tissues of patients with schizophrenia(Gallego et al. 2012; Moreau et al. 2011; Perkins et al. 2007), but the invasiveness of sampling made it impossible to apply to living patients. Therefore,

developing miRNAs as non-invasive biomarkers for blood diagnosis and treatment effect evaluation has become a popular field of interest for researchers.

There have already been findings of available miRNAs useful in differentiation of SCZ patients. Hui Wei et al. discovered dysregulation of plasma miR-130b and miR-193a-3p could be biomarkers for SCZ (Wei et al. 2015). He et al. identified that miR-34a-5p and miR-432a-5p expressed aberrantly in the serum of SCZ patients (He et al. 2019). E Gardiner et al. performed microarray analysis and qRT-PCR verified seven downregulated miRNAs in PBMCs of schizophrenia patients (Gardiner et al. 2012). However, miRNAs with altered expression in the initial stage of schizophrenia have not been fully investigated. We believe that these miRNAs are of vital importance to the understanding of the pathogenesis of SCZ and the early diagnosis. Therefore, we designed a case-control study to screen abnormally expressed miRNAs in peripheral blood of first-episode schizophrenia (FES) patients by high-throughput sequencing. Then performed the qRT-PCR analysis in an expanded sample to confirm the initial finding. We also used bioinformatics methods to predict potential target genes and integrate biomarkers information to further understand the possible biological pathways and regulatory mechanisms of the development of schizophrenia.

2. Materials And Methods

2.1 Participants

35 FES patients admitted to Changchun Psychological Hospital from July 2017 to August 2019 were recruited in this study. All patients were diagnosed by at least two consultant psychiatrists according to the Tenth Revision of International Classification of Diseases (ICD-10) for SCZ and were recruited before any antipsychotic medication. Patients with severe medical illnesses, other neuropsychiatric disorders, structural brain disorders, brain trauma, previous continuous illegal substance use, or movement disorders were excluded. In addition, 60 healthy individuals with no psychiatric disorders or family history of mental illnesses were recruited as the control group.

Among them, high-throughput sequencing samples comprised 15 SCZ patients and 15 controls matched on age and gender as the first set. On the basis of the sequencing samples, according to the above inclusion and exclusion criteria, we continue to recruit individuals with FES to 35 cases, and healthy controls to 60 cases as the second set for verification. This study was approved by the Ethics Committee of the School of Public Health, Jilin University. Informed consent of the participants was obtained after the nature of the procedures had been fully explained.

2.2 Peripheral blood collection and total RNA extraction

1.5ml whole blood was obtained from all fasting subjects, treated with 4.5ml Trizol reagent (TaKaRa,Japan), and put into a liquid nitrogen tank for quick freezing. When extracting total RNA, transfer the mixed solution to four 1.5ml EP tubes, let the sample stand for 5 minutes at room temperature, and centrifuge at 12,000 rpm at 4°C for 5 minutes. Collect the supernatant, add 0.2ml of chloroform and

shake well, then let it stand at room temperature for 5 minutes, and centrifuge at 12000rpm at 4°C for 15 minutes. Take the supernatant and add 0.8ml of isopropanol reagent to mix them well. After standing for 10 minutes at -20°C, centrifuge at 12000rpm at 4°C for 10 minutes. Subsequently, washed the total RNA pellet with 75% ethanol and centrifuge at 12,000 rpm at 4°C for 10 minutes. The supernatant was completely discarded, and the RNA pellet was dried at room temperature. Finally, the total RNA was dissolved in 15uL of DEPC and stored at -80°C.

2.3 High-throughput sequencing analysis

Sequencing was completed by Beijing Novogene Experimental Department. Briefly, after the samples were qualified, we used the Small RNA Sample Pre Kit to construct a library, and total RNA was used as the starting sample to synthesize cDNA by reverse transcription, PAGE gel electrophoresis was used to separate the target DNA fragments, and we cut the gel to recover the cDNA library. After the library was constructed, use Qubit2.0 for preliminary quantification, dilute the cDNA to 1ng/ul, and then use Agilent 2100 to detect the insert size of the library, and use qPCR to accurately quantify the effective concentration of cDNA (effective concentration > 2nM). The library preparations were then sequenced on the Illumina HiSeq 2500 platform. DNA polymerase, adaptor primers, base-specific fluorescently labeled dNTP, and buffer were added to the reaction system, and the laser was used to excite the fluorescent signal, and finally computer analysis was conducted to convert the optical signal into sequencing bases. miRNA expression levels were estimated by read counts, differential expression analysis of two samples was performed using the edgeR package. $|\log_2 \text{Foldchange}|>0.5$ and $p < 0.01$was set as the threshold for significantly differential expression by default.

2.4 qRT-PCR analysis

After checking the concentration and purity of RNA,4 μLtotal RNA was reverse transcribed to cDNA using TransScript miRNA First-Strang cDNA Synthesis SuperMix (TransGen Biotech, Beijing, China). The reverse transcription reactants were mixed according to the instruction and the reaction was performed in Mastercycler nexus PCR instrument at 37°C for 1h, and RT Enzyme Mix inactivated at 85°C for 5s. Afterward, PCR reactions were performed in a 10uL reaction mixture containing 5μL 2*TransStart® Tip Green qPCR SuperMix (TransGen Biotech, Beijing, China), 0.6μL each of forwarding primer and universal primer (Sangon Biotech，Shanghai，China, see the primer sequences in Table 1), and 2μL diluted RT cDNA product. All reactions were run in triplicate. PCR was performed on LightCycler®96 (Roche) at 94℃ for 30 seconds，followed by 44 cycles at 94℃ for 5 seconds, 64℃ for 15 seconds, and 72℃ for 10 seconds. U6 was selected as the internal reference gene for normalization, and the relative expression levels of miRNAs were calculated by the $2^{-\triangle\triangle Ct}$ method.

