Downregulated miR-383-5p contributes to the proliferation and migration of gastric cancer cells and is associated with poor prognosis

Chao Wei [1], **Jian-Jun Gao** [Corresp. 2]

[1] Department of General Surgery, the No.967 Hospital of PLA Joint Logistics Support Force, Postgraduate Culture base of Jinzhou Medical University, Dalian, China

[2] Department of General Surgery, the No.967 Hospital of PLA Joint Logistics Support Force, Dalian, China

Corresponding Author: Jian-Jun Gao
Email address: gjj1332006@126.com

AIM: The study aims to identify differentially expressed microRNAs (DEMs) in gastric cancer (GC) and explore the expression, prognosis and downstream regulation role of novel miR-383-5p in GC.

METHODS: The GC miRNA-Seq and clinical information were downloaded from Firebrowse which stores integrated data sourced from The Cancer Genome Atlas (TCGA) database. The DEMs was identified with limma package in R software at the cut-off criteria of $P<0.05$ and fold change (FC) >2.0. The expression of miR-383-5p in GC cell lines and 54 paired GC tissues was measured by quantitative real-time polymerase chain reaction (qRT-PCR). The overall survival curve of miR-383-5p and the association between its expression and clinicopathological features were explored. Wound healing and cell counting kit-8 (CCK-8) assays were performed to investigate the capacity of miR-383-5p in cell proliferation and migration. The downstream target genes were predicted by bioinformatics tools (miRDB, TargetScan and starBase). The consensus target genes were selected for gene functional enrichment analysis by FunRich v3.0 software.

RESULTS: 21 down-regulated miRNAs (including miR-383-5p) and 202 up-regulated miRNAs were identified by analyzing GC miRNA-Seq data. Survival analysis found that patients with low miR-383-5p expression had a shorter survival time (median survival time 21.1 months) than those with high expression (46.9 months). The results of qRT-PCR indicated that miR-383-5p is downregulated in GC cell lines and tissues, which is consistent with miRNA-Seq data. The expression of miR-383-5p was significantly associated with tumour size and differentiation grade. Besides, overexpression of miR-383-5p suppressed GC cells proliferation and migration. A total of 49 common target genes of miR-383-5p were obtained by bioinformatics and gene functional enrichment analysis showed that these genes participated in PI3K, mTOR, c-MYC, TGF-beta receptor, VEGF/VEGFR and E-cadherin signaling pathways.

CONCLUSION: The present study concludes that novel miR-383-5p was downregulated and may act as a tumor suppressor in GC. Furthermore, its target genes were involved in important signaling pathway. It could be a prognostic biomarker and play a vital role in exploring the molecular mechanism of GC.

KEYWORDS: Gastric cancer; Differentially expressed miRNAs; miR-383-5p; Prognosis; miRNA-Seq.

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[1] Department of General Surgery, the No.967 Hospital of PLA Joint Logistics Support Force, Postgraduate Culture base of Jinzhou Medical University, Dalian 116011, China;
[2] Department of General Surgery, the No.967 Hospital of PLA Joint Logistics Support Force, Dalian 116011, China.

Corresponding Author:
Jian-Jun Gao
Department of General Surgery, the No.967 Hospital of PLA Joint Logistics Support Force, Dalian 116011, China
Email address: gjj1332006@126.com

Abstract

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Introduction

Gastric cancer (GC) is a common malignancy of human digestive system which has a high incidence and mortality in the worldwide(Bray et al. 2018). In China, both the incidence and mortality of GC rank second among malignant neoplasms(Chen et al. 2016). The number of Chinese patients with GC increases by years, which seriously threatens the health of people. The majority of Chinese GC patients are diagnosed at advanced stage and have an unsatisfactory 5-year overall survival rate(Correa 2013). Most studies have found that the development of GC is associated with multi-factor, such as irregular diet, genetic and epigenetic influence(Carcas 2014).

