MicroRNA profiles in five pairs of early gastric cancer tissues and adjacent non-cancerous tissues

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Abstract. Approximately half of the world's gastric cancer cases and deaths occur in China. In addition, the incidence and mortality rates of gastric cancer in Gansu province in China are much higher than the average nationwide levels. The present study investigated microRNA (miRNA/miR) profiles in early gastric cancer (EGC) without specific symptoms. miRNA expression levels in five pairs of EGC tissues and adjacent non-cancerous mucosa tissues of patients from Gansu province in China were analyzed using a miRNA microarray. A total of 47 differentially expressed miRNAs (DEMs) were identified. Subsequently, mRNA expression profiles of three pairs of cancer tissues and adjacent non-cancerous tissues from 3 Asian patients with stage I or stage II gastric cancer (stage I/II; American Joint Committee on Cancer classification, Eighth Edition) were obtained from The Cancer Genome Atlas database, and differentially expressed genes (DEGs) were identified. The target genes of DEMs were filtered from the DEGs using the miRDB database and a miRNA-gene network was constructed. The functions of DEMs were evaluated using the tool for annotations of human miRNAs database, and via Gene Ontology analysis, Kyoto Encyclopedia of Genes and Genomes analysis and Gene Set Enrichment Analysis of the target genes. Finally, survival analyses of DEMs, which were in the miRNA-gene network, was performed. The results suggested that a number of miRNAs, including hsa-let-7a-5p, hsa-miR-27a-3p, hsa-miR-126-5p and hsa-miR-424-5p, may serve critical roles in EGC. The present study could provide a basis for the identification of EGC screening biomarkers. Furthermore, the present study may provide a basis for the exploration of the cause of the high incidence of gastric cancer in Gansu province from the perspective of miRNAs.

Introduction

Gastric cancer was the third most common cause of cancer-associated mortality in Asia in 2018 (1). Asia has a high incidence of gastric cancer globally, and approximately half of the world's gastric cancer cases and deaths occur in China (1). In China, gastric cancer is the second most common malignancy and the third leading cause of death from malignant neoplasms (1). In 2012, Gansu province in China had a gastric cancer age-standardized incidence rate by Chinese standard population and a mortality rate of 62.34/100,000 and 36.94/100,000 (2), respectively, which is much higher than the average levels in China of 22.06/100,000 and 15.16/100,000 (3). The prevalence of gastric cancer in Wuwei, Gansu, China is almost five times higher than that nationwide (4). Our previous study established a large-scale natural population cohort involving 24,115 individuals from Wuwei, Gansu, China, and has conducted research regarding various aspects to explore the causes of the high incidence of gastric cancer in this region and to provide a theoretical basis for the formulation of control policies (5).

Great efforts, such as the development of more effective biomarkers for diagnosis, prognosis, monitoring and prediction, have been made regarding the clinical management of patients with gastric cancer (6). Gastric cancer has atypical symptoms in the early stage and lacks effective early screening

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markers; therefore, most patients have entered the advanced stage when they are detected (7). A retrospective study revealed that the 5-year rate of relative survival of patients with early gastric cancer (EGC) with treatment is 105.0% compared with the expected survival of individuals from the general population matched for age and sex (8). By contrast, few EGCs are discovered in China and the West, leading to 5-year relative survival rates of 10-40% (9-11). In Japan, the male mortality rate of gastric cancer has fallen by more than half since a mass screening program was introduced in the early 1970s (9). Therefore, early diagnosis and treatment of gastric cancer, and screening are important. Gastroscopy is a valuable tool for reducing the mortality associated with gastric cancer (12). However, due to its acceptability and cost, large-scale screening and early detection with gastroscopy might not be easy. At present, available tumor markers for gastric cancer, including carcinoembryonic antigen and cancer antigen 19-9, are useful for detecting recurrence and distant metastasis or predicting patient survival; however, these are inadequate for gastric cancer screening due to their low sensitivity, particularly for EGC (13). Therefore, there is an urgent requirement for novel non-invasive methods for the screening of patients with gastric cancer, and microRNAs (miRNAs/miRs) have been increasingly studied for this.

miRNAs are endogenous 18-24 nucleotide RNAs, which can serve critical regulatory roles in animals and plants (14). miRNAs can combine with other associated proteins to form an active RNA-induced silencing complex (RISC), and RISC combines with the 5' untranslated region, open reading frames or 3' untranslated region of a target gene mRNA to suppress its translation or to induce its degradation (15,16). An increasing number of studies have reported that miRNAs can be used as biomarkers for gastric cancer diagnosis, as well as targets for disease treatment (17-19). For example, a study of 682 participants examined the expression levels of 578 miRNAs in serum and demonstrated that the combination of 12 miRNAs in serum has excellent diagnostic value for gastric cancer (13). Microarray technology has been widely used to investigate miRNA expression in multiple tumor types, such as gastric cancer and lung cancer (20,21).

The present study, as a part of the aforementioned Wuwei cohort research project, utilized samples collected from patients diagnosed with EGC during the screening of this disease in Wuwei. miRNA profiles in five pairs of EGC tissues and adjacent non-cancerous tissues were explored using a miRNA microarray. Bioinformatics methods were used to analyze the functions and mechanisms of the dysregulated miRNAs, as well as their potential as prognostic factors. The present study aimed to provide a basis for the identification of EGC screening biomarkers and to discuss the particularity of gastric cancer in Gansu province from the perspective of miRNAs.

Materials and methods

Patients and samples. EGC tissues and adjacent non-cancerous mucosa tissues (5 cm from the cancer tissues) were obtained from 5 patients with EGC who were treated with endoscopic submucosal dissection at the Department of Gastroenterology, The Gansu Wuwei Tumor Hospital (Wuwei, China) and

Department of Gastroenterology, The First Hospital of Lanzhou University (Lanzhou, China) between November 2014 and April 2015. The inclusion criteria were: i) Patients who agreed to participate in the study and signed the informed consent were included; ii) all patients were pathologically diagnosed as EGC. The tumor stage was confirmed according to the Eighth Edition of the The American Joint Committee on Cancer (AJCC) TNM staging system guidelines (22); and iii) the patients did not receive radiotherapy or chemotherapy before surgery. The exclusion criteria were: i) Patients who did not agree to participate in the study; ii) patients who had other malignancies; and iii) patients who had other diseases, such as diabetes mellitus and hypertension. The patients included 3 men and 2 women. Clinicopathological features of the patients are listed in Table I. The present study was approved by the Ethics Committee of The First Hospital of Lanzhou University (approval no. LDYYLL2012001; Lanzhou, China). All patients and their family members signed an informed consent form. The tissue samples were stored and transported at -80˚C.

