

Integrated miRNA profiling and bioinformatics analyses reveal upregulated miRNAs in gastric cancer

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Abstract. Gastric cancer is one of the most common malignancies in China and exhibits a poor prognosis. The most significant challenge for gastric cancer treatment is the absence of early diagnostic biomarkers. MicroRNAs (miRNAs) are small non-coding RNAs, which possess clinical value in a number of different types of cancer. The current study identified 13 miRNAs (hsa-miR-22, hsa-miR-545, hsa-let-7i, hsa-miR-15b, hsa-miR-221, hsa-miR-196a, hsa-miR-20a, hsa-miR-196b, hsa-miR-93, hsa-miR-19a, hsa-miR-503, hsa-miR-106b and hsa-miR-18a) that were significantly overexpressed in GC, by analyzing 1,000 GC samples included in four public datasets, including GSE23739, GSE78091, GSE30070 and The Cancer Genome Atlas. Furthermore, it was revealed that the expression levels of these 13 miRNAs were significantly higher in gastric cancer tissues of grades I, II and III compared with normal controls. Gene ontology analysis and Kyoto Encyclopedia of Genes and Genomes analysis demonstrated that the differentially expressed miRNAs were involved in regulating transcription, protein amino acid phosphorylation, signal transduction, protein binding, zinc ion binding, the mitogen-activated protein kinase signaling pathway and focal adhesion. In summary, the present study may provide potential new therapeutic and prognostic targets for gastric cancer.

Introduction

A number of studies have indicated that non-coding RNAs can serve as important regulators of biological processes (1). MicroRNAs (miRNAs) are small non-coding RNAs that are 18-25 nucleotides long, which are important post-transcriptional regulators of gene expression by inhibiting the stability

or translational efficiency of target genes (2). Recently studies have demonstrated that miRNAs are aberrantly expressed in different types of human cancer and serve important roles in regulating cancer initiation and progression (3). miRNAs can regulate numerous biological processes, including cancer cell proliferation, cycle arrest, apoptosis, migration and invasion (4).

Gastric cancer (GC) is one of the most common malignancies in China and exhibits a poor prognosis (5). A significant challenge for GC treatment is the absence of early diagnostic biomarkers (6). Yuan *et al* (7), reported that the 5-year survival rate of early GC is ~30-fold high compared with that of late-stage GC. A number of studies have identified aberrantly expressed miRNAs in GC, which serve regulatory roles in tumors (8). For example, miR-5590-3p has been revealed to inhibit GC growth by targeting the DDX5/AKT/mammalian target of rapamycin (mTOR) pathway (9). miRNA-223 promotes GC metastasis by targeting EPB41L3 (10). Additionally, miR-20a has been identified to be involved in the resistance of GC by targeting CYLD (11). However, these studies only investigated a small number of miRNAs and didn't perform a systems-level identification of differentially expressed miRNAs with a large sample size. Therefore, there is a requirement to understand the molecular mechanisms of miRNAs that regulate GC progression and identify miRNAs that may serve as biomarkers for the detection of GC.

The present study identified differentially expressed miRNAs in GC by analyzing four public datasets, including GSE23739, GSE26295, GSE30070 and data from The Cancer Genome Atlas (TCGA). In total, >800 GC samples were included in the present study. Additionally, to investigate the molecular mechanisms of aberrantly expressed miRNAs, gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis were performed.

Materials and methods

Microarray data and data preprocessing. The current study screened GC-associated miRNA expression profiles in National Center for Biotechnology Information (NCBI) Gene Expression Omnibus (GEO) datasets (<https://www.ncbi.nlm.nih.gov/gds/>). Candidate datasets with >50 samples were selected for further analysis. Therefore, a total of four

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miRNA expression datasets of GC were downloaded from the NCBI GEO and TCGA (<https://portal.gdc.cancer.gov/>) databases including GSE23739, GSE30070, GSE26595 and data from TCGA. GSE23739 was submitted by Oh *et al* (12), and contained 40 GC samples and 40 normal samples obtained from the National Cancer Centre and the Singhealth Tissue Repository (Singapore). GSE26595 was submitted by Lim *et al* (13), and contained 60 primary GC samples and 8 surrounding non-cancerous samples from patients who underwent a curative gastrectomy as a primary treatment method between 1999 and 2007 at Severance Hospital and Gangnam Severance Hospital, Yonsei University College of Medicine (Seoul, South Korea). GSE30070 was submitted by Kim *et al* (14), and contained 90 GC samples and 34 normal samples collected at the Hospital of Korean National Cancer Center by endoscopy between 2001 and 2006 following a protocol approved by the Institutional Review Board (IRB) of the National Cancer Center Hospital in Goyang, Korea. All patients and volunteers signed IRB-approved informed consent forms. A total of 34 healthy volunteers underwent gastroscopy for routine screening for GC and were confirmed to have normal gastric mucosa by histology. No gastritis was identified among the 34 healthy volunteers. TCGA data contained 389 GC samples and 41 normal samples.

