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Transcriptome Analyses Reveal the Important miRNAs Involved in Immune Response of Gastric Cancer

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ABSTRACT

MicroRNAs (miRNAs) are crucial factors in gene regulation, and their dysregulation plays important roles in the immunity of gastric cancer (GC). However, finding specific and effective miRNA markers is still a great challenge for GC immunotherapy. In this study, we computed and analysed miRNA-seq, RNA-seq and clinical data of GC patients from the TCGA database. With the comparison of tumour and normal tissues in GC, we identified 2056 upregulated and 2311 downregulated protein-coding genes. Based on the miRNet database, more than 2600 miRNAs interact with these genes. Several key miRNAs, including hsa-mir-34a, hsa-mir-182 and hsa-mir-23b, were identified to potentially play important regulatory roles in the expression of most upregulated and downregulated genes in GC. Based on bioinformation approaches, the expressions of hsa-mir-34a and hsa-mir-182 were closely linked to the tumour stage, and high expression of hsa-mir-23b was correlated with poor survival in GC. Moreover, these three miRNAs are involved in immune cell infiltration (such as activated memory CD4 T cells and resting mast cells), particularly hsa-mir-182 and hsa-mir-23b. GSEA suggested that the changes in their expression may possibly activate/inhibit immune-related signal pathways, such as chemokine signalling pathway and *CXCR4* pathway. These results will provide possible miRNA markers or targets for combined immunotherapy of GC.

1 | Introduction

Gastric cancer (GC) is one of the most common malignant tumours and the second-leading cause of cancer death worldwide [1–3]. Due to the characteristics of insidious onset, atypical symptoms and rapid progression, most patients with GC are already in the middle or advanced stages [4, 5]. The clinical efficacy of conventional therapies such as surgery, chemotherapy, radiotherapy and targeted therapy is limited [6, 7]. The prognosis of most advanced-stage GC patients were poor [4],

and the median overall survival is only about 1 year [8]. Immunotherapy, a major breakthrough in cancer treatment, can utilise the tumour-infiltrating immune cells (activated innate and adaptive immunity) to effectively control tumour progression [9], prevent metastasis and reoccurrence and ultimately eradicate tumour cells [10–13]. However, the immunotherapy clinical trials showed limited therapeutic effects to the overall survival of GC patients [14].

MiRNAs, an important class of noncoding RNA (ncRNA), can function as tumour suppressors or oncogenes by binding to specific target genes [15–19]. miRNAs can regulate the differentiation of various immune cells and be crucial for innate and adaptive immunity [20–22]. Several miRNAs have been found to associate with the immune system of GC and other cancer types [23–25]. For example, miR-150 and miR-34 could participate in B cell differentiation by regulating their target genes [26, 27]. miR-155 was involved in regulating the activation of T cells and macrophage functions [28, 29]. Taken miR-181a, for example, a putative biomarker, could regulate innate immune signalling and affect oncogenic transformation, which may serve as a promising therapeutic target [30]. miR-1269a is significantly highly expressed in GC cell lines and involved in different degrees of immune cell infiltration in GC [31]. miR-429 could affect the development and prognosis of GC by regulating the tumour immune infiltration [32]. Additionally, overexpression of miR-26a decreased the expression of the macrophage colony-stimulating factor and chemokine (*CCL22*, *CCL17* and *IL-10*) and suppressed the macrophage infiltration in hepatocellular carcinoma [14, 33, 34]. Although some studies have revealed the potential of certain miRNAs in tumour immunotherapy, this field is still in the early stages, and the roles of many miRNAs in the immune response of GC remain insufficiently studied [35–37]. Therefore, it is crucial to systematically investigate the relationship between miRNAs and immune system in gastric cancer. This will not only help deepen our understanding of immune mechanisms but may also provide clues for identifying specific and effective miRNA biomarkers.

In the present study, we identified several pivotal miRNAs based on the comparative analysis of RNA and miRNA transcriptome in GC tissues. In addition, we investigated the functional roles of these miRNAs in GC, as well as their correlation with clinical features and impact on patient prognosis. Our study found key miRNAs correlated with the clinical stage of GC as well as miRNA biomarkers affecting patient survival. Furthermore, we identified the effect of these key miRNAs on the immune response in GC, providing new predictive markers for GC immunotherapy.

2 | Result

2.1 | Identification of Significant miRNAs Associated With DEGs in GC

To explore the miRNAs that regulate the abnormal expression of GC-associated genes, we explored differentially expressed genes (DEGs) between GC and normal gastric tissues. The 2056 protein coding genes were defined as significantly upregulated in GC, and the 2311 genes were significantly downregulated. Based on the miRNet database, the 1795 of upregulated genes were predicted to interact with 2605 miRNAs, and the 2164 of downregulated genes were found to interact with 2600 miRNAs. Of these miRNAs, 26 miRNAs are considered particularly important because any of them can interact with at least 200 up-/downregulated genes (Table 1). More importantly, the 26 miRNAs can interact with almost all the up- and downregulated genes and may be crucial for the abnormal expression of DEGs in GC.

2.2 | Biological Functions and Signalling Pathways Involved in the 26 miRNAs

In order to understand the biological behaviour of the selected 26 important miRNAs in GC, we conducted Gene Ontology (GO) and Kyoto Encyclopaedia of Genes and Genomes (KEGG) pathway enrichment analyses to the targeted DEGs by the DAVID database. The upregulated genes targeted by the 26 miRNAs were enriched in cancer-related biological processes and signalling pathways. It mainly included cell proliferation and differentiation, inflammatory response, cell cycle, p53 signalling pathway, DNA binding and PI3K-Akt signalling

TABLE 1 | The 26 important miRNAs interacted with differentially expressed genes in GC.

miRNA ID	DEGIM number	miRNA ID	DEGIM number	miRNA ID	DEGIM number
hsa-miR-27a-3p	1348	hsa-miR-155-5p	750	hsa-miR-374a-5p	633
hsa-miR-124-3p	1205	hsa-miR-103a-3p	631	hsa-miR-20a-5p	508
hsa-miR-1-3p	1046	hsa-let-7b-5p	674	hsa-miR-128-3p	518
hsa-miR-16-5p	1098	hsa-miR-195-5p	630	hsa-miR-23b-3p	479
hsa-miR-34a-5p	921	hsa-miR-1343-3p	645	hsa-miR-101-3p	542
hsa-miR-129-2-3p	960	hsa-miR-182-5p	603	hsa-miR-7-5p	516
hsa-miR-146a-5p	1013	hsa-miR-335-5p	728	hsa-miR-26b-5p	491
hsa-miR-147a	712	hsa-miR-26a-5p	561	hsa-miR-10b-5p	416
hsa-miR-107	693	hsa-miR-210-3p	512		

Abbreviation: DEGIM: Differentially expressed genes interacting with miRNAs.