RNA extraction. Total RNA from 10 tissue samples was isolated using TRIzol® (Invitrogen; Thermo Fisher Scientific, Inc.) and purified using a RNeasy mini kit (Qiagen, Inc.) according to the manufacturer's protocols. RNA quality and quantity were measured using a NanoDrop spectrophotometer (ND-1000; NanoDrop Technologies; Thermo Fisher Scientific, Inc.). RNA integrity was determined by formaldehyde agarose gel electrophoresis in 3-(N-morpholino)propanesulfonic acid buffer with a 1.2% gel. Ethidium bromide was used as a fluorescent dye, and the GelDoc Go Gel Imaging System (Bio-Rad Laboratories, Inc.) was used for imaging.

miRNA labeling and array hybridization. miRNA microarray assays were performed by Aksomics, Inc. The miRCURY™ Hy3™/Hy5™ Power labeling kit (Exiqon; Qiagen, Inc.) was used with total RNA samples according to the manufacturer's guidelines for miRNA labeling. After termination of the labeling procedure, the Hy3™-labeled samples were hybridized on the array in miRCURY LNA™ microRNA Array Kit, 7th generation-hsa, mmu and rno (Exiqon; Qiagen, Inc.). Following hybridization, the slides were washed several times using Wash buffer kit (Exiqon; Qiagen, Inc.). The slides were scanned using an Axon GenePix 4000 B microarray scanner (Molecular Devices, LLC).

Microarray data processing. Scanned images were subsequently imported into GenePix Pro 6.0 (Molecular Devices, LLC) for grid alignment and data extraction. Data analyses were performed using R software (v3.6.3; https://cran.r-project.org/src/base/R-3/). The 'limma' package (v3.42.2) (23) in R was used for background correction and normalization between arrays. The robust multi-array average algorithm was selected when performing background correction, and the 'offset' parameter was set to 50. Expression data were normalized using median normalization. The landing lights (probe ID 13138; annotated as Hy3™) were only included for gal-file orientation, and their corresponding data points were removed prior to the normalization of the dataset, according to the manual of the miRCURY LNA™ microRNA Array Kit, 7th

Table I. Clinicopathological features of patients with early gastric cancer whose tissues underwent microRNA microarray testing.

Patient	Sex	Age, years	Location	Lesion location	Histology	T stage	N stage	M stage	AJCC pathologic stage (8th edition)
Patient 1	Female	65	Wuwei, Gansu, China	Cardia	Adenocarcinoma	T1a	0	0	IA
Patient 2	Male	72	Wuwei, Gansu, China	Gastric antrum	Adenocarcinoma	Tis	0	0	0
Patient 3	Male	46	Wuwei, Gansu, China	Gastric body	Adenocarcinoma	T1b	0	0	IA
Patient 4	Male	45	Wuwei, Gansu, China	Cardia	Adenocarcinoma	T1a	0	0	IA
Patient 5	Female	67	Lanzhou, Gansu, China	Gastric antrum	Adenocarcinoma	Tis	0	0	0

AJCC, American Joint Committee on Cancer.

generation-hsa, mmu and rno (Exiqon; Qiagen, Inc.). For the same probes, the expression data of all samples corresponding to each probe were averaged. The probe with the maximum average value was retained among the same probes. The 'limma' package was used to give a microarray linear model fit, compute moderated t-statistics, moderated F-statistic, and log-odds of differential expression by empirical Bayes moderation of the standard errors towards a common value. The cutoff criteria for differentially expressed miRNAs (DEMs) were a |$\log_2$ fold change ($\log_2$FC)| ≥1 and an adjusted P-value (adj. P. val) <0.05. Data were submitted to the Gene Expression Omnibus database (24) (dataset accession no. GSE158315).

Functional annotation of DEMs. The functions of DEMs were annotated using TAM (v2.0), a web-based program for annotations of human miRNAs (25).

mRNA expression profiles of gastric cancer at stage I/II based on data from The Cancer Genome Atlas (TCGA). The mRNA expression profiles of three pairs of gastric cancer tissues and their matched adjacent non-cancerous mucosa tissues were obtained from TCGA [project ID, TCGA-stomach adenocarcinoma (STAD)] using the Genomic Data Commons Data Transfer Tool (v1.5.0; https://gdc.cancer.gov/access-data/gdc-data-transfer-tool). The samples were obtained from 3 Asian patients with stage I/II gastric cancer (AJCC, Eighth Edition; Table II). The 'DESeq2' package (v1.26.0; http://www.bioconductor.org/packages/release/bioc/html/DESeq2.html) (26) in R was used to perform the differential analysis. Differentially expressed genes (DEGs) with |$\log_2$FC| ≥2 and adj. P. val <0.05 in cancer tissues compared with non-cancerous tissues were filtered.

Functional enrichment and pathway analysis of DEGs. Gene Ontology (GO) analysis (27), Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis (28) and Gene Set Enrichment Analysis (GSEA) (29) were performed on the DEGs in the cancer tissues and adjacent non-cancerous tissues of 3 Asian patients with stage I/II gastric cancer. First, the 'org.Hs.eg.db' package (v3.10.0; http://www.bioconductor.org/packages/release/data/annotation/html/org.Hs.eg.db.html) was used to convert the gene symbols into entrezIDs. Subsequently, 'clusterProfiler' (v3.14.3; http://www.bioconductor.org/packages/release/bioc/html/clusterProfiler.

html) (30), 'ggplot2' (v3.3.2) (31) and 'enrichplot' (v1.6.1; http://bioconductor.org/packages/devel/bioc/html/enrichplot.html) packages were utilized to find the enriched GO terms and KEGG pathways, where a false discovery rate <0.05 was considered to be statistically significant. A 'gmt' format file called 'Hallmark gene sets, NCBI (Entrez) gene IDs', which includes the information of the gene sets and their associated pathways, was downloaded from the GSEA website (https://www.gsea-msigdb.org/gsea/index.jsp) and was used to perform GSEA in R software. GO and KEGG analyses of the predicted DEM target genes were performed in the same manner.

Prediction of DEM target genes. The miRDB database (http://mirdb.org/), an online database for miRNA target prediction and functional annotation, was used to predict the target relationships between DEMs and DEGs (32). DEMs and DEGs were controlled to have opposite trends in expression level variations between cancer tissues and non-cancerous tissues. In other words, for the upregulated DEMs, target genes were predicted among the downregulated DEGs and vice versa. Subsequently, a miRNA-gene regulatory network was constructed and visualized using Cytoscape software (v3.7.2) (33).