These datasets were based on different platforms. For example, GSE23739 was based on Agilent Human miRNA Microarrays and TCGA data were based on RNA-sequencing (RNA-seq). A previous study demonstrated that RNA-seq and microarray-based methods do not exhibit a high coincidence degree, although they are both good technologies for measuring gene expression level (15). The present study used TCGA data and three GEO datasets to identify common dysregulated miRNAs in GC. A similar analysis has also been reported by a number of other groups (16-19).

GO and KEGG pathway analysis. To predict the targets of the differentially expressed miRNAs, four different databases were used, including TargetScan (<http://www.targetscan.org/>), miRWALK (<http://zmf.umm.uni-heidelberg.de/apps/zmf/mirwalk2/>), miRDB (<http://www.mirdb.org/>) and starbase (<http://starbase.sysu.edu.cn/>). A total of 605 target mRNAs were obtained. Accordingly, the network between differentially expressed targets and miRNAs was constructed using Cytoscape v3.2.1 (<http://www.cytoscape.org/>) software. Molecular Annotation System 3.0 (<http://bioinfo.capitalbio.com/mas3/>) was used to determine the biological roles of the target mRNAs. Gene functions were classified into the following three subgroups: Biological process, molecular function and cellular component. The enriched GO terms were presented as enrichment scores. KEGG pathway analysis (<https://www.genome.jp/kegg/pathway.html>) was performed to determine the involvement of differentially expressed mRNAs in different biological pathways using DAVID system (<https://david.ncifcrf.gov/tools.jsp>). $P < 0.05$ was considered to indicate a statistically significant result.

Statistical analysis. Results were analyzed using SPSS software (version 15.0; SPSS, Inc.). Numerical data are presented as the mean \pm standard deviation. Statistical comparisons between two groups of normalized data were performed using

Table I. Analysis of four miRNA expression profiles, which include 579 gastric cancer samples and 123 normal samples.

Dataset	Total cases	Normal cases	Tumor cases
GSE23739	80	40	40
GSE30070	124	34	90
GSE26595	68	8	60
TCGA	430	41	389

TCGA, The Cancer Genome Atlas.

a student's t-test or Mann-Whitney U-test according to the test condition. Statistical comparisons among multiple groups of normalized data were performed using one-way analysis of the variance followed by a Dunnett's post hoc test. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Identification of the significantly differentially expressed miRNAs in GC. To identify the significantly differentially expressed miRNAs in GC, the current study used four miRNA expression profiles including 579 GC samples and 123 normal samples, which were downloaded and analyzed from GEO and TCGA datasets, as presented in Table I and Fig. 1. A total of 10 miRNAs (hsa-miR-15b-5p, hsa-miR-21-5p, hsa-miR-93-5p, hsa-let-7i-5p, hsa-miR-25-3p, hsa-miR-185-5p, hsa-miR-181b-5p, hsa-miR-224-5p, hsa-miR-196b-5p and hsa-miR-135b-5p) were identified as upregulated and hsa-miR-204-5p was revealed as downregulated in GC in the four datasets. Clustering analysis was subsequently performed for all abnormally expressed miRNAs in GC (Fig. 1E and F; Table II).

Differentially expressed miRNAs are dysregulated in all stages of GC. To evaluate the possible prognostic value of the 11 abnormally expressed miRNAs, the present study analyzed RNA-seq data from TCGA, which included a cohort of 41 normal tissues, 7 grade I GC tissues, 140 grade II GC tissues and 232 grade III GC tissues (20). All patients were staged using the tumor-node-metastasis classification of the American Joint Committee on Cancer/International Union Against Cancer from 2009 (21). It was identified that the expression levels of hsa-miR-15b-5p, hsa-miR-21-5p, hsa-miR-93-5p, hsa-let-7i-5p, hsa-miR-25-3p, hsa-miR-185-5p, hsa-miR-181b-5p, hsa-miR-224-5p, hsa-miR-196b-5p and hsa-miR-135b-5p were significantly upregulated, and hsa-miR-204-5p expression was significantly downregulated in grade I, II and III 3 GC tissues compared with the normal controls (Fig. 2A-K). However, differential expression levels of the miRNAs were not observed between the different GC stages. This analysis demonstrated that the abnormally expressed miRNAs may be associated with GC progression and may serve as early diagnostic biomarkers. However, the dysregulation of these miRNAs could not predict the stage of GC.