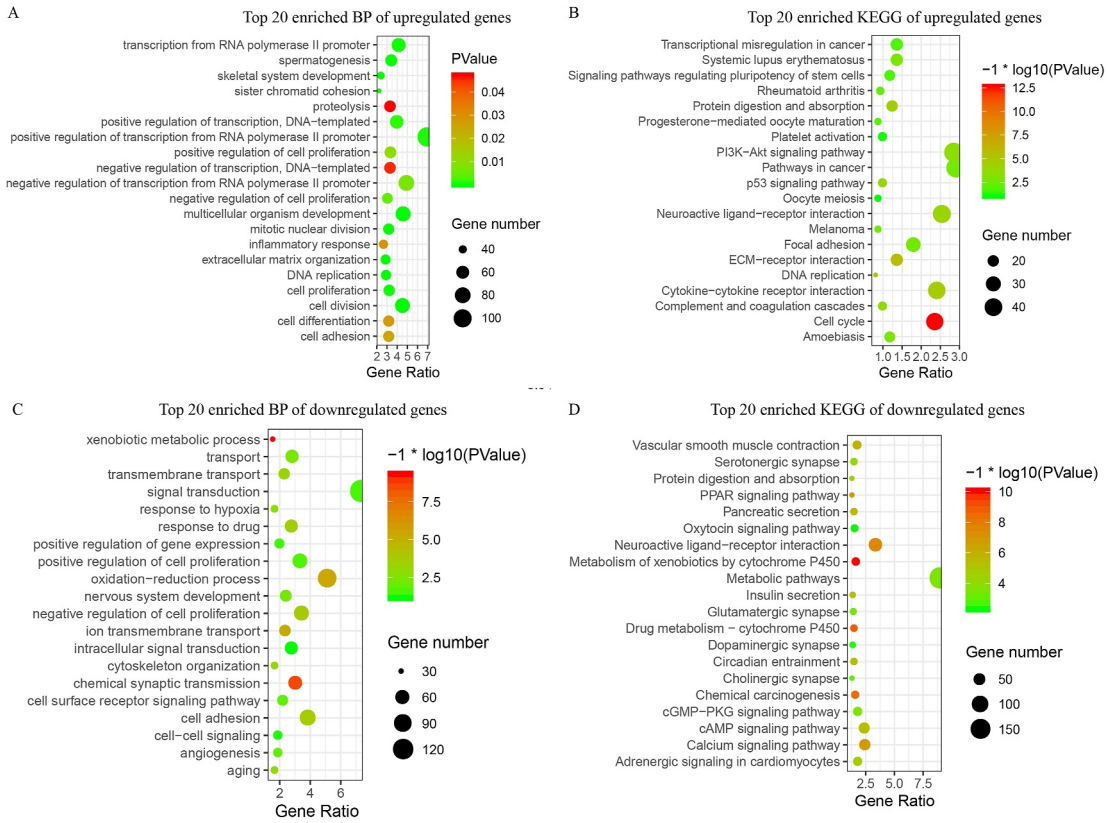

FIGURE 1 | The important 26 miRNAs are associated with several major cancer signalling pathways. (A, B) The GO biological processes and KEGG signalling pathways of significant enrichment of upregulated genes interacting with the 26 miRNAs. (C, D) The GO biological processes and KEGG signalling pathways of significant enrichment of downregulated genes interacting with the 26 miRNAs. BP: biology process; KEGG: Kyoto Encyclopaedia of Genes and Genomes.

pathway (Figure 1A, B, and Supporting Information S1: Figure S1). For the downregulated genes targeted by the 26 miRNAs, results showed that they were mainly enriched in signal transduction, oxidation–reduction process, zinc ion binding, calcium ion binding and metabolic pathways ($p < 0.05$, Figure 1C, D, and Supporting Information S1: Figure S1). Results suggested that the 26 miRNAs may be crucial for GC.

2.3 | Relationship Between miRNA Expression and Clinical Features of GC

Based on the miRNA expression profiling of GC from the TCGA database, we analysed the expression of the 26 important miRNAs in 401 cancer and 38 normal tissues. Ten miRNAs showed significant changes ($p < 0.05$, Figure 2A). Among the 10 miRNAs, expression levels of 6 miRNAs were visibly upregulated in GC tissues, including hsa-mir-335, hsa-mir-20a, hsa-mir-107, hsa-mir-34a, hsa-mir-182 and hsa-mir-210 (Figure 2A). Expression levels of 4 miRNAs were markedly downregulated, including hsa-mir-195, hsa-mir-129-2, hsa-mir-23b and hsa-mir-10b (Figure 2A). Correlation analysis showed that the expressions of 10 miRNAs were related to each other ($p < 0.05$; Figures 2B and 2C). For example, the expression of hsa-mir-182 was significantly positively correlated with the expression of hsa-mir-335 ($p < 0.05$) and was related to the expression of both hsa-mir-129-2 and hsa-mir-195. Results suggested that the 10 miRNAs may interact with each other to jointly affect the expression of tumour-related genes, thus playing an important regulatory role in GC.

We verified the expression of the 10 miRNAs in GC using the UALCAN platform. Results showed that the abnormal expression of 8 miRNAs was consistent with our analysis (Supporting Information S1: Figure S2). For hsa-mir-129-2 and hsa-mir-10b, the changing trend of their expression was consistent with our results. In addition, compared with normal tissues, expressions of hsa-mir-129-2 were changed significantly in Stage 3 and 4, Grade 2 and nodal metastasis N1 of GC ($p < 0.05$; Supporting Information S1: Figure S3 A, B and C).