Table 1
primer sequences for RT-PCR

miRNA	Primer sequence (5'-3')
hsa-miR-9-5p	F: CGGGTCTTTGGTTATCTAGCTGTAT
hsa-miR-328-3p	F: CTGGCCCTCTCTGCCCTT
has-miR-144-3p	F: CGGCGGTACAGTATAGATGATGTACT
has-miR-4467	F: TGGCGGCGGTAGTTATGGG
U6	F: CTCGCTTCGGCAGCACA
Universal Primer:	R: GATCGCCCTTCTACGTCGTAT

2.5 Bioinformatics Analysis

Bioinformatics methods were used to predict potential target genes of miRNAs with differential expression in SCZ patients and controls. We first take the intersection of the prediction results of TargetScan (http://www.targetscan.org/vert_72/) and miRDB (http://mirdb.org/), and then take the union set withresults from miRTarbase (http://mirtarbase.cuhk.edu.cn/php/index.php) as the target gene set of miRNAs. We used DAVID (https://david.ncifcrf.gov/home.jsp) to perform GO and KEGG pathway enrichment analysis on the target genes, and use FDR (false discovery rate) corrected Padj < 0.05 as standard to screen and sort out biological functions and signaling pathways which are significantly related to target genes. The protein and protein interaction networks were constructed by String (http://www.string-db.org), and Hub genes were screened by Cytoscape MCC algorithm.

2.6 Statistical analysis

For the high-throughput sequencing analysis in the first set, the statistical significance of readcount was assessed using Student's t-test. Mann-Whitney U test and Chi-square test were used to compare continuous and categorical variables of basic demographic data between groups, respectively. One sample Kolmogorov-Smirnov test was used to test the normal distribution of variables. The relative expression of miRNA was expressed by the median and quartile of $2^{-\triangle\triangle Ct}$, Mann-Whitney U test was used to compare the expression of miRNAs between groups. ROC curve was drawn to assess the diagnostic value of miRNAs for schizophrenia. G*Power 3.1.9.2 was used to calculate the minimum sample size. Statistical analysis and graphs plotting were completed using SPSS 24.0 and GraphPad Prism 8.0.1. $p < 0.05$ (two-tailed) was considered statistically significant.

3. Results

3.1 Characteristics of subjects

There was no statistical difference between the FES group and the control group in gender, age, years of education, and residence. However, the family history of mental illness ($p = 0.002$) and marital status ($p <$

0.001) were significantly different between the two groups (Table 2).

Table 2
Basic demographic characteristics of the subjects

	FES (n = 35)	Control (n = 60)	t/χ^2	*p*
Gender			0.163	0.687
Male	16(45.7)	30(50.0)		
Female	19(54.3)	30(50.0)		
Age	32.89 ± 11.71	32.25 ± 11.48	-0.961	0.339
Family history			-	0.002[a]
With	6(17.1)	0(0)		
Without	29(82.9)	60(100.0)		
Marital status			20.307	< 0.001
Married	6(17.1)	39(65.0)		
Unmarried	29(82.9)	21(35.0)		
Education(years)			0.001	1.0
<=9 years	10(28.6)	17(28.3)		
> 9 years	25(71.4)	43(71.7)		
Residence			2.441	0.118
Rural	13(38.2)	33(55.0)		
Urban	21(61.8)	27(45.0)		

Note: [a] *p* value was calculated by Fisher's exact test; FES, First-episode schizophrenia

3.2 High-throughput sequencing analysis of the first sampke set.

176 altered miRNAs were identified by high-throughput sequencing in 15 SCZ patients and 15 age- and gender-matched healthy controls, of which 96 miRNAs were up-regulated in schizophrenia patients and 80 miRNAs were down-regulated. The volcano plot showed in Fig. 1. Based on the results of high-throughput sequencing and literature review, we selected 4 miRNAs (miR-9-5p, miR-144-3p, miR-328-3p, miR-4467) that the $|\log_2 Foldchange|<0.5$, $p < 0.01$ (Table 3), and have been extensively studied in psychiatric disorders, for further qRT-PCR experiment verify.

Table 3
The expression levels of miRNAs in the first set

miRNA		Readcount		log_2FoldChange	*p*
		FES (n = 15)	Control (n = 15)		
miR-9-5p	Downregulated	13.222	35.658	-1.441	< 0.001
miR-328-3p	Upregulated	2528.228	1429.964	0.822	0.002
miR-144-3p	Upregulated	830.124	302.067	1.458	0.003
miR-4467	Upregulated	45.990	15.203	1.594	< 0.001
Note: FES, First-episode schizophrenia					

3.3 Results of qRT-PCR analysis of the second set and ROC curve

We first conducted pre-experiments on 4 miRNAs in 10 cases and 10 controls. The effect size *d* was 0.79. The G*Power 3.1 software calculated that the minimum sample size required 35 cases and 35 controls (power = 0.9). Thus, the study finally included 35 SCZ patients and 60 healthy controls for further qRT-PCR verification.