Construction of a differentially expressed miRNAs-mRNAs network for GC. Four different databases were used, including

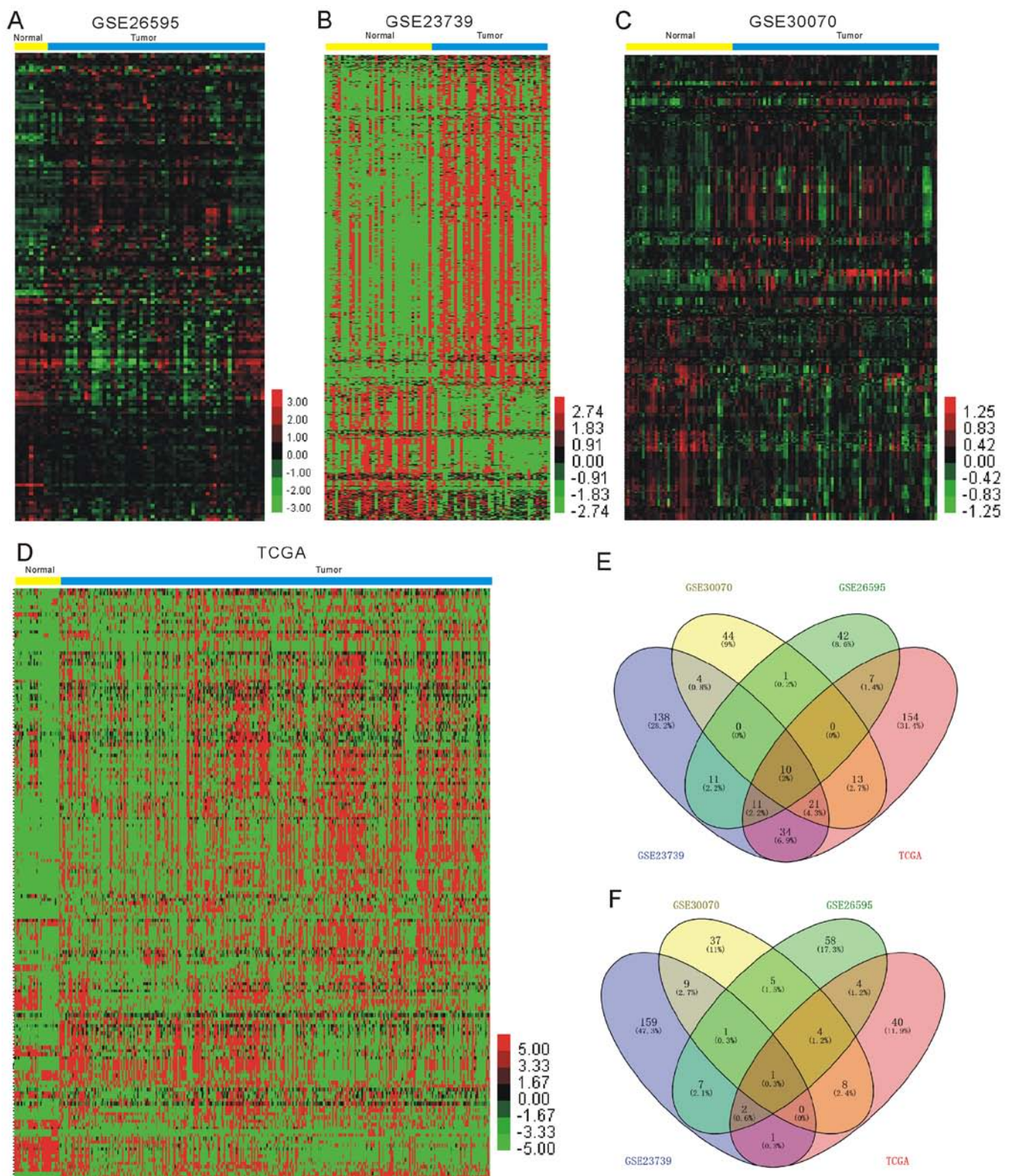


Figure 1. Identification of the significantly differentially expressed miRNAs in gastric cancer. Heatmaps of the differentially expressed miRNAs in (A) GSE26595, (B) GSE23739, (C) GSE30070 and (D) TCGA data. Clustering analysis of the (E) upregulated miRNAs and (F) downregulated miRNAs in the four datasets. miRNA, microRNA; TCGA, The Cancer Genome Atlas.