Construction of the target gene protein-protein interaction (PPI) network and hub genes association network. The target genes were input into the Search Tool for the Retrieval of Interacting Genes/Proteins database (v11.0; https://string-db.org) (34), an online protein interaction search tool for the retrieval of interacting genes/proteins, to construct a PPI network. 'Experiments', 'Databases', 'Co-experiment' and 'Co-occurrence' were set as the parameter of 'active interaction sources', and the interactions with a combined score >0.7 were selected for the PPI network. The PPI network was visualized using Cytoscape software, and the tight link hub mRNAs within the PPI network were calculated using Molecular Complex Detection in Cytoscape (v2.0.0; http://apps.cytoscape.org/apps/mcode) (35) with default parameters.

Survival analysis validation. To verify the results obtained, clinical data and miRNA expression data of 80 Asian patients with gastric cancer were downloaded from TCGA (project ID, TCGA-STAD). For each miRNA, which was in

Table II. Clinicopathological features of patients with gastric cancer at stage I/II whose gene expression profiles were obtained from TCGA.

Patient	Sex	Age, years	Ethnicity	Lesion location	Histology	T stage	N stage	M stage	AJCC pathologic stage (8th Edition)
TCGA-HU-A4GH	Male	75	Asian	Gastric body	Adenocarcinoma	T1b	N0	M0	IA
TCGA-HU-A4GP	Female	62	Asian	Gastric antrum	Adenocarcinoma	T2	N1	M0	IIA
TCGA-HU-A4HB	Male	68	Asian	Gastric antrum	Adenocarcinoma	T2	N2	M0	IIB

AJCC, American Joint Committee on Cancer; TCGA, The Cancer Genome Atlas.

the miRNA-gene network, the 'survminer' package (v0.4.8; https://cran.r-project.org/web/packages/survminer/index.html) was used to find a best separation cutoff value, and then patients were divided into two groups according to the cutoff value. The prognostic values of partial DEMs were identified by Kaplan-Meier analysis with the 'survival' package (v3.2.3) (36) in R software, and P<0.05 (log-rank test) was considered to indicate a statistically significant difference.

Statistical analysis. The statistical analyses were performed using R software. A moderated t-test was performed to screen the DEMs with the 'limma' package, and a paired samples t-test was performed to screen the DEGs with the 'DESeq2' package. The expression levels of miRNAs and genes are presented as $\log_2$ FC. The log-rank test was performed in the survival analysis with the 'survival' package. P<0.05 was considered to indicate a statistically significant difference.

Results

Microarray data processing and identification of DEMs. A flowchart of the research design is shown in Fig. 1. The box and density plots indicated the normalization results of miRNA microarray data for 10 tissue samples (Fig. 2A and B). A total of seven upregulated and 40 downregulated human miRNAs were identified using the criteria of $\log_2$FC ≥1 and adj. P. val <0.05 [seven downregulated miRNAs of human viruses and one downregulated small nuclear RNA (snRNA) were also detected]. The heatmap demonstrated that the samples could be separated into two groups based on the 55 dysregulated RNA molecules (Fig. 2C). The volcano plot revealed the expression distribution of miRNAs (Fig. 2D), and the principal component analysis plot is shown in Fig. 2E.

Functional annotations of DEMs. The functions of the DEMs were annotated using TAM. 'Hormones regulation', 'Human embryonic stem cell (hESC) regulation', 'Immune response', 'Inflammation', 'Cell cycle related', 'Epithelial-mesenchymal transition', 'Cell death', 'Hematopoiesis' and 'MiRNA tumor suppressors' were the most enriched function terms (Table III).

Identification of DEGs using samples from TCGA. To further investigate the functions of DEMs via their target genes, the present study aimed to obtain DEGs between EGC and non-cancerous tissues. Since there was only 1 Asian patient
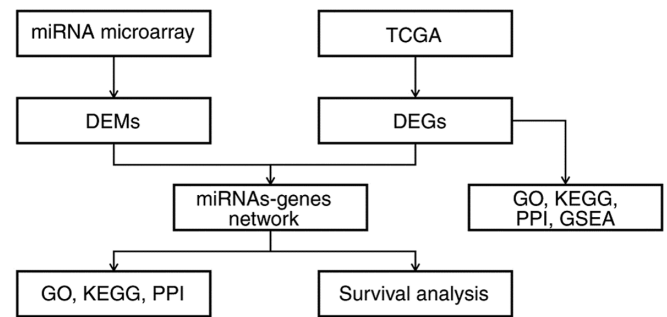

Figure 1. Flowchart of the study design. DEGs, differentially expressed genes; DEMs, differentially expressed miRNAs; GO, Gene Ontology; GSEA, Gene Set Enrichment Analysis; KEGG, Kyoto Encyclopedia of Genes and Genomes; miRNA, microRNA; PPI, protein-protein interaction; TCGA, The Cancer Genome Atlas.

with EGC, the scope was expanded to stage I/II. A total of 3 Asian patients with stage I/II gastric cancer were included in the present study. The clinicopathological features of the selected patients are shown in Table II. A total of 2,097 upregulated genes and 2,131 downregulated genes were identified, and are shown in a heat map and a volcano plot (Fig. 3A and B). Principal component analysis indicated that the gastric cancer tissue samples at stage I/II and their matched non-cancerous tissue samples could be divided into two groups based on the gene profile (Fig. 3C).