Furthermore, we explored the relationship between abnormally expressed miRNAs and the clinical features of GC based on the Mann–Whitney *U* test. Results showed that the expressions of hsa-mir-34a, hsa-mir-182, hsa-mir-195, hsa-mir-129-2 and hsa-mir-210 were closely linked to the age of GC patients, whereas expressions of hsa-mir-34a and hsa-mir-182 were correlated with tumour stages (Table 2). Additionally, expressions of hsa-mir-34a and hsa-mir-182 showed significant changes in all stages of GC compared with normal tissues ($p < 0.05$, Figures 3A and 3B). Expressions of hsa-mir-34a and hsa-mir-182 may affect the development of GC.

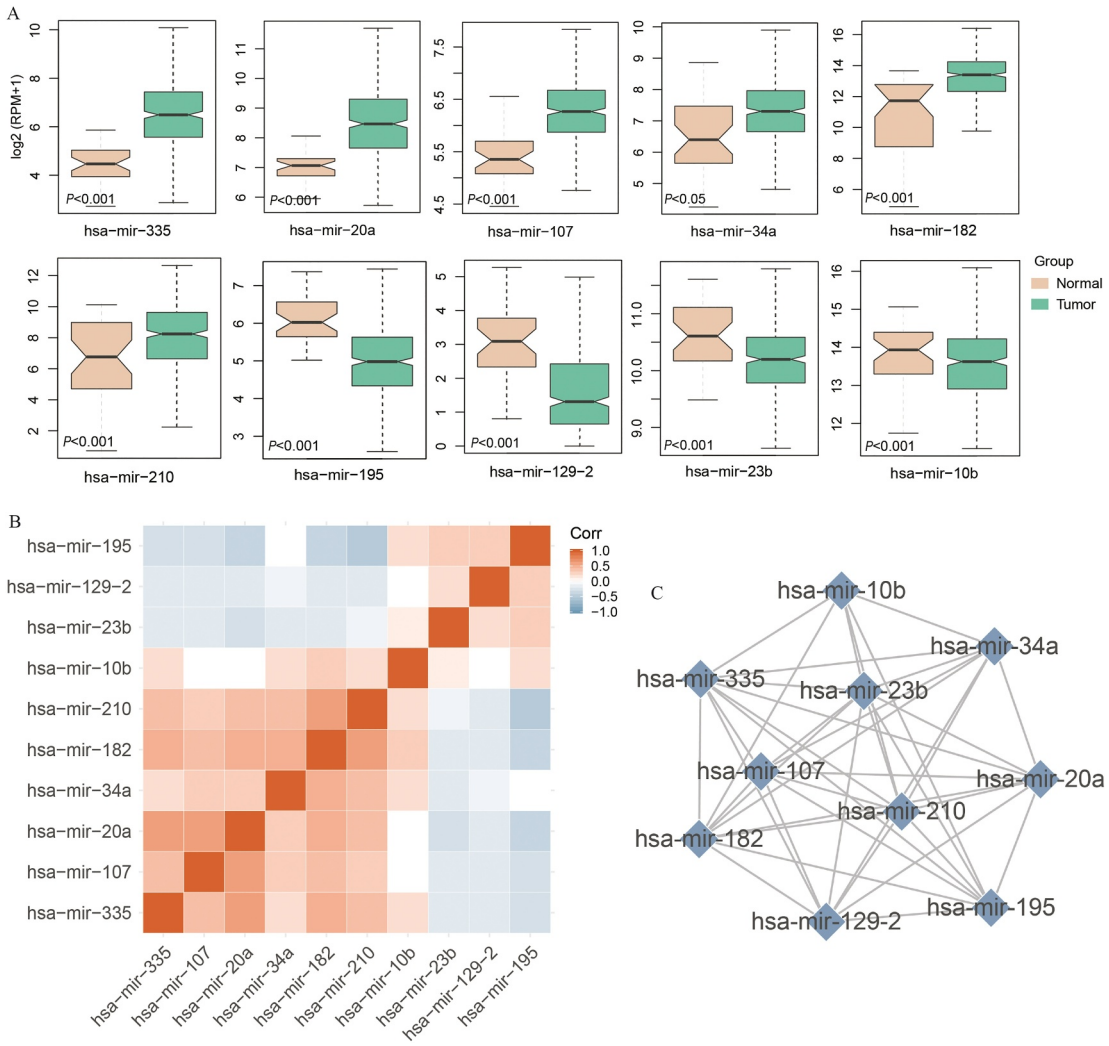

FIGURE 2 | (A) Differential expression of the 10 important miRNAs between the 401 cancer and 38 normal tissues from GC patients. (B) Pearson correlation between the expressions of these 10 miRNAs. A blank in the heat map represents no statistical correlation ($p > 0.05$). (C) The link between these 10 miRNAs.

TABLE 2 | Associations between miRNAs and clinical features of gastric cancer patients.

miRNA	*p*-value 1 (age < = 65 vs. > 65)	*p*-value 2 (tumour_stage i/ii vs. iii/iv)
hsa-mir-107	0.090	0.975
hsa-mir-129-2	**0.021**	0.150
hsa-mir-182	**0.001**	**0.039**
hsa-mir-195	**0.000**	0.082
hsa-mir-20a	0.066	0.681
hsa-mir-210	**0.001**	0.131
hsa-mir-23b	0.682	0.518
hsa-mir-335	0.202	0.491
hsa-mir-34a	0.516	**0.039**

Note: Bold indicates statistical correlation.

We analysed the relationship between these miRNAs and GC survival using the Kaplan–Meier (KM) method. Patients with GC were divided into low- and high-expression groups by the median expression value of the miRNAs in tumour samples. Only the expression of hsa-mir-23b was associated with the survival of patients with GC (Figure 3C; log-rank test $p < 0.0046$). Results indicated that hsa-mir-23b may be a prognostic factor for GC.