TargetScan, miRWALK, miRDB and starbase. A total of 605 target mRNAs were obtained by combining the analyses of these databases (Fig. 3A). Furthermore, the TCGA dataset was analyzed to investigate the expression pattern of the 605 target mRNAs in GC (Fig. 3B). In the present study,

the downregulated genes were selected as potential targets of the upregulated miRNAs and the upregulated genes were selected as potential targets of the downregulated miRNAs. As presented in Fig. 3C, a total of 11 miRNAs and 267 mRNAs were included in this network. Four miRNAs,

Table II. Expression of miRNAs in gastric cancer samples vs. normal tissue samples.

miRNA	GSE23739			GSE26595			GSE30070			TCGA		
	P-value	T	N	P-value	T	N	P-value	T	N	P-value	T	N
hsa-miR-15b-5p	0.01	173.40	81.74	0.01	12.96	12.67	0.03	0.22	0.13	<0.01	272.81	138.62
hsa-miR-21-5p	<0.01	21106.46	5068.67	<0.01	14.88	14.51	0.01	-0.26	-0.47	<0.01	28,4397.89	61,871.29
hsa-miR-93-5p	<0.01	481.12	177.25	<0.01	12.84	12.43	<0.01	-0.22	-0.39	<0.01	6,766.76	1,685.58
hsa-let-7i-5p	<0.01	1787.97	496.38	<0.01	13.44	13.00	0.01	-0.11	-0.19	<0.01	559.93	404.31
hsa-miR-25-3p	0.01	254.91	107.19	<0.01	13.04	12.40	<0.01	-0.07	-0.30	<0.01	9,512.39	4104.21
hsa-miR-185-5p	<0.01	179.77	92.53	<0.01	11.25	10.46	<0.01	0.04	-0.06	<0.01	75.59	28.86
hsa-miR-181b-5p	<0.01	109.79	67.29	<0.01	10.90	9.77	0.03	0.15	0.03	<0.01	279.87	83.34
hsa-miR-224-5p	0.03	29.75	2.54	<0.01	9.89	7.99	<0.01	0.70	0.18	0.04	36.37	13.50
hsa-miR-196b-5p	<0.01	30.49	7.97	<0.01	10.42	8.34	<0.01	-0.20	-0.35	<0.01	637.67	26.86
hsa-miR-135b-5p	0.01	32.91	2.34	<0.01	11.85	9.36	0.01	0.30	0.05	<0.01	66.81	6.74
hsa-miR-204-5p	<0.01	3.61	11.63	<0.01	8.25	10.73	<0.01	0.06	0.52	<0.01	7.31	21.90

T, tumor; N, normal; miRNA, microRNA; TCGA, The Cancer Genome Atlas.

including hsa-miR-93-5p, hsa-miR-15b-5p, hsa-let-7i-5p and hsa-miR-204-5p were identified as key regulators in GC as they were connected to >40 mRNAs in the network.

GO analysis of differentially expressed miRNAs. GO analysis was performed for the differentially expressed miRNAs using the target mRNAs. We hypothesized that the potential functions of target mRNAs could reflect the possible roles of differentially expressed miRNAs. The present study only presented the GO analysis results for the biological processes and molecular functions (Fig. 4). According to the GO analysis, differentially expressed miRNAs were enriched in ‘positive regulation of transcription’, ‘anterior/posterior pattern specification’, ‘proteasome-mediated ubiquitin-dependent protein catabolic process’, ‘embryonic hemopoiesis’, ‘positive regulation of G1/S transition of mitotic cell cycle’, ‘canonical Wnt signaling pathway’, ‘regulation of cyclin-dependent protein serine/threonine kinase activity’, ‘regulation of apoptotic process’ and ‘positive regulation of small GTPase mediated signal transduction’ (Fig. 4A).

In addition, the differentially expressed miRNAs were involved in regulating several molecular functions, including ‘receptor signaling protein serine/threonine kinase activity’, ‘ATP binding’, ‘transcription factor activity’, ‘sequence-specific DNA binding’, ‘sequence-specific DNA binding’ and ‘coreceptor activity’ (Fig. 4B).

KEGG analysis of differentially expressed miRNAs. KEGG pathway analysis revealed that the differentially expressed miRNAs predominantly participate in the regulation of ‘Insulin resistance’, ‘Transcriptional misregulation in cancer’, ‘FoxO signaling pathway’, ‘mTOR signaling pathway’, ‘Glucagon signaling pathway’, ‘Neurotrophin signaling pathway’ and ‘cGMP-PKG signaling pathway’ (Fig. 4C).

Notably, it was identified that the differentially expressed miRNAs serve crucial roles in regulating the forkhead box protein O (FoxO) signaling pathway and the mTOR signaling pathway. Nine genes in the FoxO signaling pathway, including AKT3, FBXO32, CCND2, CCNG2, PTEN, PRKAB2, SGK1, STAT3 and TGFBR2, and six genes in the mTOR signaling pathway, including AKT3, RICTOR, EIF4B, PTEN, RPS6KA3 and RPS6KA6, were identified to be targets of the differentially expressed miRNAs (Fig. 5).