GO analysis, KEGG analysis and GSEA of DEGs. To explore the functions and pathways of DEGs in the cancer tissues and non-cancerous tissues of 3 Asian patients with stage I/II gastric cancer, GO analysis, KEGG analysis and GSEA were performed. Functional enrichment results revealed that DEGs were mainly associated with 'Regulation of membrane potential' and 'Muscle system process' in the biological process category. In the cellular component category, the DEGs were mainly enriched in 'Neuronal cell body', 'Collagen-containing extracellular matrix' and 'Cell-cell junction'. 'Channel activity' and 'Passive transmembrane transporter activity' were enriched terms in the molecular function (MF) category (Fig. 4A). In addition, KEGG pathway analysis indicated that the enrichment of DEGs was associated with 'Neuroactive ligand-receptor interaction' and 'Cytokine-cytokine receptor interaction'. 'Cell adhesion molecules (CAMs)' and 'Calcium signaling pathway' were
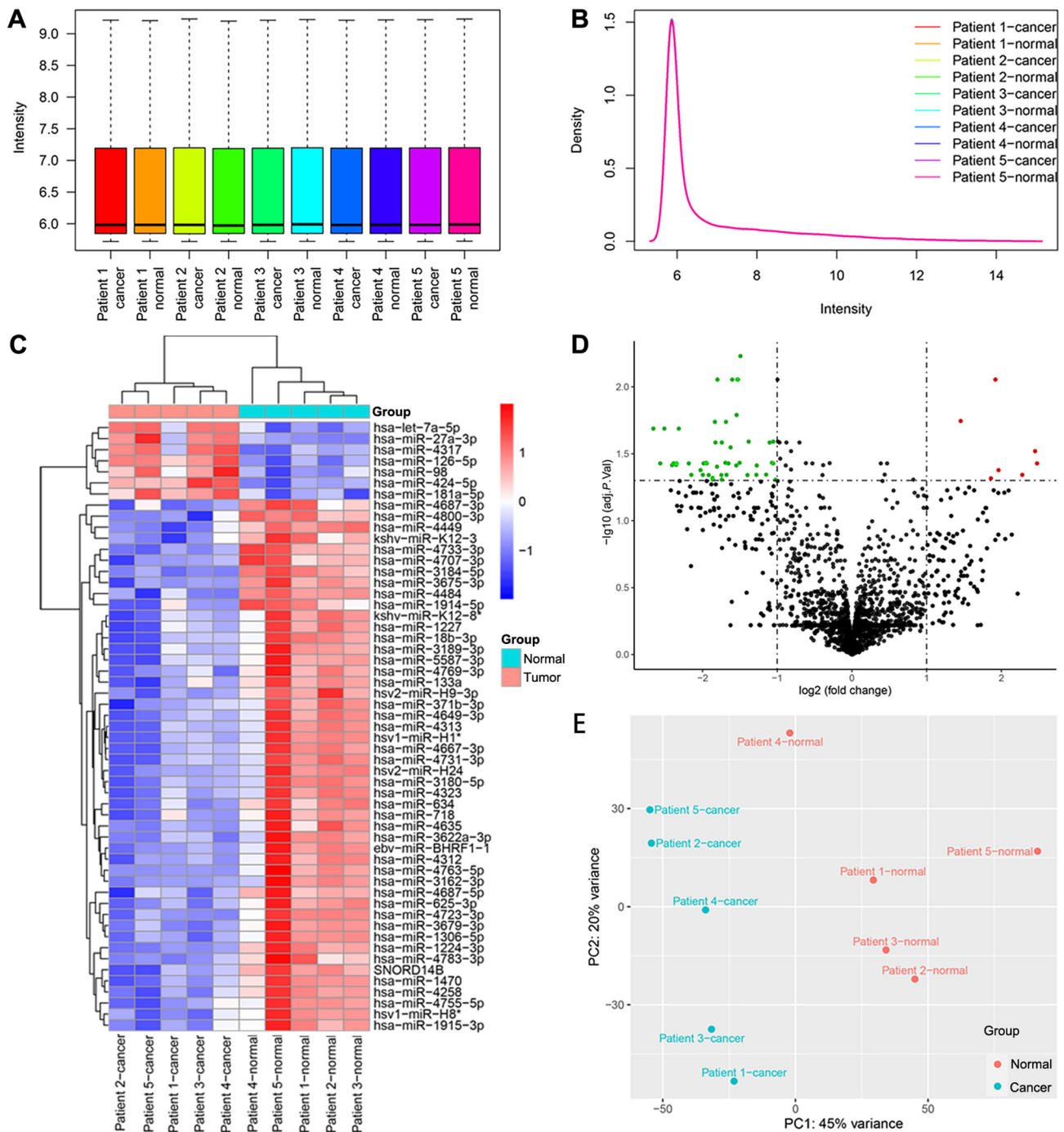
Figure 2. Data analysis of the miRNA microarray using five early gastric cancer tissues and five adjacent non-cancerous tissues, and the identification of DEMs. (A) Box plot after normalization, which shows the maximum, upper quartile, median, lower quartile and minimum of different datasets. (B) Density plot after normalization, which shows a probability distribution at different miRNA expression levels. (C) Heat map generated by unsupervised hierarchical clustering for DEMs between two groups. The color varies from red to blue, indicating upregulated or downregulated expression of DEMs, respectively. (D) Volcano plot of the DEMs in the miRNA microarray. Red and green dots represent upregulated and downregulated molecules (54 miRNAs and one small nuclear RNA), respectively. (E) Principal component analysis result of the tissue samples. adj. P. Val., adjusted P-value; DEMs, differentially expressed miRNAs; miRNA/miR, microRNA; PC, principal component.

also enriched (Fig. 4B). Finally, GSEA results suggested that pathways of the 'cell cycle', 'DNA replication', '*P53* signaling pathway' and 'natural killer cell-mediated cytotoxicity' tended to be activated in the cancer group (Fig. 4C-F).

miRNA-gene network construction and functions of target genes. After the DEMs and DEGs were identified, miRDB was used to predict the possible target relationships between

them. For the upregulated miRNAs, target genes were predicted among the downregulated DEGs and vice versa. Subsequently, a miRNA-gene network was constructed (Fig. 5A).