In the verification part, compared with healthy controls, the expression of miR-9-5p was significantly down-regulated in the peripheral blood of patients with schizophrenia ($p = 0.046$), and miR-4467 was significantly up-regulated ($p < 0.001$), which was consistent with high-throughput sequencing analysis. However, no significant difference in the expression of miR-144-3p and miR-328-3p between the two groups was found in the expanded sample (Table 4), which were excluded for subsequent analysis. To further assess the diagnostic value of miRNAs, ROC curves were plotted, and AUC was calculated to evaluate the predictive power of these two miRNAs for schizophrenia. The results showed that miR-4467 (AUC = 0.719, 95%CI: 0.613–0.824) showed a greater diagnostic value than miR-9-5p (AUC = 0.623, 95%CI: 0.509–0.737) (Fig. 2).

Table 4
The expression level of miRNAs in the second set for validation

miRNA	FES (n = 35)	Healthy control (n = 60)	Z	*p*
miR-9-5p	0.23 (0.06–1.84)	0.85 (0.13-5.00)	-1.994	0.046
miR-328-3p	1.00 (0.35–1.74)	1.29 (0.26–3.57)	-0.698	0.485
miR-144-3p	0.28 (0.03–2.83)	1.33 (0.10–5.36)	-1.829	0.067
miR-4467	2.57 (0.51–4.98)	0.59 (0.10–1.62)	-3.545	< 0.001
Note: FES, First-episode schizophrenia				

3.4 Target gene prediction and functional enrichment of miR-9-5p and miR-4467

Taken the intersection and union of the results as planned, 1116 target genes for miR-9-5p and 92 target genes for miR-4467 were predicted using Targetscan, miRDB, and miRTarbase. Figure 3 showed the biological processes (BP), cell components (CC) and molecular functions (MF) the target genes of miR-9-5p and miR-4467 mainly involved in, including locomotory behavior, nervous system development, and regulation of cardiac muscle contraction. regulation of neuron differentiation and development, negative regulation of Schwann cell proliferation; KEGG pathway analysis showed that the potential functions of miR-9-5p target genes are mainly enriched in neurotrophin, MAPK, Estrogen, and Phosphatidylinositol signaling systems (Fig. 4).

3.5 Hub genes and significant gene model screening by Protein-Protein Interaction

STRING (https://string-db.org/cgi/input.pl) was used to construct interaction networks of proteins encoded by miR-4467 and miR-9-5p target genes (Fig. 5). CytoHubbs plug-in of Cytoscape was used to screen the most important modules in the network composed of miR-4467 and miR-9-5p target genes. 20 Hub genes were screened, namely *H2AFX, FBXO44, WHSC1, EIF4G1, BCL2L1, RUNX1, FBXL16, CPLX1, CSTF2* and *FEM1A* for miR-4467; *FBXL3, FBXW2, KLHL42, FBXL16, ASB7, RNF111, SMURF2, NEDD4, TRIM71, HECW2* for miR-9-5p (Fig. 6A,6B). And 3 significant modules were identified (Fig. 6C-E).

4. Discussion

In recent years, the view that miRNAs participate in the pathophysiological process of mental diseases by regulating hundreds of target transcripts has been widely accepted. Evidence showed that the dysregulation of many miRNAs in postmortem brain samples was associated with schizophrenia (Moreau et al. 2011). However, it is difficult to exclude the effects of long-term treatment and medication for the explanation of the altered miRNA expression in the postmortem brain or peripheral circulation(Luoni and Riva 2016; Santarelli et al. 2013; Seo et al. 2014; Wang et al. 2019). In an article studying the association between miR-195 level and cognitive impairment in patients with schizophrenia of different genders, Huang et al. also demonstrated that recruiting first-episode and drug-free patients as the object could rule out the effects of antipsychotics on cognition and miRNA expression levels (Huang et al. 2020).

Therefore, this study recruited FES patients who were diagnosed for the first time and had not been treated with antipsychotic drugs, analyzed the alteration of miRNA expression profiles in their peripheral blood, predicted target mRNAs and integrated their common functions. We initially screened the differentially expressed miRNAs in schizophrenia patients through high-throughput sequencing globally, subsequently performed a qRT-PCR procedure on 4 miRNAs (miR-9-5p, miR-328, miR-144, and miR-4467)

in an expanded sample for verification to avoid false-positive results. We found that compared with healthy controls, the level of miR-9-5p in peripheral blood of FES patients was down-regulated, and miR-4467 was up-regulated. Moreover, the ROC curve indicated that, as a non-invasive biomarker of FES, the expression level of miR-9-5p in peripheral blood showed a better diagnostic value than miR-4467.

There have been reported cases earlier in researches focusing on the two miRNAs and mental diseases. miR-4467 was first found in the cerebrospinal fluid of Alzheimer's patients and was identified as a potential biomarker for Alzheimer (Denk et al. 2015). Disorders of the brain can exhibit similar symptoms(Anttila et al. 2018), which made us wonder if the etiology of schizophrenia and Alzheimer would overlap. It is worth mentioning that this study is the first attempt that the alteration of miR-4467 expression was explored in SCZ. As for miR-9-5p, Mehmet et al. detected a significant up-regulation of miR-9-5p in blood samples of patients with schizophrenia using qRT-PCR(Camkurt et al. 2016). Studies have also shown that miR-9-5p is an important regulator of neurogenesis(Krichevsky et al. 2006; Leucht et al. 2008; Shibata et al. 2011), nerve cell proliferation and differentiation (Delaloy et al. 2010; Sun et al. 2013; Zhao et al. 2009), and axon development (Dajas-Bailador et al. 2012; Otaegi et al. 2011). A genomic analysis published in JAMA Psychiatry revealed that among several miRNAs that regulate schizophrenia risk genes, the miR-9-5p target is the most enriched, provided evidence for its role in the etiology of schizophrenia (Hauberg et al. 2016).