Discussion

The most significant challenge for the treatment of GC is the absence of early diagnostic biomarkers (1). miRNAs are small non-coding RNAs that demonstrate clinical value in a number of types of cancer (2). Numerous studies have identified certain differentially expressed miRNAs in GC (22-24); however, these studies only investigated a small number of miRNAs and lacked large sample sizes to systematically identify numerous differentially expressed miRNAs.

Previous studies regarding GC have indicated that hsa-miR-93 (25), hsa-miR-19a (26), hsa-miR-20a (27) and hsa-miR-221 (28) might act as oncogenic miRNAs. To identify differentially expressed miRNAs in GC, the present study analyzed four public datasets, including GSE23739, GSE26595, GSE30070 and data from TCGA.

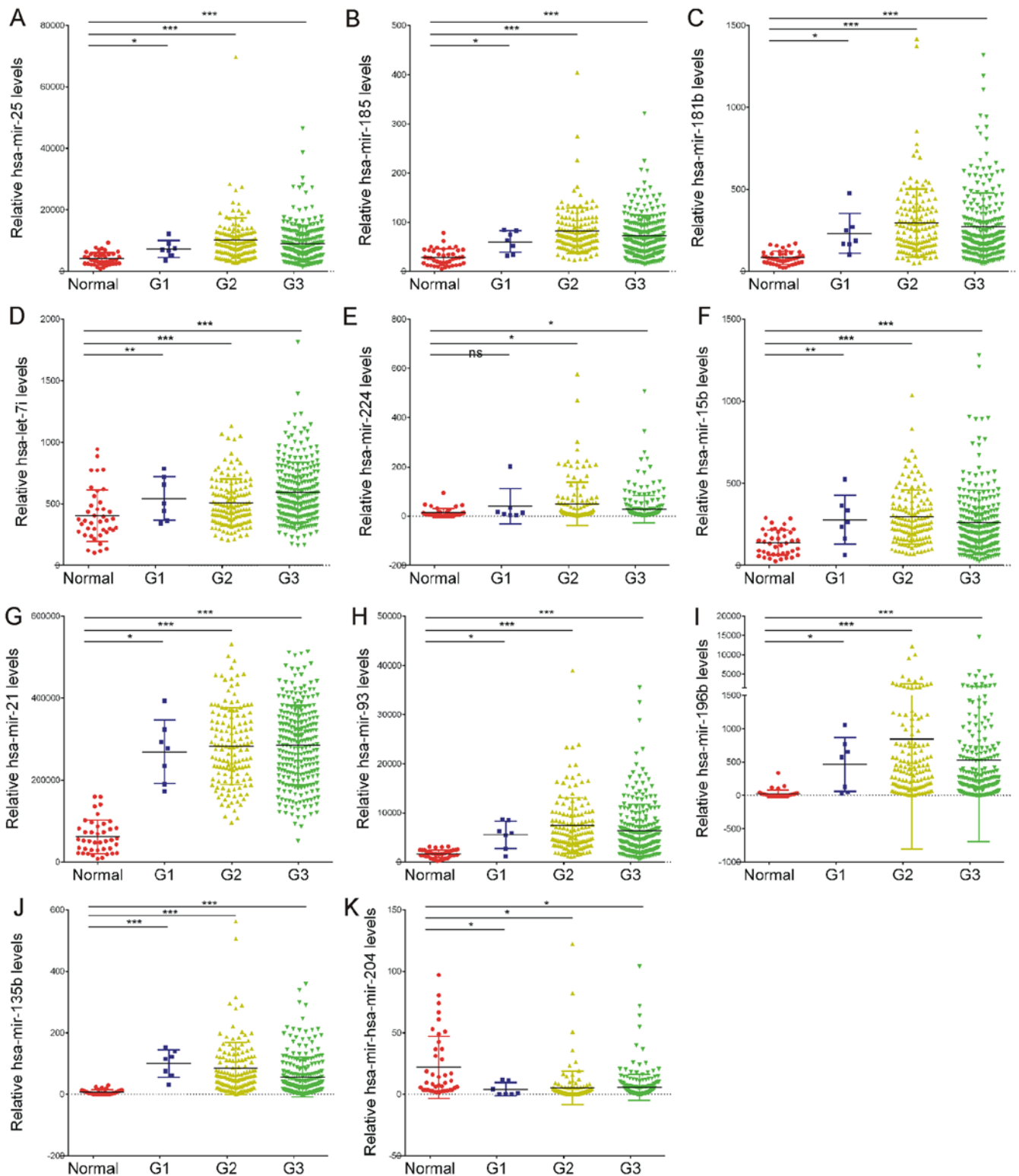
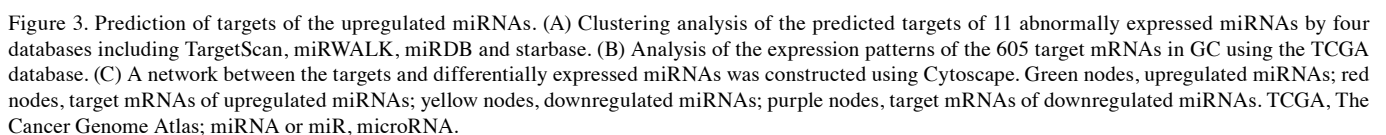