GO and KEGG analyses of the predicted target genes were performed to learn more about the functions of the DEMs. In the MF category of GO analysis, the terms with the highest gene counts were 'Metal ion transmembrane
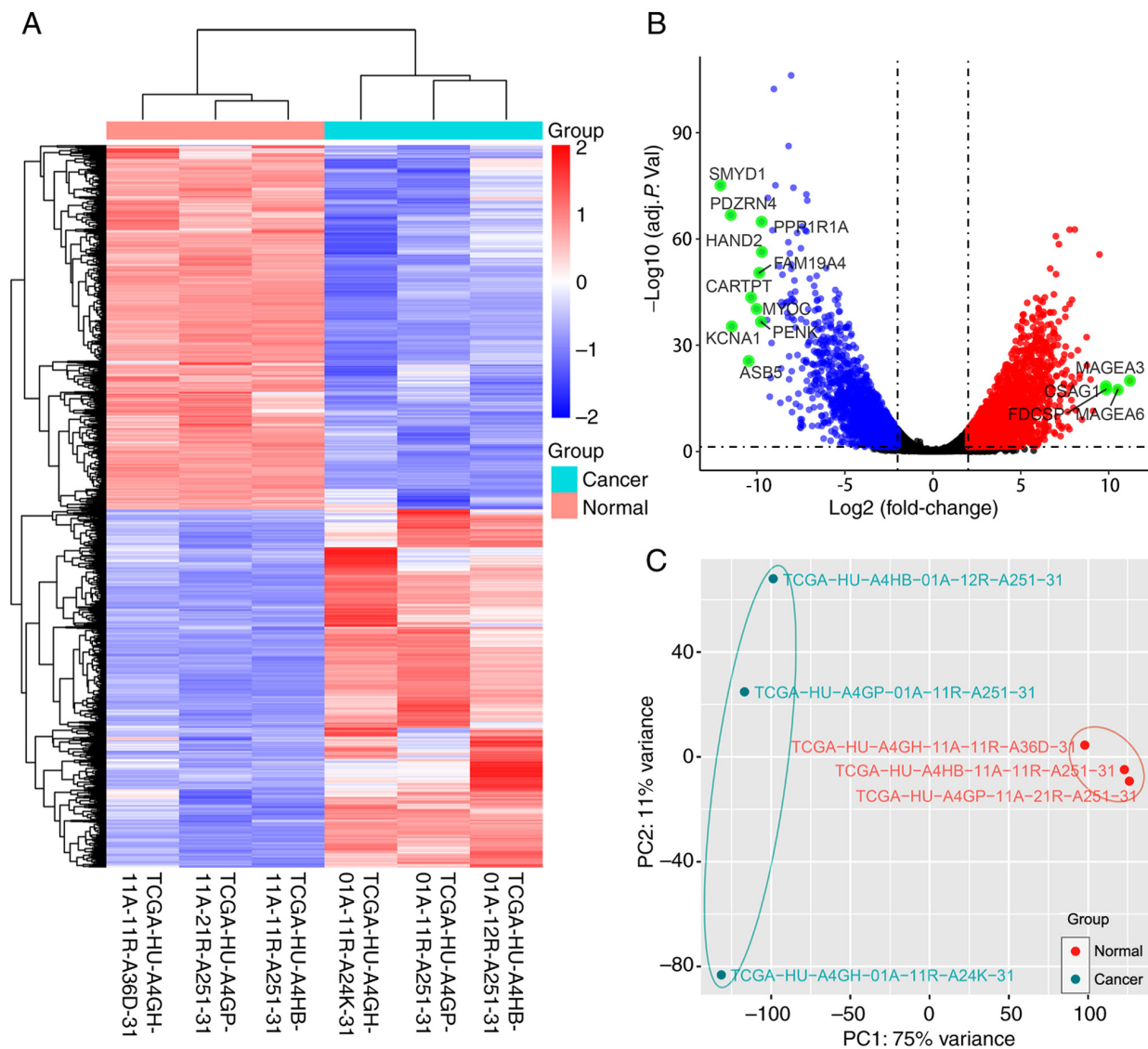
Figure 3. Gene expression profiles of three pairs of gastric cancer tissue samples and the adjacent normal mucosa tissue samples from 3 Asian patients with gastric cancer at stage I/II based on data obtained from TCGA. (A) Heat map generated by unsupervised hierarchical clustering for DEGs between the two groups. The color varies from red to blue, indicating upregulated or downregulated expression of DEGs, respectively. (B) Volcano plot of the DEGs. Red dots represent upregulated genes, and blue dots represent downregulated genes. Green dots represent genes with |log$_2$ (fold change)| ≥9.5 (C) Principal component analysis plot of the six samples. DEGs, differentially expressed genes; adj. P. Val., adjusted P-value; TCGA, The Cancer Genome Atlas; PC, principal component.

transporter activity', 'Actin binding' and 'Ion channel activity' (Fig. 5B). KEGG terms, such as 'Neuroactive ligand-receptor interaction', 'Pathways in cancer' and 'Focal adhesion', were significantly enriched (Fig. 5C).

The results of PPI analysis are shown in Fig. 5D. A total of 13 genes, including ADRB3, ADCYAP1, POMC, CALCB, PTHLH, ADCY5, GNB4, VIP, PTGDR, LHCGR, ADRB2, GPR83 and GIPR were filtered as hub genes in the network, which indicated that the DEMs may mainly work by regulating these hub genes.

Survival analysis validation. Clinical data and RNA expression data of Asian patients with gastric cancer (irrespective of the stage) from TCGA were used for survival analysis. A total of four miRNAs (hsa-let-7a-5p, hsa-miR-27a-3p, hsa-miR-126-5p and hsa-miR-424-5p) were identified to be significantly associated with the prognosis of patients with gastric cancer (Fig. 6).

Discussion

A total of seven upregulated and 40 downregulated human miRNAs were identified in EGC tissues compared with adjacent non-cancerous tissues. The functions of the DEMs were annotated using the TAM webtool. 'Hormones regulation' was the most enriched function term, which indicated that hormones may be regulated in EGC by miRNAs. A population-based matched cohort study in Sweden suggested that menopausal hormone therapy users are at a decreased risk of gastric adenocarcinoma (37). In addition, gonadotropin-releasing hormone (38), corticotropin-releasing hormone (39), steroid hormones (40) and growth hormone (41) have been reported to be associated with gastric cancer. However, more evidence is required to verify the role of hormone regulation in EGC.

Expression profiles of three pairs of cancer tissues and adjacent non-cancerous tissues from patients with

Table III. Functional annotation of differentially expressed miRNAs using the tool for annotations of human miRNAs.

Function term	Count	Percent	Fold	P-value
Hormones regulation	9	0.14516129	3.27217742	0.00062950
Human embryonic stem cell (hESC) regulation	8	0.09411765	2.12156863	0.02291040
Immune response	8	0.16666667	3.75694444	0.00052898
Inflammation	8	0.19512195	4.39837398	0.00016338
Cell cycle related	7	0.10606061	2.39078283	0.01856736
Epithelial-mesenchymal transition	5	0.12195122	2.74898374	0.02820963
Cell death	5	0.09090909	2.04924242	0.08525479
Hematopoiesis	5	0.16129032	3.63575269	0.00867962
MiRNA tumor suppressors	5	0.13513514	3.04617117	0.01854272
Lipid metabolism	4	0.20000000	4.50833333	0.00898287
Angiogenesis	3	0.12500000	2.81770833	0.08400821
Cell proliferation	3	0.10714286	2.41517857	0.12100311
HIV latency	3	0.14285714	3.22023810	0.06027038
Adipocyte differentiation	3	0.11111111	2.50462963	0.11122976
Onco-miRNAs	3	0.09677419	2.18145161	0.15217690
Apoptosis	2	0.04545455	1.02462121	0.59774380
Bone regeneration	2	0.05882353	1.32598039	0.45394525
Folliculogenesis	2	0.28571429	6.44047619	0.03458783
Anti-cell proliferation (Hwang etal BJC2006)	2	0.18181818	4.09848485	0.08125429
Cell division	2	0.11764706	2.65196078	0.17103576
Immune system (Xiao's Cell2009)	2	0.11111111	2.50462963	0.18736736
Cell differentiation	1	0.05882353	1.32598039	0.54311212
Chemosensitivity of tumor cells	1	0.25000000	5.63541667	0.16641762
DNA repair	1	0.10000000	2.25416667	0.36724517
Granulopoiesis	1	0.10000000	2.25416667	0.36724517
Carbohydrate metabolism	1	0.14285714	3.22023810	0.27345270
Cell fate determination	1	0.03846154	0.86698718	0.70138621
Heart development	1	0.14285714	3.22023810	0.27345270

miRNA, microRNA.