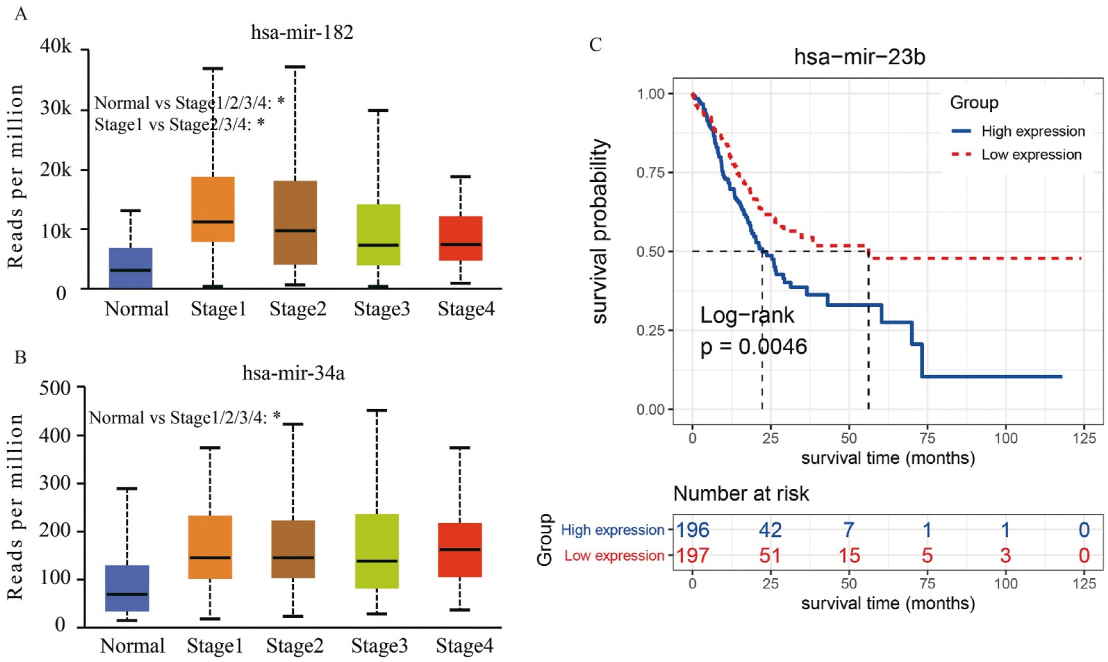

FIGURE 3 | (A, B) The expression levels of hsa-mir-34a and hsa-mir-182 in normal and tumour tissues at different stages from gastric cancer based on the UALCAN platform. * indicates that the statistical difference (p-value) is less than 0.05. (C) Association between the expression of hsa-mir-23b and the survival in gastric cancer.

2.4 | Screening of Hub miRNA-mRNA Networks in GC

As hsa-mir-34a, hsa-mir-182 and hsa-mir-23b were significantly correlated with tumour stages and survival in patients with GC, the potential molecular mechanisms were inferred by regulatory network analysis with protein coding genes. We predicted the upregulated and downregulated genes interacting with the three miRNAs based on the miRNet database. A total of 720 upregulated and 763 downregulated genes were identified to bind the three miRNAs. Furthermore, based on protein–protein interaction networks and topological degree method of CytoHubba in Cytoscape plug-in, 30 hub genes were found in 720 upregulated and in 763 downregulated genes, respectively (Supporting Information S1: Figure S4A and B, and Table 3). Of the 30 upregulated genes, *CDK1* and *CCNA2* seem more important because their degrees were larger. *CDK1* has been demonstrated to have important effects on cell apoptosis and cell cycle in GC [38]. *CCNA2* is regarded as an oncogene of multiple cancer types, which is significantly related to immune infiltration and immune checkpoint suppressor genes, and can affect the prognosis of cancer [39].

Interaction networks between the 3 miRNAs and 30 upregulated/downregulated hub genes were constructed, respectively (Figure 4A and C). Results showed that hsa-mir-34a-5p could interact with most hub genes, and the degree of hsa-mir-34a-5p was the highest than that of hsa-mir-23b-3p and hsa-mir-182-5p (Figure 4B and D). Hsa-mir-34a played a key role in a variety of cancers, which could affect almost the entire development of tumour [40]. Our results suggested that hsa-mir-34a seemed to be particularly important in GC. Hsa-mir-34a played important roles in the progression of GC possibly by regulating the expression of the hub genes.

2.5 | Effect of miRNA Expression Change on Immune Response in GC

miRNAs, an important regulator of the interaction between cancer cells and immune cells, played an important role in the immune process and are considered as key partners of immunotherapy. To further understand whether the three important miRNAs (hsa-mir-34a, hsa-mir-182 and hsa-mir-23b) could affect the immune process in GC, we performed immune-related analysis through cell-type identification by estimating relative subsets of RNA transcripts (CIBERSORT) and gene set enrichment analysis (GSEA) methods. GC patients were divided into high- and low-expression groups using the median expression level of each of the three miRNAs. Compared with distributions of multiple immune cells, the three miRNAs (particularly hsa-mir-182 and hsa-mir-23b) were significantly associated with several tumour-filtrating immune cells, including activated memory CD4 T cells and resting mast cells ($p < 0.05$, Figure 5A, Supporting Information S1: Figures S5A, and Figure S6A). For hsa-mir-182 and hsa-mir-34a, resting memory CD4 T cells were strongly negatively correlated with activated memory CD4 T cells, follicular helper T cells and CD8 T cells, respectively (Figure 5B and Supporting Information S1: Figure S6B). However, for hsa-mir-23b, activated memory CD4 T cells were strongly positively correlated with gamma delta T cells, M1 macrophages and CD8 T cells, respectively (Supporting Information S1: Figure S5B). Naive CD4 T cells were strongly positively correlated with naive B cells (Supporting Information S1: Figure S5B).

The vast majority of immune-related gene sets were upregulated in the low-expression group of hsa-mir-182 based on GSEA analysis (normalised enrichment score (NES) < 0, Figures 6A and 6B). The expression of hsa-mir-182 was

TABLE 3 | The 30 upregulated and downregulated hub genes interacting with hsa-mir-23b/34a/182.