MicroRNAs (miRNAs, 20-24 nucleotides in length) are a series of non-coding RNAs and play important roles in the regulation of gene expression at post-transcriptional level(Zhang et al. 2013). Mechanistically, miRNAs negatively regulate gene expression through binding to sites in the 3'-untranslated regions of messenger RNAs(Zen & Zhang 2012). Over the past years, mounting studies have confirmed that miRNAs can act as oncogenes or anti-oncogenes in the initiation and development of GC by regulating the downstream target genes(Chen et al. 2018; Hui et al. 2018; Wang et al. 2019b). Thus, exploring the expression and regulation role of miRNAs may be in favor of uncovering the tumorigenesis mechanism of GC.

In present study, we first identified that miR-383-5p was down-regulated in GC tissues by analyzing GC miRNA-Seq data. In order to confirm this finding, qRT-PCR experiment was performed to measure the expression of miR-383-5p in GC cell lines and tissues. Kaplan–Meier survival analysis also found that patients in high miR-383-5p expression group have longer overall survival time than those in low miR-383-5p expression group. Overexpression of miR-383-5p suppressed GC cells proliferation and migration. All the results showed that miR-383-5p was downregulated and it may play an anti-oncogene role in GC. The potential target genes of miR-383-5p were predicted through online bioinformatics tools. The functional enrichment analysis of target genes indicated that miR-383-5p may take part in PI3K, mTOR, c-MYC, TGF-beta receptor, VEGF/VEGFR and E-cadherin signaling pathways. Above all, miR-383-5p can be a meaningful target in understanding the potential molecular mechanism of GC tumorigenesis and progression.

Materials & Methods

MiRNA-Seq data and clinical information

The GC cliniacl information and miRNA-Seq data, which contain 389 cancer tissue samples and 41 gastric normal tissue samples, were downloaded from Firebrowse website (TCGA data version 2016_01_28). At the cut-off criterion of fold change (FC) > 2.0 and $P < 0.05$, the DEMs were identified using limma package in R software. The follow-up days and vital status of patients were extracted from clinical information data. Patients meeting the following criteria were included for overall survival: (1) patients have integrated follow up days and vital status; (2) patients have both follow-up days and expression value of miR-383-5p. Totally, 382 patients were respectively divided into low and high expression group according to the median value of miR-383-5p expression. The overall survival curve of low and high miR-383-5p expression groups was analyzed with the method of Kaplan−Meier and log-rank test.

GC cell lines culture

Human GC cell lines (SGC-7901, BGC-823, SGC-803, and MKN-45) and a normal gastric mucous membrane cell line (GES-1) were purchased from the Institute of Biochemistry and Cell Biology of the Chinese Academy of Sciences (Shanghai, China), and all cell lines were cultivated in RPMI 1640 medium (GIBCO-BRL) supplemented with 10% fetal bovine serum (FBS; Gibco, Grand Island, NY, USA), 100 U/mL penicillin and 100 mg/mL streptomycin. All cells were cultured in a humidified air at 37 °C and 5% CO2.

Patient tissues collection

All the GC tissues and the corresponding adjacent normal tissues were collected from 54 patients who received surgical resection at the No.967 Hospital of PLA Joint Logistics Support Force and the Northern Theater Command General Hospital. Tissues were histologically confrmed and immediately stored at -80˚C after resection. The clinicopathological features of 54 GC patients were recorded and preserved. All patients signed the informed consent and this study was appvoed by the Research Ethics Committee of Jinzhou Medical University.

RNA extraction and qRT-PCR assays

The TRIzol reagent (Invitrogen, CA, USA) was utilized for extracting total RNA of tissues and cells. Reverse Transcription Kit (GenePharma, China) was used for obtaining cDNA reverse transcribed from RNA. qRT-PCR assay was performed with SYBR-Green Hairpin-itTM MicroRNAs Kit (GenePharma, China), which was conducted on ABI 7500 FAST Real-Time PCR System. The expression level was determined using $2^{-\Delta\Delta Ct}$ method and normalized to U6. All the sequences of primers used in present study were summaried in **Table 1**.

RNA oligonucleotide and cell transfection

The miR-383-5p mimics and mimics NC were designed and synthesized by GenePharma Co., Ltd (Shanghai, China). According to the manufacturer's protocols, the GC cells were transfected using Lipofectamine™ 3000 reagent (Invitrogen; Thermo Fisher Scientific.). All the cells were cultivated for 48h after transfection.