stage I/II gastric cancer were downloaded. Gastric cancer exhibits biological and epidemiological differences between Asian and non-Asian populations (42,43). To improve the understanding of the roles of DEMs, the present study aimed to identify target mRNAs among DEGs in Asian patients. Since only 1 Asian patient with EGC has been included in the TCGA database to date, the scope was expanded to patients at stage I/II (AJCC, Eighth Edition). Notably, 'Neuroactive ligand-receptor interaction' and 'Calcium signaling pathway' were enriched KEGG terms for DEGs. 'Neuroactive ligand-receptor interaction', 'Axon guidance' and 'Neurotrophin signaling pathway' were enriched KEGG terms for the target genes of DEMs. Furthermore, 'muscle contraction' was an enriched biological process term in GO analysis of DEGs. These results suggest that pathways associated with nerves, muscle contraction and calcium signaling may serve a role in gastric cancer. There is much evidence regarding the association between the calcium signaling pathway and gastric cancer (44,45). Regarding nerve and muscle contraction, certain proteins involved in

neurodegenerative events are considered to be associated with gastric cancer, according to previous reports (46-48). Based on the target gene results, 'pathways in cancer' and other pathways associated with cancer, such as 'Focal adhesion' (49) and 'Melanoma', were also affected. Since the results obtained by this method are closely associated with the gene set, the two methods of detecting functional enrichment complement each other.

The miRNA-gene network was constructed. However, >90% of the target genes were identified to be potentially modulated by hsa-let-7a-5p, hsa-miR-27a-3p, hsa-miR-126-5p, hsa-miR-424-5p, hsa-miR-181a-5p and hsa-miR-1915-3p in the network. Survival analysis of Asian patients demonstrated that four of the miRNAs (hsa-let-7a-5p, hsa-miR-27a-3p, hsa-miR-126-5p and hsa-miR-424-5p) were significantly associated with the prognosis of patients with gastric cancer. Multiple studies have demonstrated that hsa-miR-27a-3p (50-53) and hsa-miR-424-5p (54-56) are upregulated in gastric cancer and act as tumor activators. The present results demonstrated that hsa-miR-27a-3p and
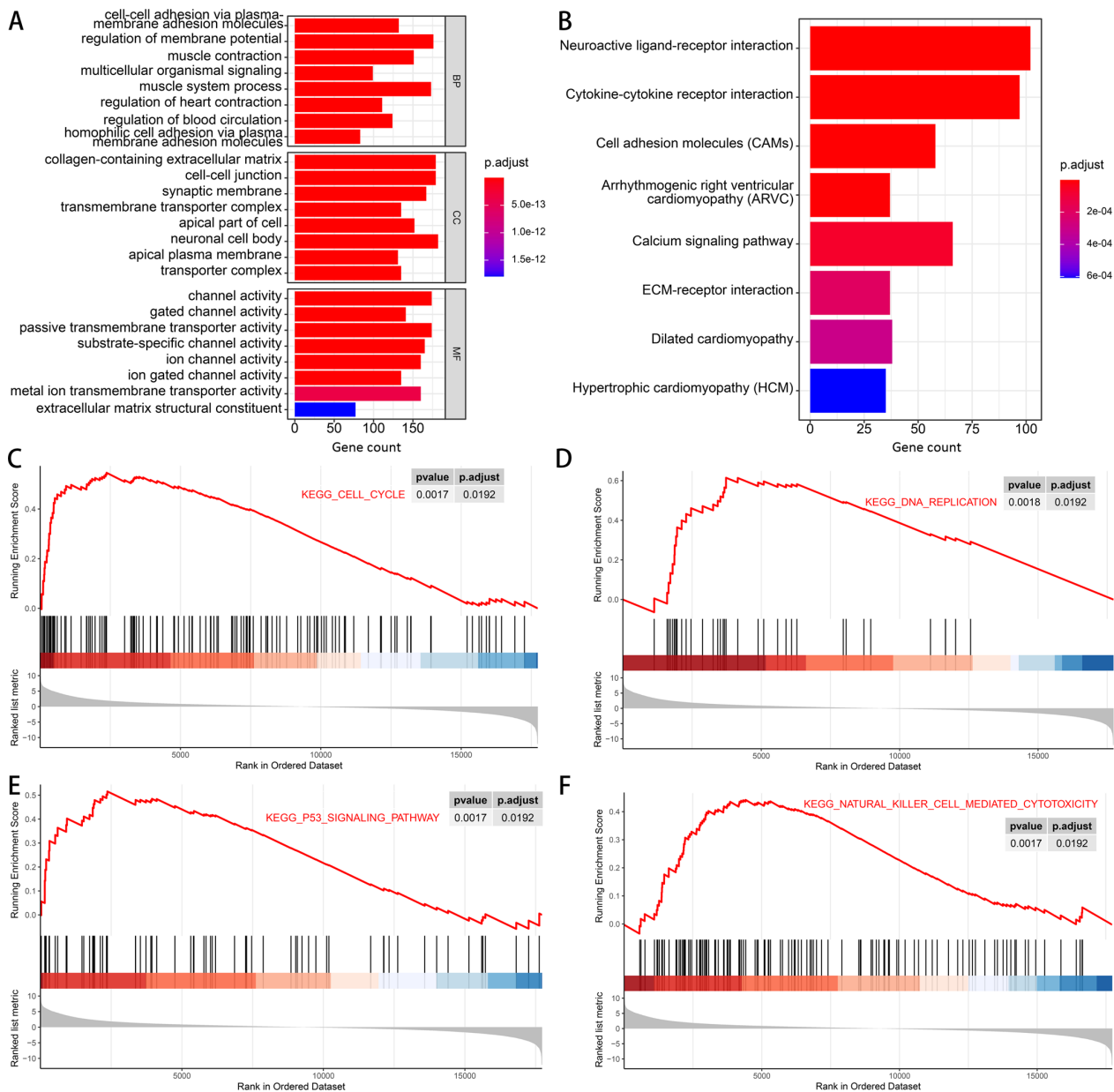
Figure 4. GO analysis, KEGG analysis and GSEA of the differentially expressed genes between the cancer tissues and non-cancerous tissues of 3 Asian patients with stage I/II gastric cancer. (A) Results of GO analysis, including BP, CC and MF aspects. (B) Results of KEGG analysis. (C-F) Results of GSEA revealing that the KEGG pathways of (C) 'cell cycle', (D) 'DNA replication', (E) 'P53 signaling pathway' and (F) 'natural killer cell-mediated cytotoxicity' were activated in the gastric cancer tissues at stage I/II. BP, biological process; CC, cellular component; GO, Gene Ontology; GSEA, Gene Set Enrichment Analysis; KEGG, Kyoto Encyclopedia of Genes and Genomes; MF, molecular function; p.adjust, adjusted P-value.