Top 30 upregulated genes			Top 30 downregulated genes		
Rank	**Name**	**Degree**	**Rank**	**Name**	**Degree**
1	CDK1	114	1	MAPK3	22
2	CCNA2	89	2	JUN	19
3	CCNB1	87	3	FOS	18
4	CDC20	78	4	AR	9
5	BUB1	75	4	GNAI1	9
6	TOP2A	74	6	CXCL12	8
7	CCNB2	73	6	CAV1	8
8	KIF11	72	6	JUNB	8
9	PLK1	69	9	CYP1A1	7
10	AURKB	67	9	GNAO1	7
10	ASPM	67	9	EGR1	7
12	BUB1B	63	9	IL6	7
12	NDC80	63	13	APOA1	6
14	NCAPG	59	13	ME1	6
15	CDCA8	58	13	MAPT	6
15	MAD2L1	58	16	SMARCD3	5
17	KIF20 A	56	16	KAT2B	5
18	DLGAP5	54	16	MYLK	5
18	CDC45	54	16	PRKAR2B	5
18	AURKA	54	16	AKR1C3	5
21	TTK	53	16	VAMP2	5
22	TPX2	52	16	SFN	5
22	CENPE	52	16	FLNA	5
22	NUSAP1	52	16	FKBP5	5
25	RRM2	50	16	ARC	5
25	MCM2	50	16	FOSB	5
27	CENPF	49	16	ATF3	5
28	MCM4	48	16	IRF4	5
28	UBE2C	48	16	PKLR	5
28	CHEK1	48	16	ILK	5

significantly correlated with multiple immune-related biological processes and signalling pathways, mainly including immune response, phagocytosis recognition, regulation of immune system process, chemokine binding and chemokine signalling pathway (Figure 6C and D, Supporting Information S1: Table S1 and S2). In addition, CXCR4 pathway, BCR pathway and calcineurin pathway were significantly enriched in the high-expression group of hsa-mir-23b ($p < 0.05$, false discovery rate (FDR) < 25%, and NES > 1, Supporting Information S1: Figure S7 A, B and C). Furthermore, the proteasome pathway was significantly enriched in the low-expression group of hsa-mir-23b ($p < 0.01$, FDR < 25% and NES < (−1), Supporting Information S1: Figure S7D and E). The results indicated that the expression of hsa-mir-23b was related to the immune system in GC, and the expression changes of hsa-mir-23b may activate or inhibit the immune signal pathways.

Moreover, GSEA showed that hsa-mir-34a expression was closely linked to GC immunity. Most of the immune-related gene sets were upregulated in the high-expression group of hsa-mir-34a (NES > 0; Supporting Information S1: Figure S8A and B). Immune response to tumour cell, T cell mediated immunity, T helper 17 type immune response, antigen binding, TH1TH2 pathway, proteasome, innate immune system and intestinal immune network for IGA production were significantly enriched in the high-expression group of hsa-mir-34a (Supporting Information S1: Table S3).

Overall, results revealed that the expression of hsa-mir-182, hsa-mir-23b and hsa-mir-34a were closely related to various immune cells and signal pathways, and they may be important regulators in the immune system of GC.

3 | Discussion

With the breakthrough of immunology in cancer biology research and clinical practice, cancer immunotherapy has changed to be a promising way to treat cancer [41, 42]. However, the efficacy of this treat way is limited. Studies have shown that miRNAs were significantly important in the regulation of tumour immune responses and could affect antitumour immune responses [43, 44]. Thus, miRNAs are considered as promising diagnostic or prognostic markers or therapeutic targets for future combined immunotherapy [45, 46]. In the present study, some crucial miRNAs were essential in regulating the abnormal expression of protein coding genes in GC. More importantly, several of the miRNAs showed a significant closely relationship with the GC immune system.

In the present study, the obtained core miRNAs could be likely used as potential biomarkers and targets for immunotherapy of GC. Therefore, based on the bioinformatics method and miRNA-mRNA interaction network, we found that some important miRNAs were crucial for the expression of GC-related genes, such as hsa-mir-23b and hsa-mir-34a. These important miRNAs could interact with most of the upregulated and downregulated genes in GC, thereby affecting their expression. Results showed that hsa-mir-34a was significantly related to the stage of GC (Table 2) and participated in multiple immune-related biological processes and signal pathways, such as immune response, regulation of immune system process and chemokine signalling pathway. A change in the expression of hsa-mir-34a was likely to affect activated memory CD4 T cells, neutrophils and resting mast cells in GC (Supporting Information S1: Figure S6). Results were consistent with previous studies that the low expression of miR-34a was closely related to the damage of the immune function in GC [47, 48].

Furthermore, hsa-mir-182 and hsa-mir-23b were also found to be strongly associated with the development of GC. Expressions of