In order to better understand the role of miR-9-5p and miR-4467 in schizophrenia, we performed bioinformatics analysis to predict their target genes. In addition to the top 10 functions of potential target genes shown in Fig. 3, the SCZ-related biological processes that miR-9-5p may participate in also include regulation of astrocyte, oligodendrocyte differentiation, regulation of neural precursor cell and mesenchymal cell proliferation, regulation of neuron differentiation, development, and apoptotic, the biosynthetic process of ceramide and sphingomyelin, neuron-neuron synaptic transmission, regulation of dendritic spine development, the neuronal stem cell population maintenance, brain development, motor learning, branching morphogenesis of a nerve, axonogenesis (all $p < 0.05$). And predicted cell components also include neuron projection, dendritic spine synapse, synaptic vesicle membrane, and postsynaptic membrane. Enrichment of the KEGG pathway showed that the target genes of miR-9-5p were enriched in the neurotrophin signaling pathway and estrogen signaling pathway. It is worth mentioning that, A number of recent studies have demonstrated that SCZ is a neurodevelopmental disorder (Kranz et al. 2015; Li et al. 2020; Su et al. 2021). And Estrogen is believed to play a vital role in the pathophysiology of neurodevelopmental disorders (Crider and Pillai 2017), including involvement in brain development, synaptic plasticity and neuroprotection, while neurons are the primary site of estrogen synthesis in the brain (Azcoitia et al. 2011; Cui et al. 2013). Therefore, the potential of the pathways as targets for the treatment of SCZ can be further explored. Since there were only 92 target genes of miR-4467 predicted in this study, no statistically significant pathway enrichment results were produced. Nevertheless,the BP of miR-4467 target gene was obviously enriched in regulation of neuron differentiation and development, and negative regulation of Schwann cells proliferation.

We further screened the hub genes of miR-9-5p and miR-4467 by the Maximal Clique Centrality algorithm (MCC). The hub genes can be classified according to their functions, resulting in psychosis susceptibility genes (*NEDD4*, *EIF4G1*, *FBXL16*)(Deng et al. 2015; Han et al. 2019; Smith et al. 2019), neurodevelopment-related genes (*EIF4G1*, *FBXL3*) (Ansar et al. 2019; Dong et al. 2020), mental disease phenotype-related genes (*CPLX1*) (Glynn et al. 2007; Glynn et al. 2005), neuroprotective genes (*FEM1A*) (Fujikawa et al. 2016), and antipsychotic drug-sensitive gene (*BCL2L1*) (Fatemi et al. 2012).

Taken together, the enrichment analysis and hub genes indicated that miR-9-5p and miR-4467 are likely to play a role in neurodevelopment and synaptic transmission via the regulation of target genes, which could contribute to the occurrence and development of schizophrenia. In addition, considering the involvement of target gene groups in the regulation of neurodevelopment, it further proves the significance of selecting first-episode schizophrenia patients to explore the etiology and pathogenesis of schizophrenia.

For miR-328-3p and miR-144-3p whose expression changes were also detected in the sequencing samples, studies have been verified in autistic spectrum disorder patients (Kichukova et al. 2017; Nt et al. 2018) and Alzheimer's disease (Zhou et al. 2019). However, there is no evidence to support the abnormal expression of miR-328-3p or miR-144-3p in FES patients, and no significant changes in the expression of the two were found in our validation set, the trends were different from the sequencing results ($p > 0.05$). In this regard, we considered that high-throughput sequencing and qRT-PCR are two different experimental platforms. High-throughput sequencing is used for large-scale screening, reflecting the overall gene expression trend of the samples, and there is a certain probability of false positives. There is no guarantee that the trend of the gene expression is exactly the same as that of qRT-PCR.

In spite of our novel findings of alterations of miR-4467 and miR-9-5p in first-episode schizophrenia patients. This study has some limitations that need to be acknowledged. First of all, our case-control study design has determined the fact that it cannot reflect the causal relationship between miRNA expression level and schizophrenia. Secondly, although we have enrolled enough samples based on the pre-experiment results and statistical power requirements, larger sample will definitely help us provide more valuable data, we are still recruiting. Of further interest are the expression level of miR-9-5p and miR-4467 predicted targets in the peripheral blood of FES patients, as well as the binding sites of miRNA and target genes. Are these hub genes directly regulated by miR-9-5p and miR-4467? What are their binding fragments? These problems need to be explored through subsequent investigations at the cellular level.

In conclusion, miR-9-5p and miR-4467 in peripheral blood are found to be potential biomarkers for early diagnosis of schizophrenia, and might affect the onset and development of SCZ by target regulation of neurodevelopment-related mRNAs.

Declarations

Author contributions

Mengdi Jin: Experimentation, Data curation, Visualization, Writing - original draft; Xiaojing Zhu: Investigation, Experimentation, Methodology; Yaoyao Sun: Experimentation, Validation, Methodology; Zhijun Li: Supervision; Xinwei Li: Writing - review & editing; Yang He: Software, Visualization; Yane Liu: Investigation, Data curation; Ningning Jia: Investigation; Writing - review & editing; Guoyan Hu: Investigation, Formal analysis; Xingyao Cui: Investigation; Qiong Yu: Conceptualization, Funding acquisition, Writing - review & editing.

Declarations of interest:

The authors declare no conflict of interest.