Figure 2. Dot-plot assay for the expression levels of the 11 abnormally expressed miRNAs in G1, G2 and G3 gastric cancer tissues compared with the normal controls. (A) hsa-miR-25-3p, (B) hsa-miR-185-5p, (C) hsa-miR-181b-5p, (D) hsa-let-7i-5p, (E) hsa-miR-224-5p, (F) hsa-miR-15b-5p, (G) hsa-miR-21-5p, (H) hsa-miR-93-5p, (I) hsa-miR-196b-5p and (J) hsa-miR-135b-5p were identified to be significantly upregulated in G1, G2 and G3 gastric cancer tissues compared with the normal samples. (K) hsa-miR-204-5p was revealed to be downregulated in G1, G2 and G3 gastric cancer tissues compared with the normal samples. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. miRNA or miR, microRNA; G1, grade I; G2, grade II; G3, grade III.

A total of 10 miRNAs (hsa-miR-15b-5p, hsa-miR-21-5p, hsa-miR-93-5p, hsa-let-7i-5p, hsa-miR-25-3p, hsa-miR-185-5p, hsa-miR-181b-5p, hsa-miR-224-5p, hsa-miR-196b-5p and hsa-miR-135b-5p) were revealed to be upregulated in GC

and hsa-miR-204-5p was identified to be downregulated in GC, which suggests these miRNAs serve key roles in GC. To evaluate the possible prognostic value of these abnormally expressed miRNAs, the current study analyzed RNA-seq



Considering the important roles of miRNAs in human disease, a number of studies have investigated the functions and molecular mechanisms of miRNAs by performing loss or gain of function assays (29-32). However, these experimental validations are expensive and time-consuming. Therefore,