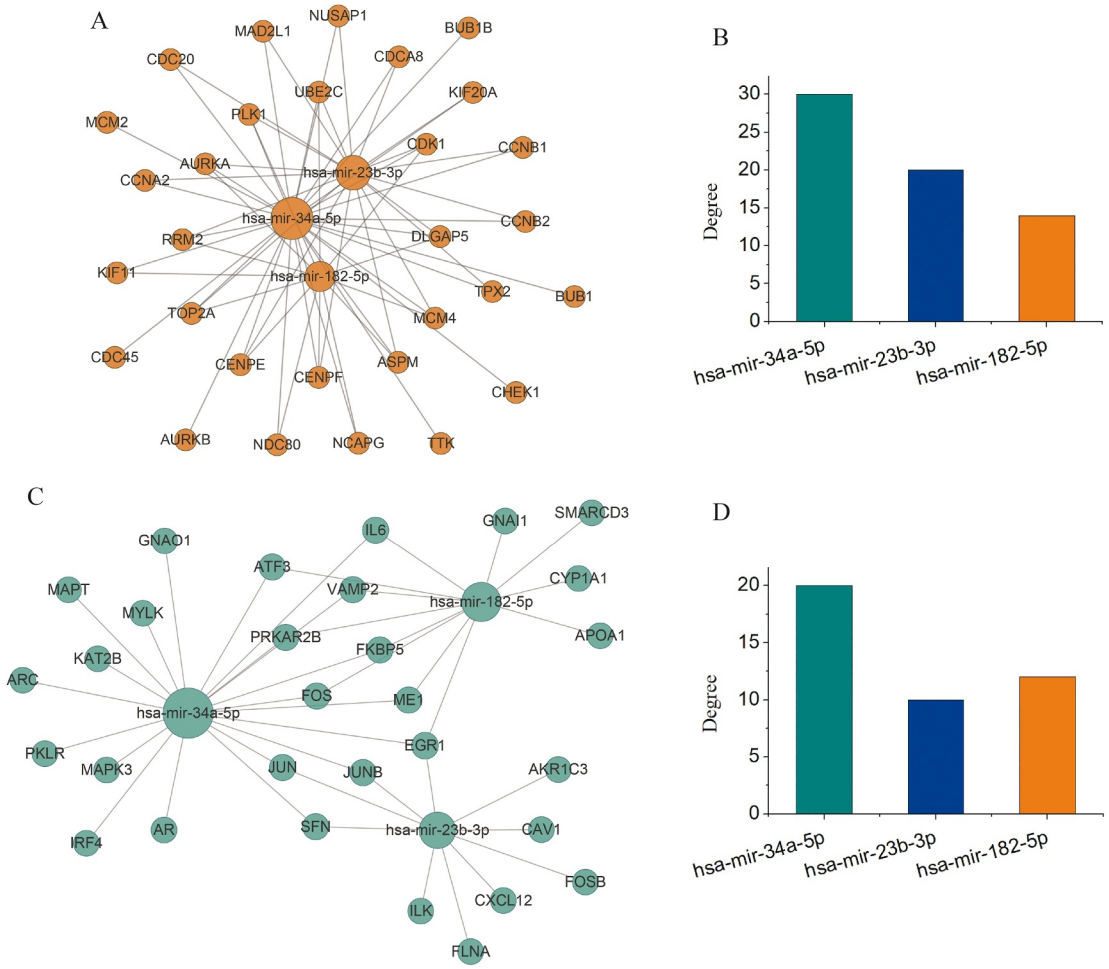

FIGURE 4 | The interaction networks between three important miRNAs (hsa-mir-34a, hsa-mir-182 and hsa-mir-23b) and up-/downregulated hub genes. (A) The interaction network between 30 upregulated hub genes and three miRNAs. (B) The degree of miRNAs in the upregulated gene network. (C) The interaction network between 30 downregulated hub genes and three miRNAs. (D) The degree of miRNAs in the downregulated gene network.

hsa-mir-182 were correlated with clinical stages in GC, whereas the high expression of hsa-mir-23b was associated with poor prognosis in patients with GC. In addition, miR-182-5p was likely to act as a diagnostic biomarker to differentiate GC tissues from normal tissues and positively related to the patient's age [49]. Similar results were also mentioned in our work, which further demonstrated the reliability of our results. Our study found that there were significant differences in the relative proportion of multiple immune cells between the high-expression group and low-expression group of hsa-mir-182 and hsa-mir-23b, respectively. Particularly, CIBERSORT analysis revealed that the expression of hsa-mir-182 was significantly correlated with 13 types of tumour-infiltrating immune cells, including memory B cells, CD8 T cells, resting mast cells, activated NK cells, eosinophils, neutrophils and macrophages M0 (Figure 5). MiR-182 had a dominant role in the regulation of adaptive immune responses [50], which was strongly induced in B cell activation and can affect extrafollicular B-cell antibody responses [51, 52]. This study indicated that miR-182 was correlated with immune responses. However, in previous studies, as far as we know, correlations between miR-182 expression and immune cell infiltration in GC were not been studied.

MiR-23b, as a pleiotropic regulator of different organs, played a key role in the development of cancer [53, 54]. Experiments have verified that miR-23b was downregulated in colon cancer and can strongly mediate tumour angiogenesis, growth and invasion in vivo [55]. In pancreatic cancer cells, decreased levels of miR-23b can enhance levels of ATG12 and autophagy, thereby promoting radioresistance of pancreatic cells [56, 57]. Microarray profile analysis revealed that miR-23b could be used as a predictive biomarker for the prognosis of GC patients [58, 59]. It is worth noting that our analysis further showed that expression changes of miR-23b possibly influenced immune cell infiltration in GC (Supporting Information S1: Figure S5A). Moreover, GSEA analysis indicated that high expression of miR-23b was prominently enriched in CXCR4 pathway, calcineurin pathway and BCR pathway in GC (Supporting Information S1: Figure S7 A, B and C). Recent studies showed that miR-23b could restrain cGAS-mediated innate immune responses and autoimmunity [60]. In liver cancer, miR-23b was correlated to activated memory CD4/CD8 T cells [61]. Studies suggested that miR-23b was likely to serve as an immune-related biomarker or therapeutic target in GC and other cancers.