Cell proliferation assay

After transfection, the MGC-803 or MKN-45 cells were seeded into a 96-well plate with 4×10^3 cells per well in triplicate. At 0, 24, 48, and 72 h, each well was added with 10 μL of CCK-

8 reagent (Dojindo, Japan) and then incubated at 37°C for 3 h. The absorbance at 450 nm was measured using a spectrophotometer.

Wound healing assay

The MGC-803 or MKN-45 cells were seeded into 6-well plate. When the cells were cultured to a density of 90%, a 100 μL pipette tip was used to draw a straight wound. Then, the cells were cultured with serum-free medium in the humidified incubator. At 0 and 48h, an inverted microscope was utilized to visualize the wound healing and photograph.

Target genes prediction of miR-383-5p and functional enrichment analysis

The bioinformatics websites of TargetScan(Agarwal et al. 2015) (http://www.targetscan.org/vert_71/), miRDB (Wang 2016) (http://www.mirdb.org/) and starBase(Li et al. 2014) (http://starbase.sysu.edu.cn/) were applied for predicting potential target genes of miR-383-5p. The consensus results of three tools were selected for further analysis. Genes functional enrichment analysis was performed by FunRich v3.0 software which is a widely used tool for the gene functional enrichment and interaction network analysis(Pathan et al. 2015). All the procedures were conducted according to official protocols and default parameters.

Statistical analysis

MiRNA-Seq data was processed by the limma(Ritchie et al. 2015) package in R software. The survival curve was described by Kaplan-Meier survival plot and analysed with log-rank test. The differences between groups were analyzed by paired or unpaired Student's t-test and chi-square test. $P<0.05$ was recognized as statistically significant and all statistical analysis were conducted by IBM SPSS software 19.0.

Results

Identification of DEMs in GC

At the cut-off criterion of fold change (FC) > 2.0 and $P < 0.05$, 223 DEMs were identified by screening GC miRNA-Seq data. A volcano plot was drawn to visualize the 21 down-regulated and 202 up-regulated miRNAs (**Figure 1**). The top 20 of down- and up-regulated miRNAs ranked by FC was listed in **Table 2**. After literature retrieval, we first found that miR-383-5p was down-regulated in GC.

MiR-383-5p is confirmed to be downregulated in GC

The expression value of miR-383-5p was extracted from miRNA-Seq data. After excluding unavailable value, there were 235 GC samples and 35 normal samples. The expression of miR-383-5p in GC was significantly lower than normal tissues (**Figure 2A**). The results of qRT-PCR showed that miR-383-5p was significantly down-regulated in GC at the level of cell and tissue (**Figure 2B and 2C**). Combining the miRNA-Seq data and qRT-PCR assay, we confirmed that miR-383-5p was down-regulated and may be a novel tumor suppressor gene in GC.

Association between miR-383-5p and prognosis, clinicopathological features

A total of 382 GC TCGA samples with necessary data were selected to investigate the prognostic role of miR-383-5p. According to the median expression value of miR-383-5p, patients were equally distributed to the low and high expression groups. The Kaplan-Meier

survival analysis indicated that patients with low miR-383-5p expression had a shorter survival time (median survival time 21.1 months) than those with high expression (46.9 months) (**Figure 3**). Furthermore, we explored the association between miR-383-5p expression and clinicopathological features. The results manifested that low miR-383-5p expression was significantly associated with large tumor size and poor differentiation grade (**Table 3**). Nevertheless, the features of age, gender, lymph node metastasis and TNM stage were found to be of no significant difference.

Overexpression of miR-383-5p inhibits GC cell proliferation and migration

The CCK-8 and wound healing assays were performed to assess the effect of miR-383-5p on the proliferation and migration of GC cells. Compared with NC group, transfection with miR-383-5p mimics weakened the migration capacity of MGC-803 and MKN-45 cells (**Figure 4A**). Besides, the CCK-8 assay showed that miR-383-5p mimics inhibited GC cells proliferation (**Figure 4B**).