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Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Figures

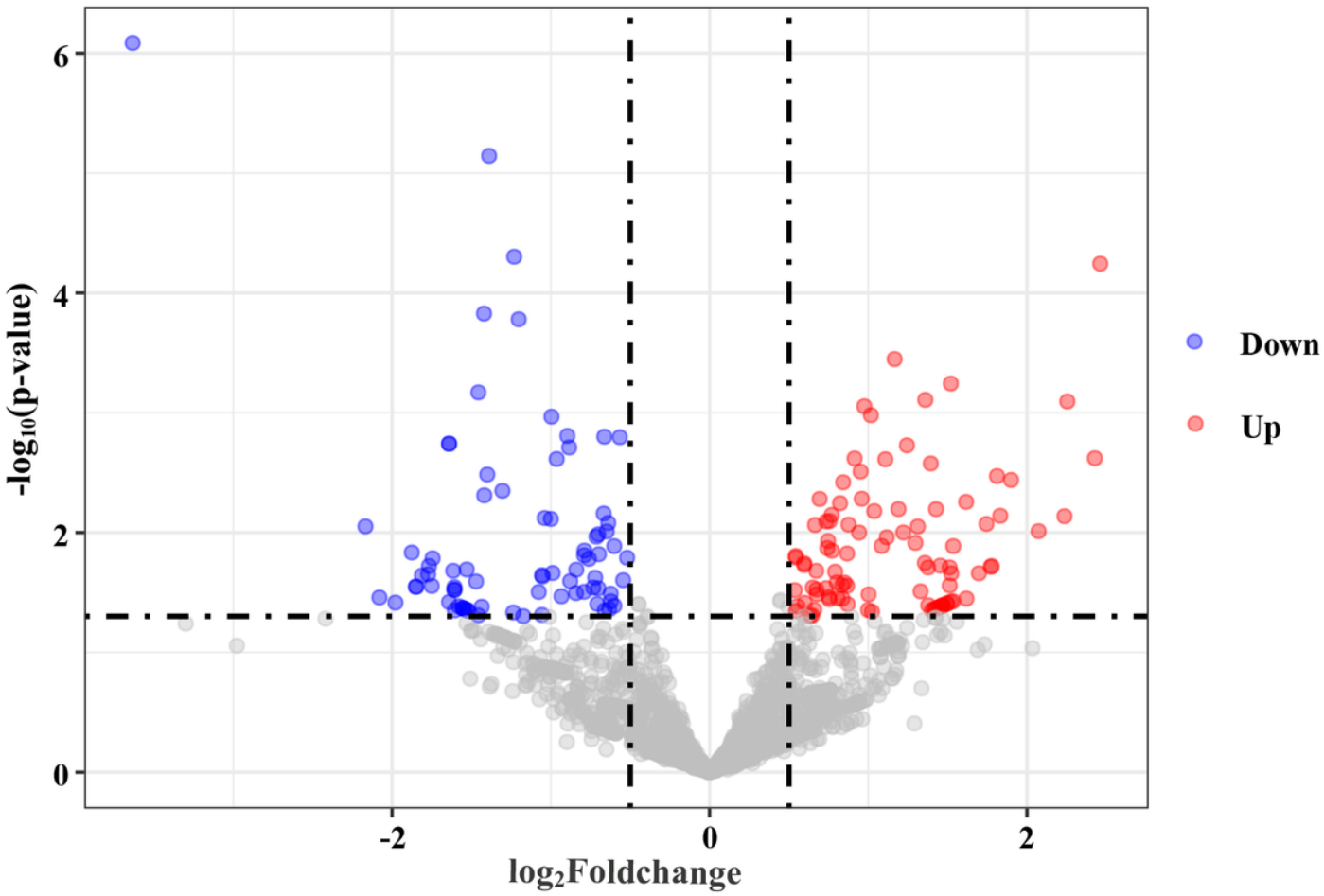

Figure 1

The volcano plot of differentially expressed miRNAs.