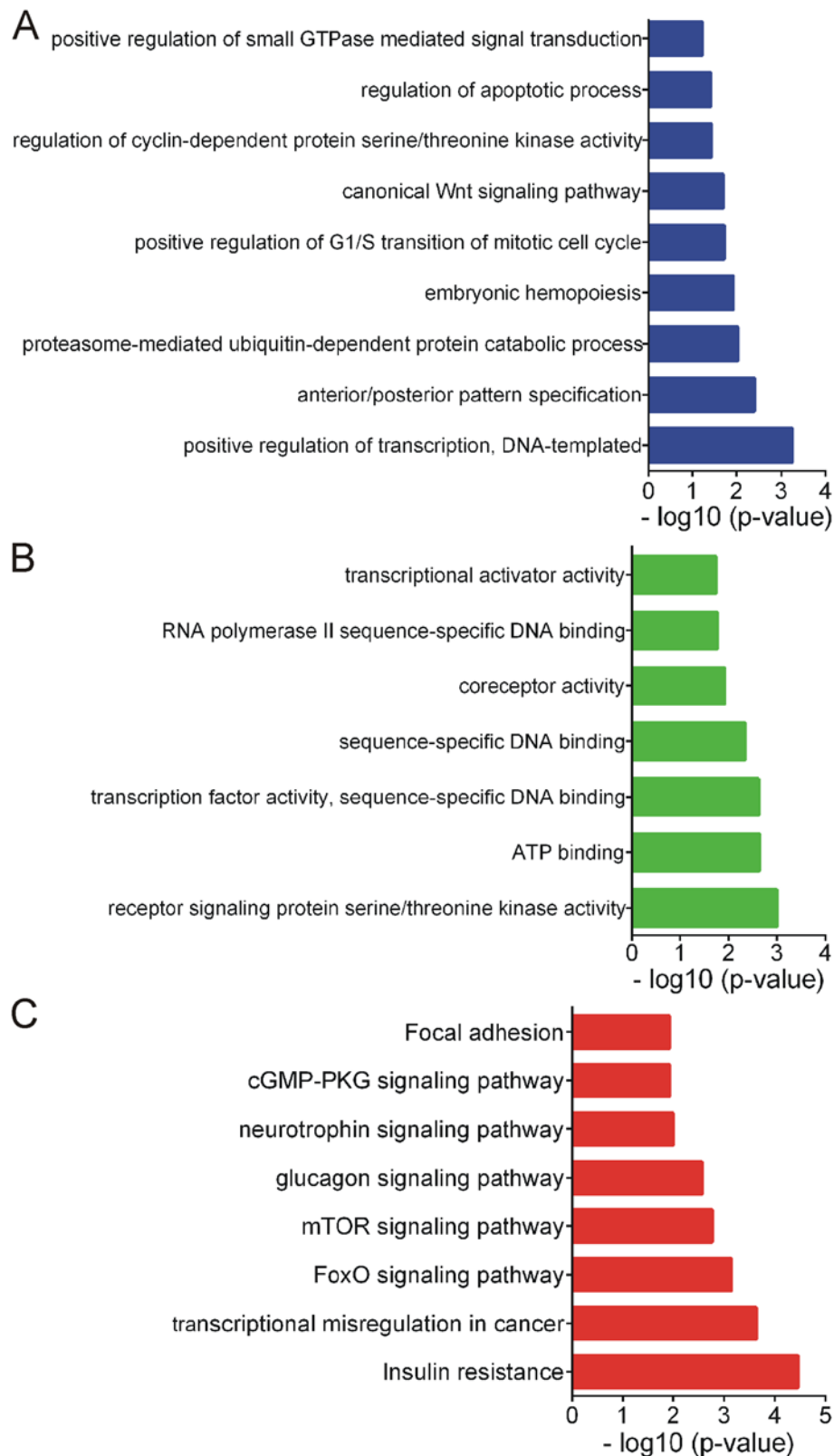


Figure 4. GO and KEGG analysis of the target mRNAs of differentially expressed miRNAs. (A) Biological processes analysis of the differentially expressed miRNAs using the target mRNAs. (B) Molecular function analysis of the differentially expressed miRNAs by using the target mRNAs. (C) KEGG pathway analysis of the differentially expressed miRNAs. The bioinformatics analyses were performed using Molecule Annotation System. GO, gene ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes; miRNA, microRNA.

a number of effective and feasible computational methods have been developed to assist with the prediction of potential associations between disease and miRNAs. For example,

Chen *et al* (33) developed the Path-Based miRNA-Disease Association model for miRNA-disease association prediction. In addition, Chen *et al* (33,34) developed the Bipartite