Target prediction and genes functional enrichment analyses

Three bioinformatics websites (TargetScan, miRDB, and starBase) were selected for predicting target genes of miR-383-5p. In view of that each website has diverse bioinformatics algorithm, we took the consensus results of different predictions. As described in the venn plot, 49 consensus target genes were obtained **(Figure 5**). To comprehend the function of miR-383-5p target genes, the 49 genes were used for functional enrichment analysis by FunRich. At the aspect of biological pathway analysis, we found that these genes participate in PI3K, mTOR, c-MYC, TGF-beat receptor, VEGF/VEGFR and E-cadherin signaling pathways (**Figure 6**).

Discussion

GC has the malignant features of terrible proliferation, invasion, metastasis and multiple drug resistance, which lead to high mortality and poor prognosis. Increasing studies have proved that miRNAs are aberrantly expressed and involved in the initiation and development of GC(Chen et al. 2019; Kang et al. 2018; Maruyama et al. 2018; Wang et al. 2019a). Thus, identifying DEMs and exploring the biological function of miRNAs can be useful for finding novel biomarkers and understanding the mechanism of GC progression.

In this study, we first downloaded and analyzed the GC miRNA-Seq and clinical data from Firebrose website(Deng et al. 2017), which conserves integrated gene expression profiles and clinical information data from TCGA. Through screening for DEMs, we found that miR-383-5p was down-regulated in GC tissues. Besides, we also investigated the prognostic role of miR-383-5p and Kaplan-Meier survival analysis indicated that patients with low miR-383-5p expression had a shorter survival time than those with high expression. All these results inspired us that miR-383-5p may play an important role in GC.

Furthermore, we retrieved literatures published worldwide to comprehend the studies about miR-383-5p. Zhao et al. found that miR-383-5p was significantly decreased in lung adenocarcinoma and overexpression of miR-383-5p inhibited cell proliferation by G1 cell cycle phase arrest and induced apoptosis in vitro(Zhao et al. 2017). In hepatocellular carcinoma, miR-383-5p was proved to be a tumor suppressor and to modulate hepatocellular carcinoma

tumorigenesis and progress by targeting AKR1B10 (Wang et al. 2018) and LDHA(Fang et al. 2017). Besides, Jiang's study reported that overexpression of miR-383-5p could inhibit ovarian cancer cell proliferation and enhance chemosensitivity of cells by regulating TRIM27 (Jiang et al. 2019). MiR-383-5p could also suppress ovarian cancer cell proliferation, invasion and aerobic glycolysis through regulating LDHA(Han et al. 2017). However, the expression and clinical significance of miR-383-5p in GC have not been studied. The present study investigated that miR-383-5p was decreased in GC and its expression was associated with tumor size and differentiation grade. Furthermore, the CCK-8 and wound healing assays demonstrated that overexpression of miR-383-5p could inhibit GC cells proliferation and migration.

We further explore the downstream regulation role by predicting the potential target genes of miR-383-5p. The consensus target genes were obtained by integrating the results from three bioinformatics tools, which improved the accuracy of prediction. The functional enrichment analysis demonstrated that miR-383-5p may be involved in PI3K, mTOR, c-MYC, TGF-beta receptor, VEGF/VEGFR and E-cadherin signaling pathways through regulating the target genes. It is well known that mTOR pathway regulates tumor growth and metastasis by mediating tumor metabolic homeostasis(Xia & Xu 2015). Multiple miRNAs were reported to participate in the regulation of PI3K/AKT/mTOR signaling pathway(Riquelme et al. 2016). Yu' study showed that miR-106b is overexpressed in CD44(+) GC stem-like cells and retains cancer stem cell characteristics through modulating TGF-β/Smad signaling pathway(Yu et al. 2014). MiR-372 negatively targets KIF26B to suppresses GC cells proliferation and metastasis by regulating VEGF pathway(Zhang et al. 2017). Above all, miR-383-5p may act as a novel tumor suppressor in taking part in the biological function of GC.

Conclusions

In summary, we found that a novel miR-383-5p may act as a tumor suppressor in GC. It is of important clinical significance and prognostic value, which could contribute to revealing the molecular mechanism of GC tumorigenesis and progress.

Acknowledgements

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Figure 1

The volcano plot

The red spots represent 202 up-regulated miRNAs and the green spots represent 21 down-regulated miRNAs (blue imaginary lines represent Fold change: ±2 and P-value: 0.05).