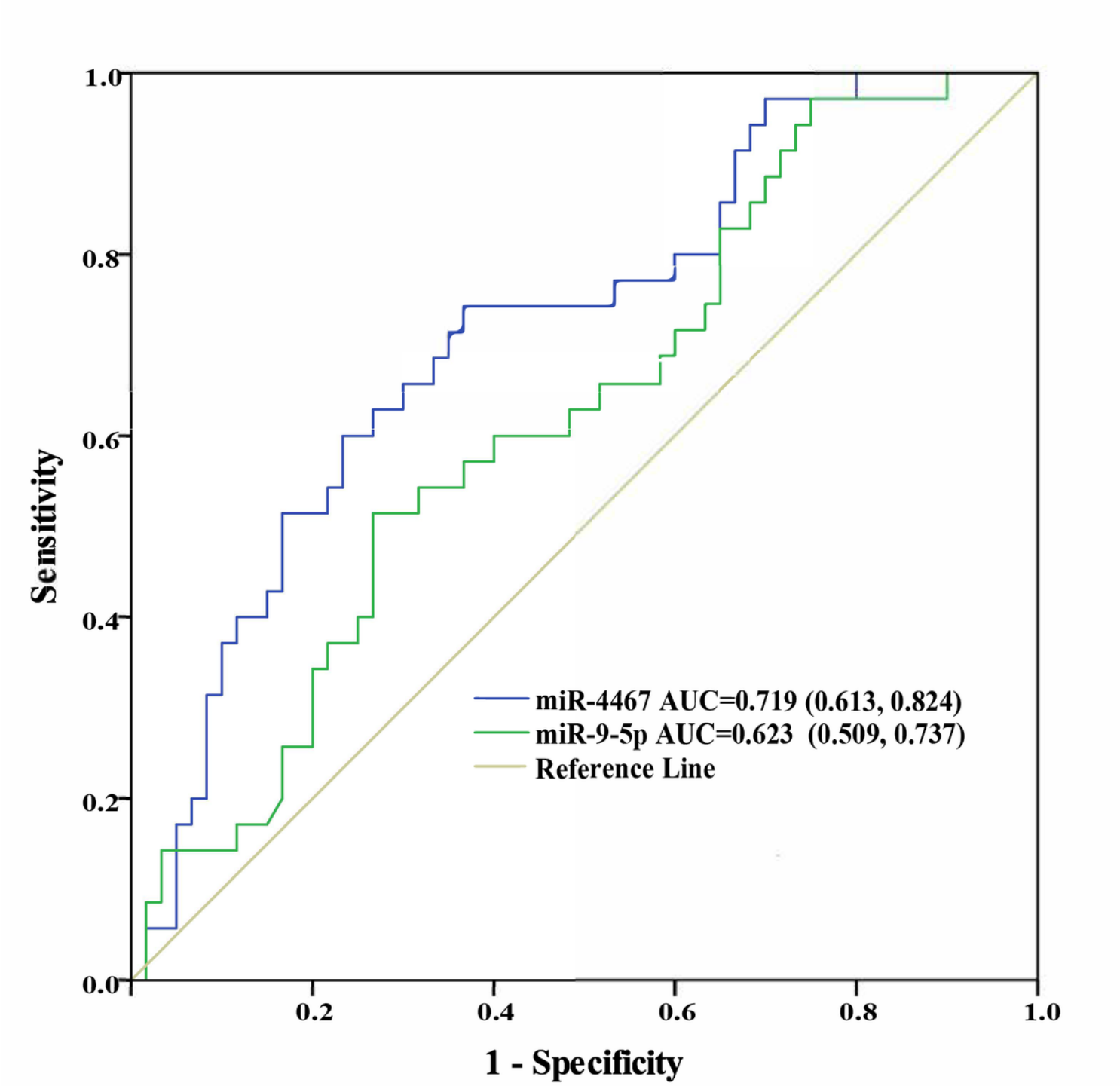

Figure 2

ROC curves of miR-4467 and miR-9-5p. The ROC analysis showed an AUC of 0.719 (95%CI: 0.613-0.824) for miR-4467, and an AUC of 0.623 (95%CI: 0.509-0.737) for miR-9-5p.

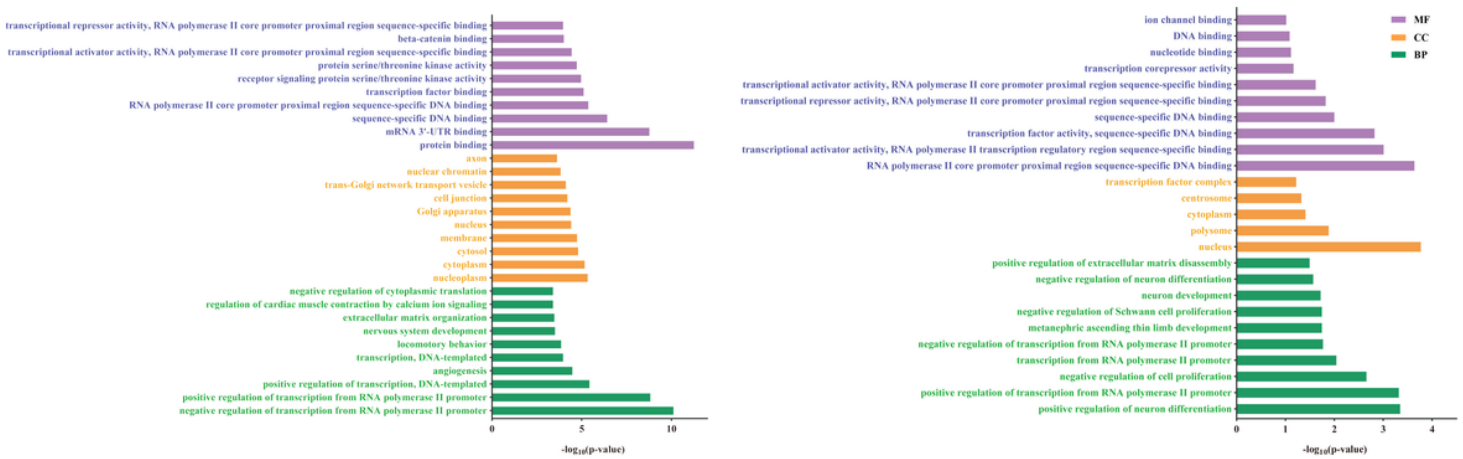

Figure 3

Gene ontology analysis of miR-9-5p (A) and miR-4467 (B) target genes.