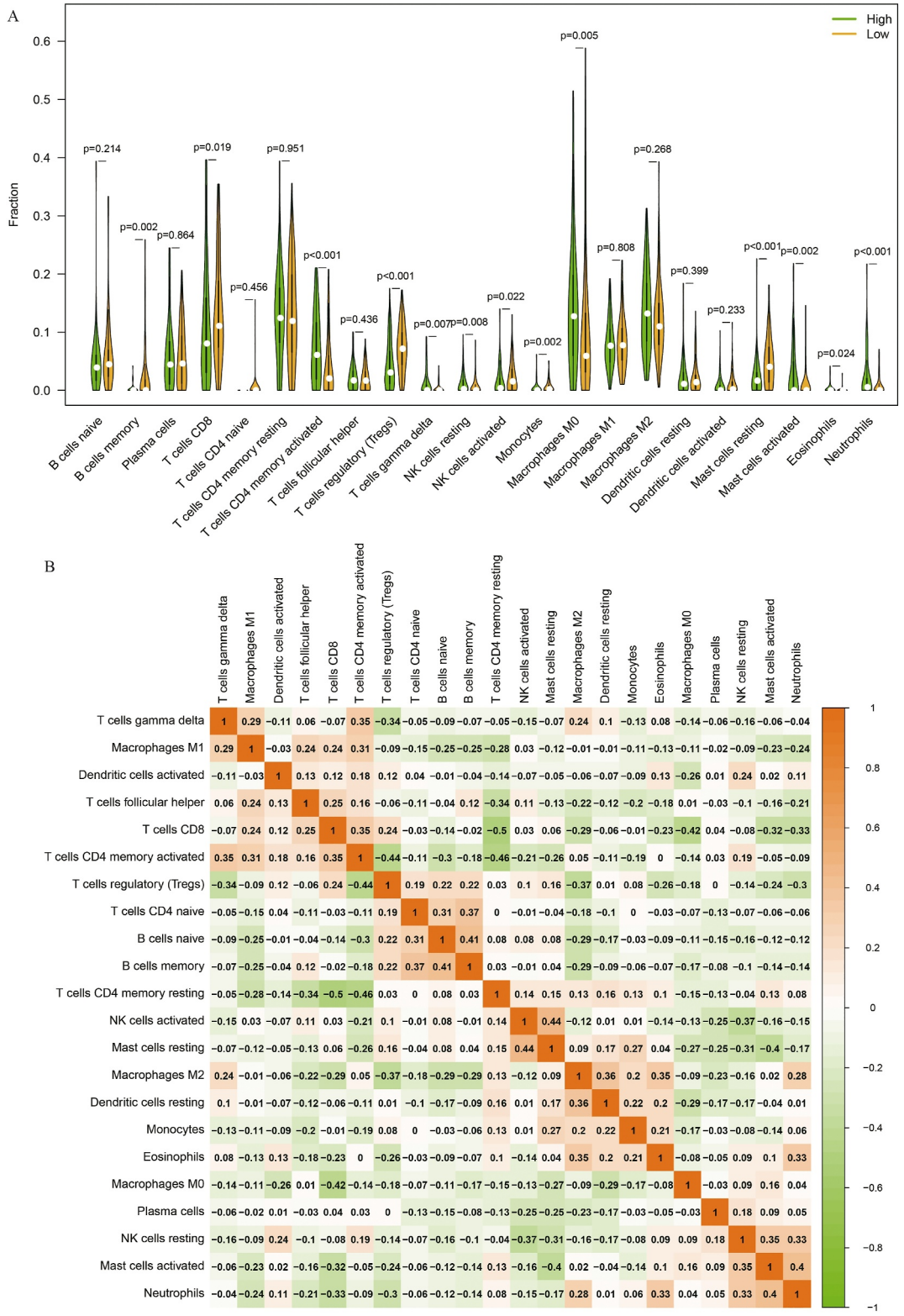

FIGURE 5 | (A) The relative fraction distribution of 22 types of immune cells between the high- and low-expression groups of hsa-mir-182. (B) Correlations between diverse immune cells.

In conclusion, we have identified 3 key miRNAs (hsa-mir-182, hsa-mir-34a and hsa-mir-23b) related to GC and established the interaction networks between the 3 miRNAs and differentially expressed hub genes. Analysis showed that the three miRNAs were likely to play a crucial role in regulating differentially expressed genes in GC. The expressions of 3 key miRNAs could affect the progression or prognosis of GC and be closely related to immune cell infiltration, particularly hsa-mir-182 and hsa-mir-23b. To the best of our knowledge, the present study highlighted the potential clinical significance of miRNAs in

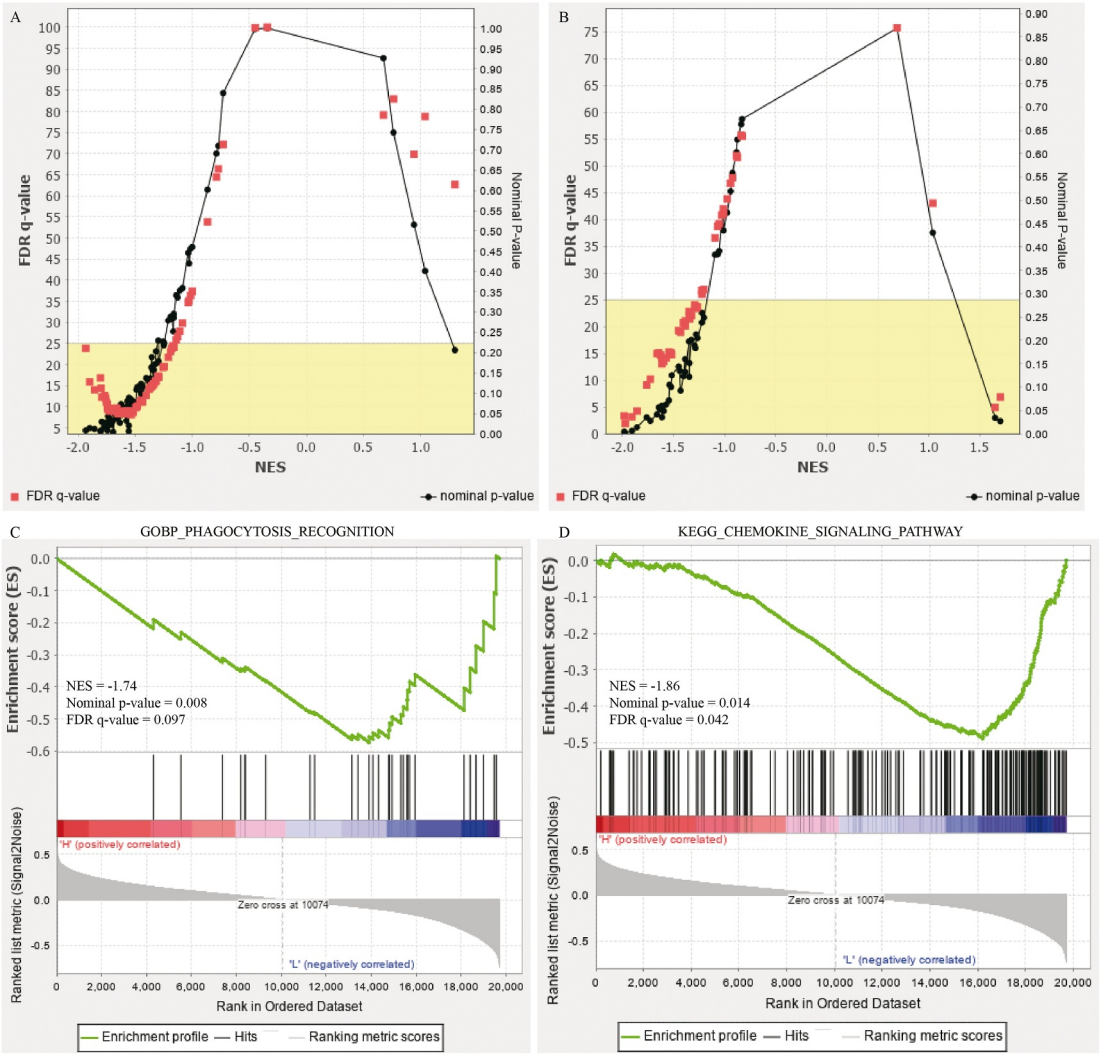

FIGURE 6 | Enrichment of immune-related biological processes and signalling pathways associated with hsa-mir-182 expression in GC. (A) Plots of *p*-values versus NES in GO analysis (B) and canonical pathways analysis. (C) GOBP phagocytosis recognition and (D) KEGG chemokine signalling pathway were significantly enriched in the low-expression group of hsa-mir-182. NES, normalised enrichment score.

immunotherapy strategies for GC patients. But findings need to be verified and supplemented by further basic experiments and clinical trials.