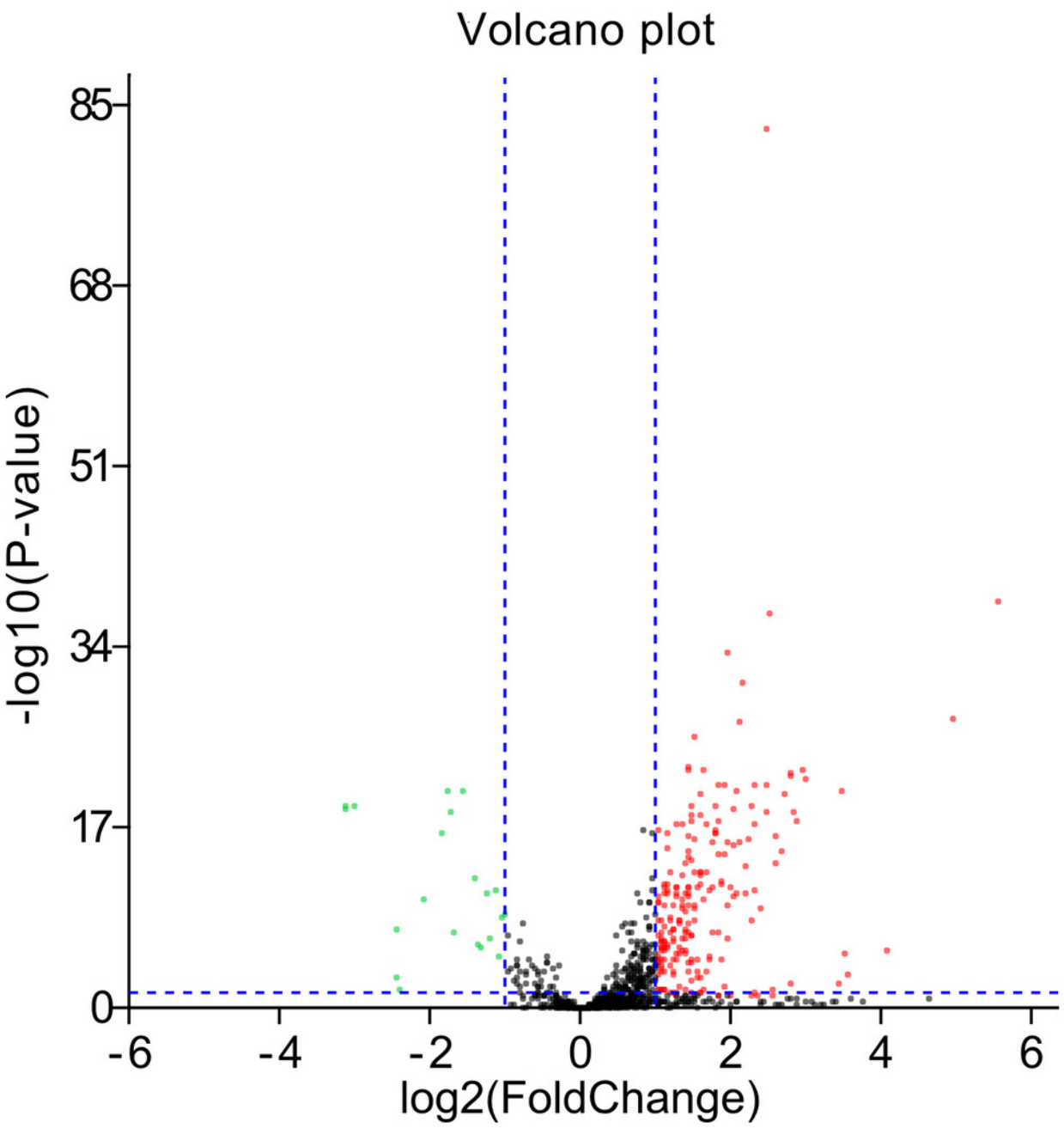
Volcano plot
-log10(P-value)
85
68
51
34
17
0
-6
-4
-2
0
2
4
6
log2(FoldChange)

Figure 2

The expression of miR-383-5p in GC tissues and cells.

A: miRNA-Seq data indicated that miR-383-5p was down-regulated in GC tissues; B, C: qRT-PCR showed that miR-383-5p was significantly down-regulated in GC cells and tissues. * P<0.05 compared with GES-1 group.