has-miR-424-5p could be upregulated in EGC and may affect the prognosis of patients with gastric cancer. Liang *et al* (57) reported that the hsa-let-7 family inhibits tumor invasion and metastasis by targeting myosin heavy chain 9 in human gastric cancer, which is different from the results of the present study. Since the sample size included in the present study was small, the results should be verified with larger sample sizes. To the best of our knowledge, there have been no studies on hsa-miR-126-5p in gastric cancer. Although a study using expression profile analysis of miRNAs in prostate cancer by next-generation sequencing demonstrated that hsa-miR-126-5p is highly expressed in tumor tissues (58), other studies came to different conclusions and have suggested that it may be a tumor suppressor (59,60). The functions of

hsa-miR-126-5p in gastric cancer and EGC need to be verified. Experiments have demonstrated that hsa-miR-181a-5p can promote the progression of gastric cancer via Ras association domain family member 6-mediated mitogen activated kinase-like protein signaling activation (61) or by regulating protein tyrosine phosphatase non-receptor type 9 (62). Furthermore, hsa-miR-1915-3p inhibits Bcl-2 expression in the development of gastric cancer (63). hsa-miR-1915-3p serves a role in breast cancer inhibition (64) and increases drug sensitivity in colorectal cancer (65). The present results support the role of hsa-miR-181a-5p as a tumor activator and the role of hsa-miR-1915-3p as a tumor suppressor. Overall, the studies of miRNAs in EGC are still limited, and the present study provided some evidence for this. There are also
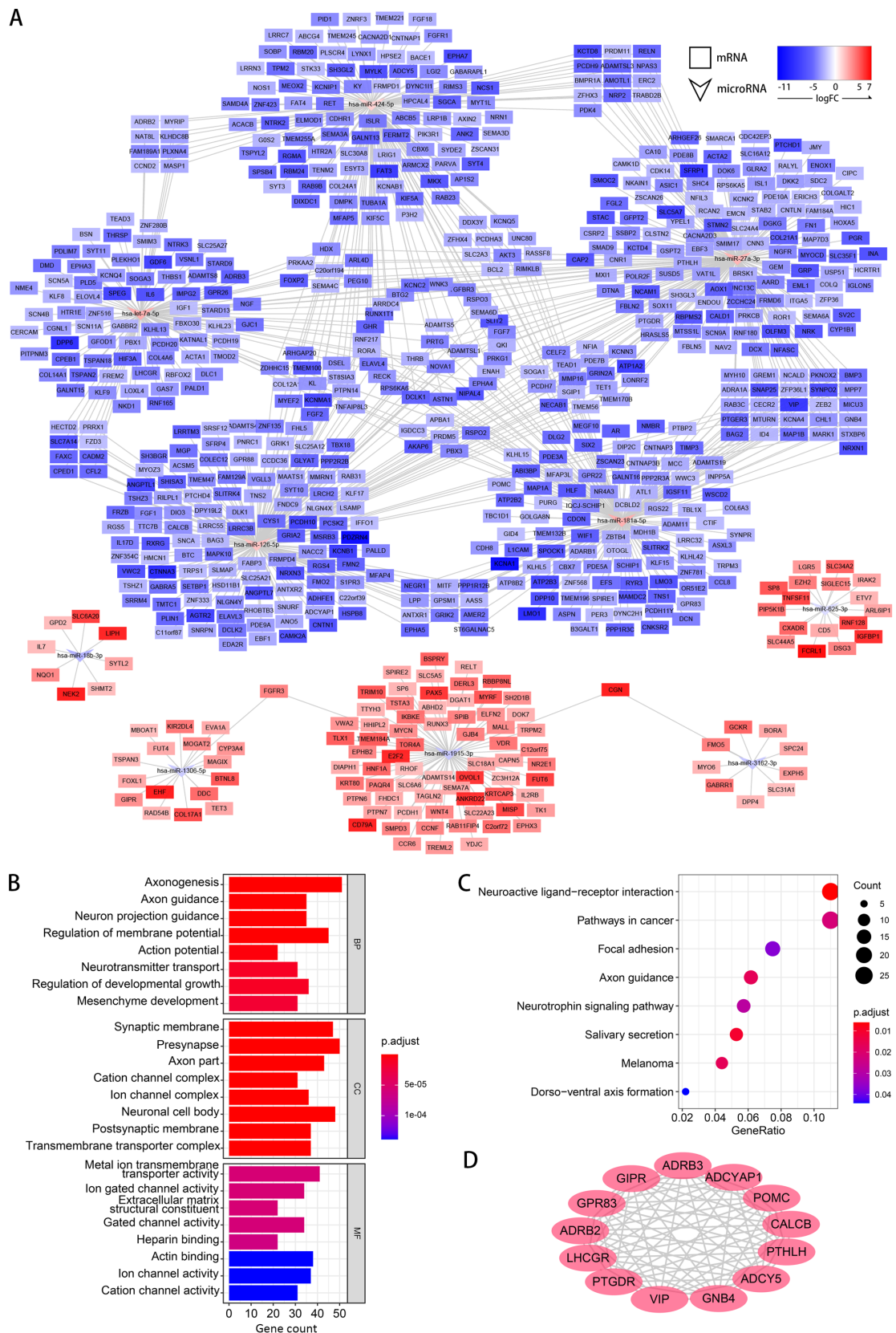
Figure 5. Construction of the miRNA-gene network and functions of the target genes. (A) Predicted miRNA-gene network in gastric cancer tissues at stage I/II. (B) Gene Ontology analysis and (C) Kyoto Encyclopedia of Genes and Genomes analysis of the predicted target genes of miRNAs. (D) Hub genes that interact closely with each other, which were filtered from the protein-protein interaction results. BP, biological process; CC, cellular component; FC, fold change; MF, molecular function; miRNA, microRNA; p.adjust, adjusted P-value.

differentially expressed viral miRNAs and snRNAs. Further research is required to clarify their functions.