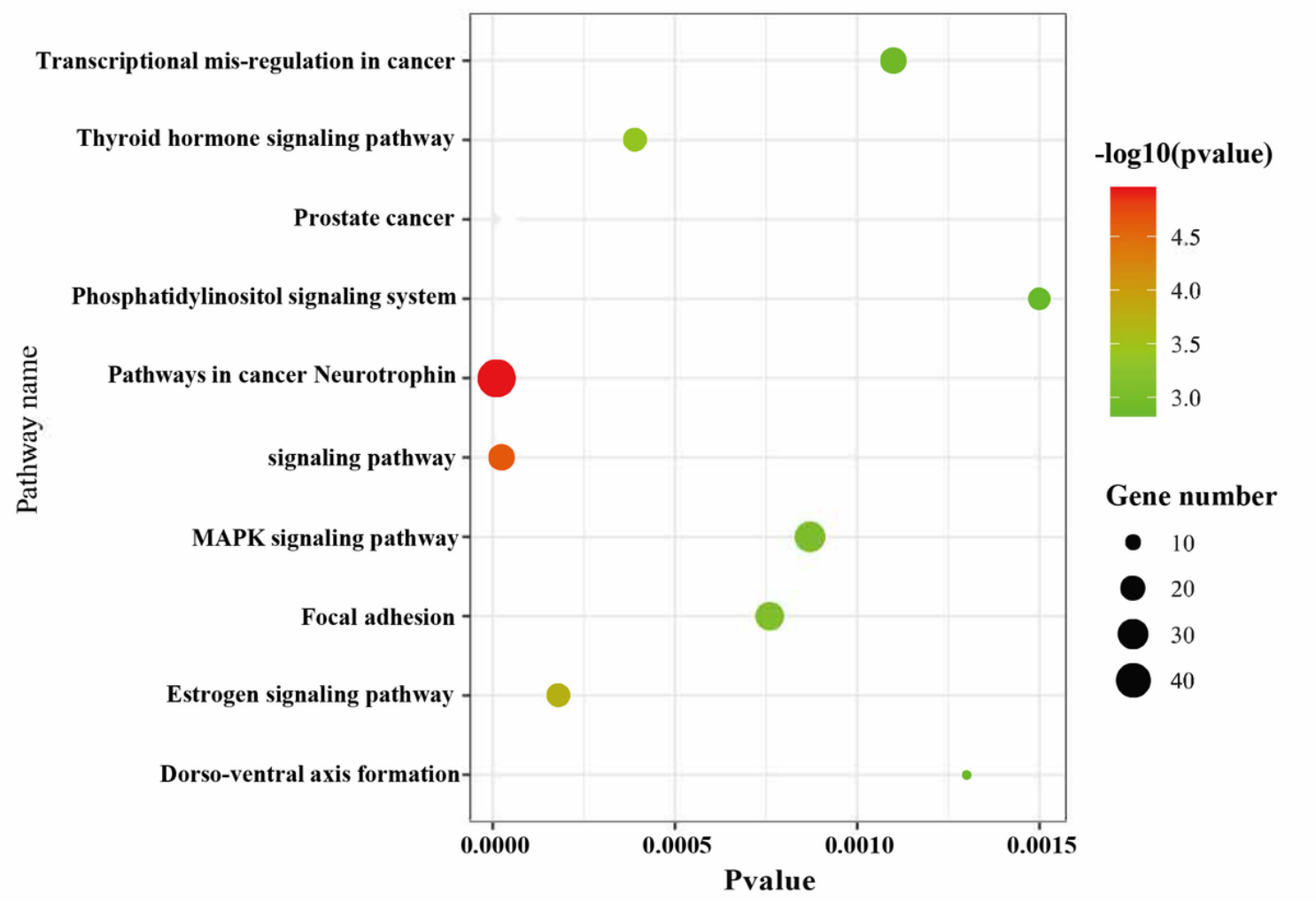

Figure 4

Top 10 KEGG pathway of miR-9-5p target genes.