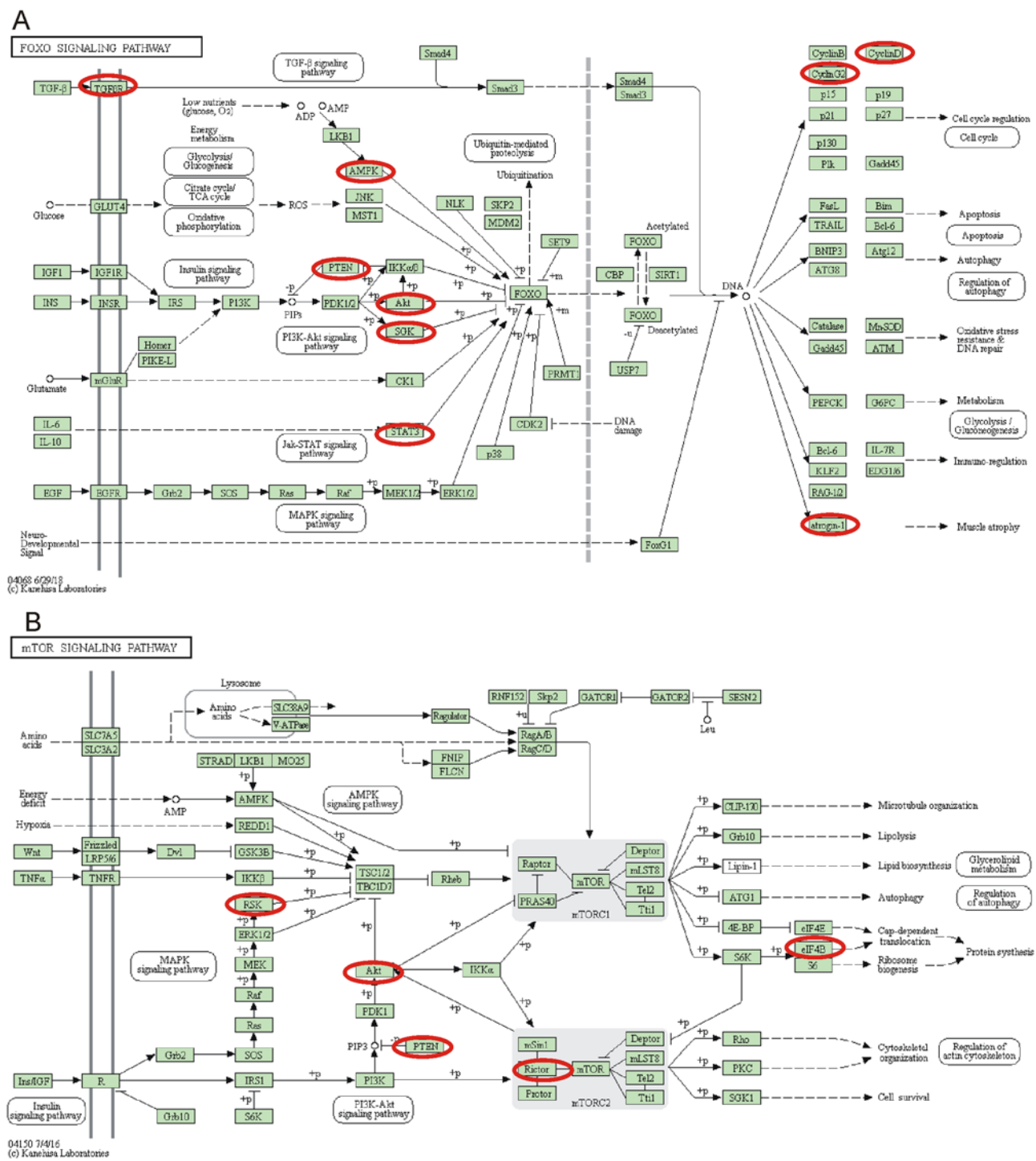


Figure 5. Key Kyoto Encyclopedia of Genes and Genomes pathways associated with the differentially expressed miRNAs. Gene targets of the differentially expressed miRNAs that are associated with the (A) FoxO and (B) mTOR signaling pathways are indicated by a red circle. miRNA, microRNA; FoxO, forkhead box O; mTOR, mammalian target of rapamycin.

Network Projection for miRNA-Disease Association and Hybrid Approach for miRNA-Disease Association prediction models for miRNA-disease prediction. It can be suggested that a combination of experimental validations, and effective and feasible computational prediction methods is a useful strategy to investigate the potential roles of miRNAs in human disease. The present study constructed a differentially expressed miRNAs-mRNAs network, and performed GO and KEGG analysis with the target mRNAs. A total of 11 miRNAs and

267 mRNAs were included in this network. Four miRNAs, including hsa-miR-93-5p, hsa-miR-15b-5p, hsa-let-7i-5p and hsa-miR-204-5p were identified as key regulators in GC as there were connected with >40 mRNAs in the network. Bioinformatics analysis revealed that the differentially expressed miRNAs are associated with ‘positive regulation of transcription’, ‘positive regulation of G1/S transition of mitotic cell cycle’, ‘canonical Wnt signaling pathway’ and ‘regulation of apoptotic process’. KEGG pathway

analysis identified that the differentially expressed miRNAs predominantly participate in regulation of 'Insulin resistance', 'Transcriptional misregulation in cancer', 'FoxO signaling pathway', 'mTOR signaling pathway', 'Glucagon signaling pathway', 'Neurotrophin signaling pathway' and 'cGMP-PKG signaling pathway'. Notably, nine genes associated with the FoxO signaling pathway, including AKT3, FBXO32, CCND2, CCNG2, PTEN, PRKAB2, SGK1, STAT3 and TGFB2, and six genes associated with the mTOR signaling pathway, including AKT3, RICTOR, EIF4B, PTEN, RPS6KA3 and RPS6KA6, were identified as targets of the differentially expressed miRNAs. Previous studies have demonstrated the important roles of FoxO and mTOR signaling in GC progression. For example, downregulation of FOXO4 was revealed to inhibit tumor proliferation and metastasis in GC (35). In addition, mTOR signaling serves crucial roles in regulating GC proliferation, apoptosis and metastasis (36,37). Nadauld *et al* (38) identified that TGFB2 acts an oncogene in diffuse GC. Furthermore, PTEN has been demonstrated to be involved in regulating apoptotic cell death, metastasis, angiogenesis and chemoresistance in GC (39). These results suggest that dysregulation of these miRNAs have important roles in GC progression.

In conclusion, the present study identified 11 miRNAs that are significantly differently expressed in GC by analyzing 1,000 GC samples from four public datasets, including GSE23739, GSE26595, GSE30070 and data from TCGA. hsa-miR-93-5p, hsa-miR-15b-5p, hsa-let-7i-5p and hsa-miR-204-5p were revealed as key regulators in GC. GO analysis and KEGG analysis demonstrated that the differentially expressed miRNAs are involved in the positive regulation of transcription, positive regulation of the G1/S transition in the mitotic cell cycle, the canonical Wnt signaling pathway, the FoxO signaling pathway and the mTOR signaling pathway. In summary, the current study may provide potential new therapeutic and prognostic targets for GC.

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Availability of data and materials

All data generated or analyzed during the present study are included in this published article.

Authors' contributions

CY and YG conceived and designed the study. CY, YZ and WT developed the methodology. CY and YZ analyzed and interpreted the data. CY, YZ, WT and YG drafted, reviewed and revised the manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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