The present study intended to perform preliminary screening of miRNAs and the results need to be verified in
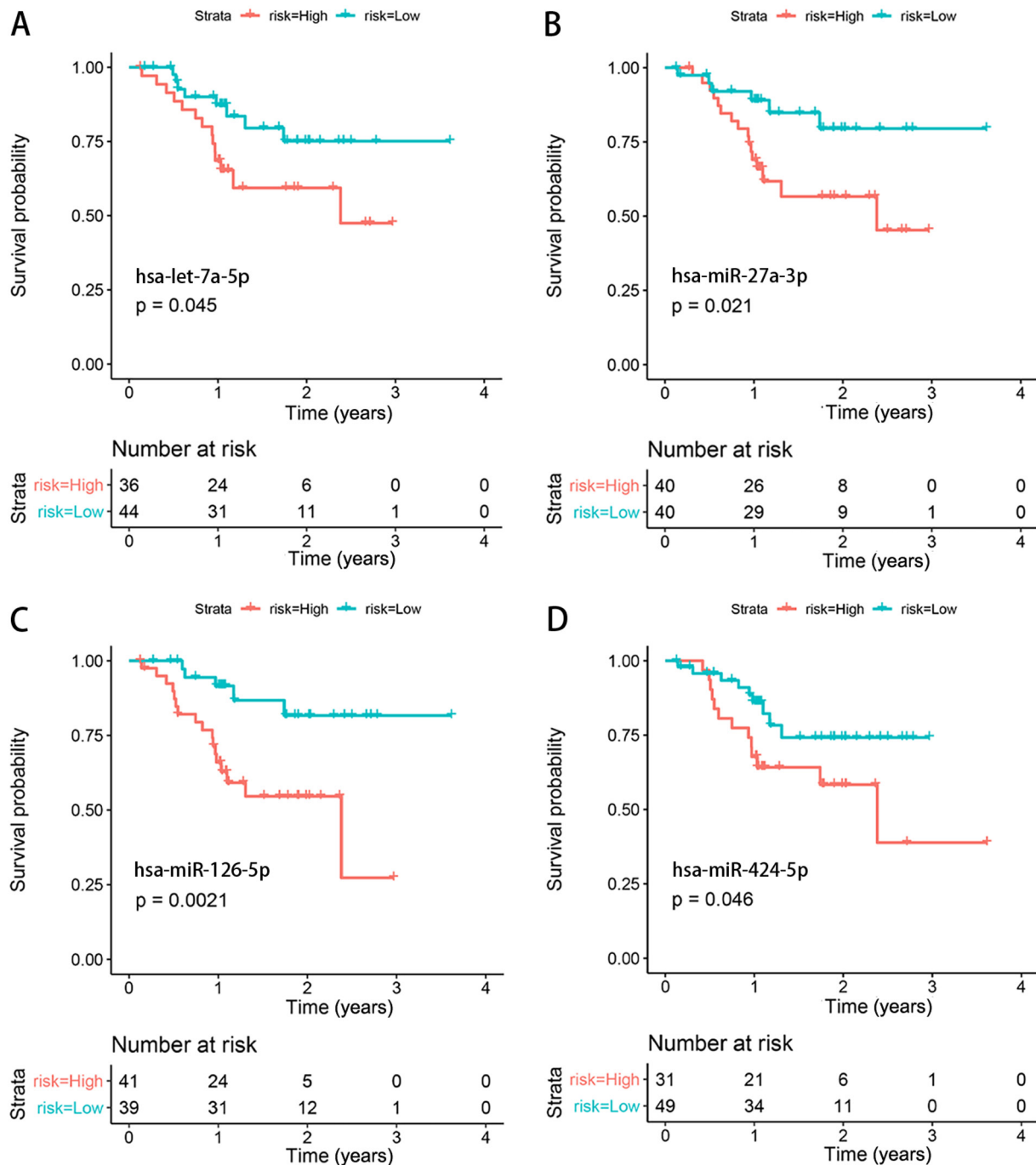
Figure 6. Survival analysis of the differentially expressed miRNAs. Prognostic value of (A) hsa-let-7a-5p, (B) hsa-miR-27a-3p, (C) hsa-miR-126-5p and (D) hsa-miR-424-5p in Asian patients with gastric cancer based on data from The Cancer Genome Atlas database. The x-axis indicates survival time in years, and the y-axis indicates survival probability. P-values, which are shown in each figure, refer to the comparison between the high and low miRNA expression groups, which were divided according to the best separation cutoff value. miR/miRNA, microRNA.

a larger cohort. Additionally, the present study was based on individuals in Gansu province and Asia, which is helpful to explore the particularity of gastric cancer in these regions. However, the population is also a limitation of the present study when the results need to be extended to other regions or other high incidence areas of gastric cancer. Comparing the results with other population cohorts should be a direction of future research.

In conclusion, in the present study, the miRNA profiles in five pairs of EGC tissues and adjacent non-cancerous tissues were investigated using a miRNA microarray. The possible mechanisms and abilities as prognostic factors of DEMs were assessed using bioinformatics methods. The present study revealed that certain miRNAs, including hsa-let-7a-5p, hsa-miR-27a-3p, hsa-miR-126-5p and hsa-miR-424-5p, were upregulated in EGC, and were associated with the prognosis of gastric cancer based on analysis of the miRNA expression profiles of patients from Gansu province, China. The present study could provide a basis for the identification of EGC screening biomarkers and for exploring the cause of the high incidence of gastric cancer in Gansu province, China from the perspective of miRNAs.

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Availability of data and materials

The datasets generated and analyzed during the current study are available in the Gene Expression Omnibus repository (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?&acc=GSE158315).

Authors' contributions

YL, WS and YW conceived the study, and YZ and LL designed the study. YZ, HW and HY were involved in the sample and data collection. YL, LL and TH performed the RNA extraction experiments and the quality control. YL, LL and HW performed the bioinformatics analysis and prepared the figures. YZ, HZ, HY and XC performed the statistical analysis. YL, YZ and TH drafted the manuscript. HZ, XC and WS contributed substantially to the revision of the manuscript. The authenticity of all the raw data has been assessed by YL, WS and YW. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The study protocol was approved by the Ethics Committee of The First Hospital of Lanzhou University (approval no. LDYYLL2012001; Lanzhou, China). Written informed consent forms were signed by the patients and their family members.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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