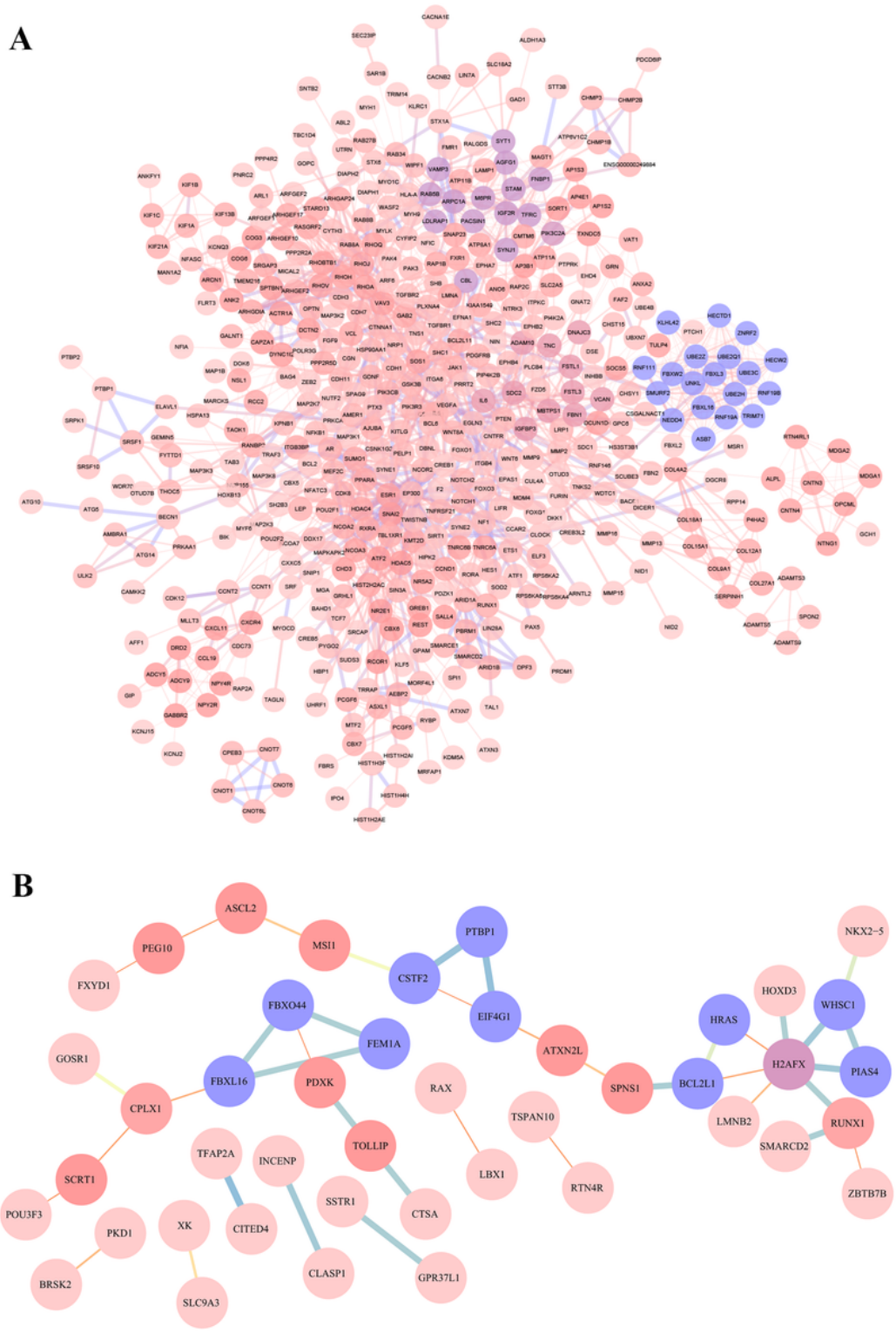

Figure 5

Interaction between proteins encoded by miR-9-5p (A) and miR-4467 (B) target genes. The size and color of each edge are determined by combined-score, and the size of node is determined by MCODE-score.

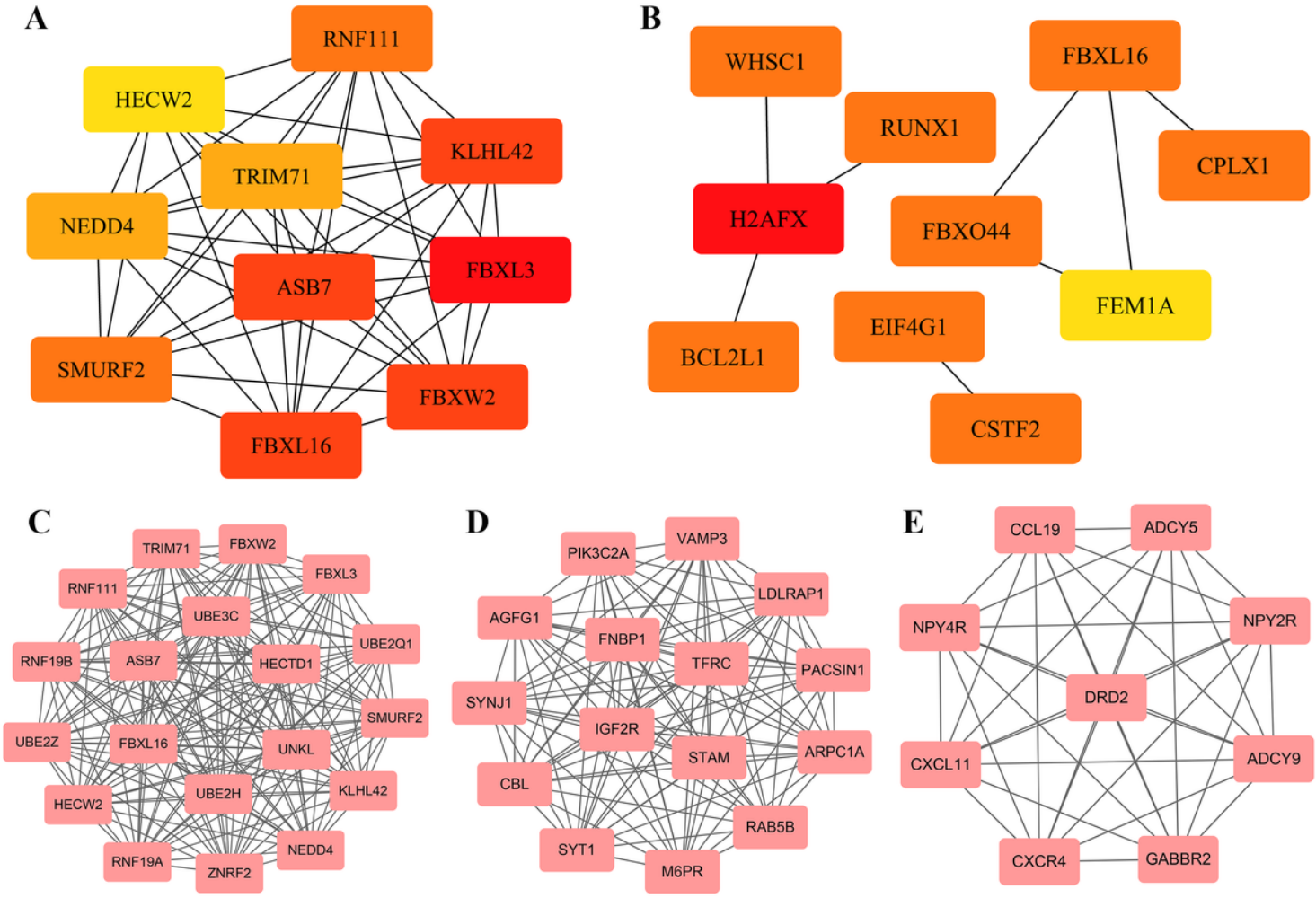

Figure 6

Hub genes and significant target gene modules for miR-9-5p and miR-4467. (A) Hub genes in miR-9-5p target genes. (B) Hub genes in miR-4467 target genes. The darker the node color, the greater the importance of the gene in the module. (C-E) Significant MCODE gene modules of miR-9-5p.