4 | Methods and Materials

4.1 | Data Sources and Processing

Transcriptome profiling and clinical information of GC were obtained by the Cancer Genome Atlas (TCGA, https://portal.gdc.cancer.gov/repository). The clinical information of GC patients, including sex, age, race, stage and survival status, is presented in Supporting Information S1: Table S4. The obtained expression data include FPKM (fragments per kilo base per million mapped reads) and raw read count values of gene expression data [62, 63] as well as RPM (reads per million mapped reads) and raw read count values of miRNA expression data. For miRNA data, the 410 tumour and 42 normal tissue samples from human gastric adenomas and adenocarcinomas were downloaded in this study. Meanwhile, for gene expression data, 343 tumour and 30 normal tissue samples were downloaded. In addition, miRNA target genes were obtained from the miRNet database [64].

4.2 | Selection of GC-Associated Protein-Coding Genes

To obtain differentially expressed genes (DEGs) between GC and normal gastric tissues, the R package 'DSEeq2' was utilised based on the raw count data from RNA-Seq of the 343 tumour and 30 normal tissues [65]. The padj (*p* value adjusted for Benjamini–Hochberg correction) < 0.05 and $|\log2\text{FoldChange}| > 1$ were used as the threshold. The padj < 0.05 and $\log2\text{FoldChange} > 1$ were indicated as upregulated genes and padj < 0.05 and $\log2\text{FoldChange} < (-1)$ as downregulated genes. The log2FoldChange was calculated using the following equation.

$$\log2\text{FoldChange} = \log_2\left(\frac{E_T}{E_N}\right)$$

Where E_T is the average expression level of the gene in tumour samples, E_N is the average expression level of the gene in normal samples.

The protein-coding genes were obtained by the GENCODE database [66]. DEGs that can encode proteins were considered to be the GC-associated protein-coding genes.

4.3 | GO and KEGG Enrichment Analyses

Gene Ontology (GO) and Kyoto Encyclopaedia of Genes and Genomes (KEGG) analyses were performed by the DAVID database [67]. GO analysis included biological process (BP), cellular component (CC) and molecular function (MF) [68]. A $p < 0.05$ was considered statistically significant in GO and KEGG enrichment analyses. The 'ggplot2' R package was used to visualise the GO and KEGG enrichment results.

4.4 | Relationship Between miRNA Expression and Survival in GC Patients

To assess the effect of changes in miRNA expression on patient survival, the Kaplan–Meier (KM) method was applied for survival analysis. The survival rates and median survival times of GC patients in the high-expression and low-expression miRNA groups were determined. The log-rank test was used to evaluate the significance of differences in survival rates between the groups. The 'survival' R package was used for KM survival analysis, whereas the 'survminer' R package was utilised to visualise the results. Additionally, the Mann–Whitney U test was used to determine the relationship between the miRNA expression and clinical features of GC.

4.5 | CIBERSORT Method

Cell-type identification by estimating relative subsets of RNA transcripts (CIBERSORT) is a new algorithm used for calculating the proportion of immune cells based on the gene expression data of large numbers of tumour samples [69]. In the present study, we utilised the CIBERSORT method to estimate the proportion of 22 types of tumour-infiltrating immune cells in GC samples. This analysis was performed using the R function 'CIBERSORT' and R package 'e1071', and 1000 permutations were performed to achieve statistical rigour.

4.6 | Gene Set Enrichment Analysis

Gene set enrichment analysis (GSEA) was applied to obtain immune-related gene sets [70]. GO gene sets and canonical pathways related to human immune responses were downloaded from molecular signatures database v7.5.1 (MSigDB). In the GSEA analysis, gene set size filters were set to a minimum of 15 and a maximum of 2000, and the number of permutations was set to 1000. The false discovery rate (FDR) < 25% and absolute value of the normalised enrichment score (NES) > 1 were set as the significance threshold.

4.7 | Identification of Hub Factors in miRNA-Gene Interaction Networks

Based on the STRING (search tool for the retrieval of interaction gene/proteins) database [71], we established gene–gene interaction networks. Then, the topological degree approach in Cytoscape plugin cytoHubba was used to identify hub genes [72]. Additionally, based on the miRNet database, miRNA–gene interaction networks were constructed. Similarly, using the topological degree approach, relatively important miRNAs were found in the interaction networks.

4.8 | Validation of Key miRNAs

The UALCAN platform was applied to analyse the relative expression of key miRNAs across tumour and normal samples from GC patients. The UALCAN platform is a comprehensive database, which is used to analyse the omics data of various cancers and is often used to verify the expression of genes/miRNAs of interest [73]. Here, we also analysed the expression of miRNAs in the four cancer stages and tumour grades of GC. The original p-value less than 0.05 indicates statistical significance.

4.9 | Statistical Analysis

We performed data analysis and computations using Windows 10, Intel i5 processor and 8 GB RAM. Statistical analysis and result visualisation were performed mainly through R software (R 4.1.2 and R 4.3.1 version). Correlations between miRNA expressions were assessed by Pearson correlation coefficients. The correlation heatmap was conducted using the 'ggcorrplot' R package. MiRNA–mRNA interaction networks and miRNA co-expression networks were constructed using Cytoscape software (Cytoscape 3.6.1). In the present study, the original $p < 0.05$ is considered statistically significant.

Author Contributions

Wen Jin and **Lan Yu:** conceptualisation, writing – review and editing, funding acquisition, resources. **Yongchun Zuo:** writing – review and editing, supervision. **Wen Jin:** writing – original draft, data curation, formal analysis, methodology, investigation, software. **Jianli Liu**, **Tingyu Yang**, and **Zongqi Feng:** investigation, writing – review and editing. **Jie Yang**, **Lei Cao** and **Chengyan Wu:** writing – review and editing. All authors read and approved the final manuscript.

Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The datasets analysed during the present study are publicly available at the TCGA data portal (https://portal.gdc.cancer.gov/repository).

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Supporting Information

Additional supporting information can be found online in the Supporting Information section.