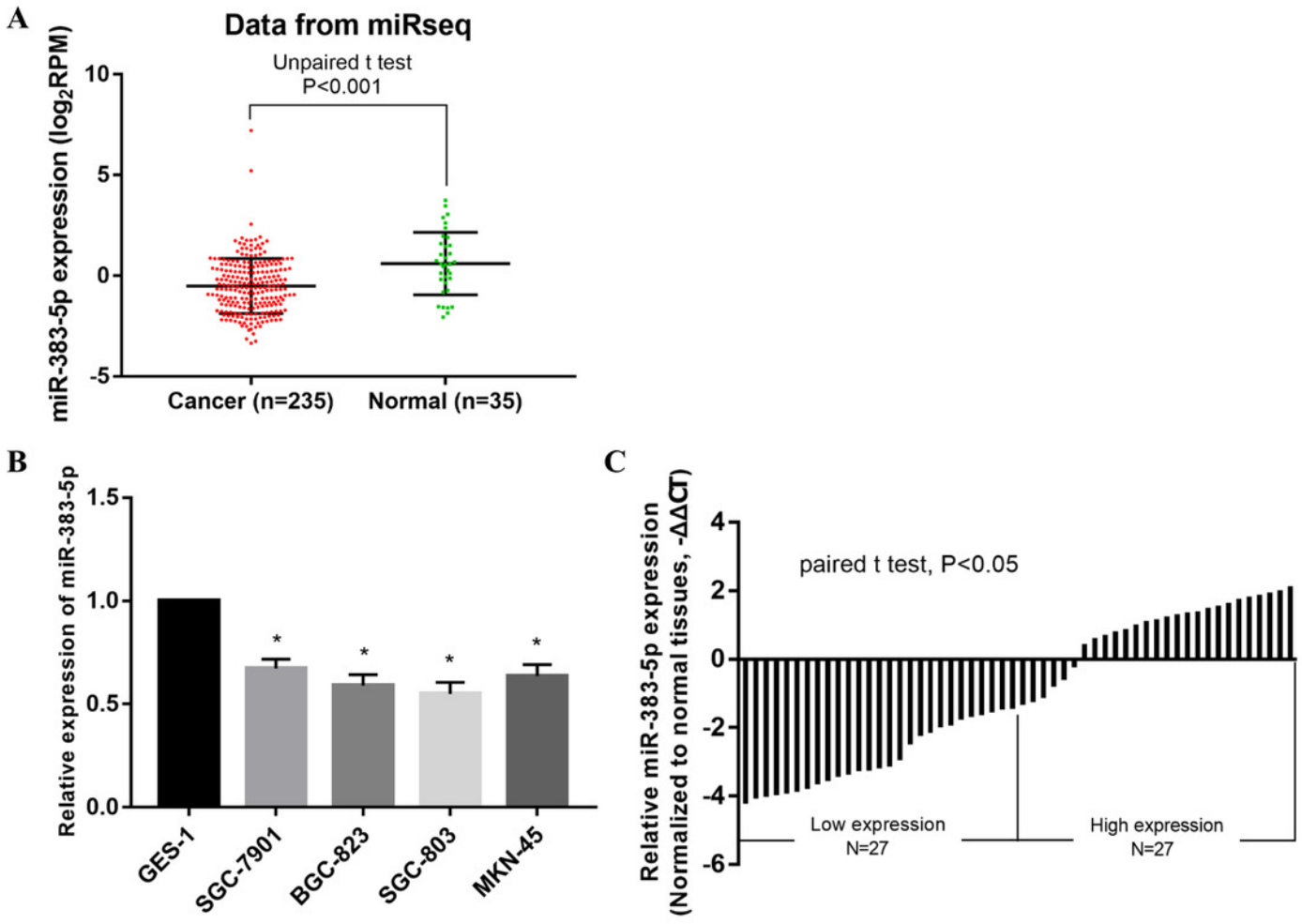

Figure 3

The Kaplan-Meier overall survival curve

The group with low miR-383-5p expression had a significantly less survival time (median survival time 21.1 months) than that with high expression (46.9 months).

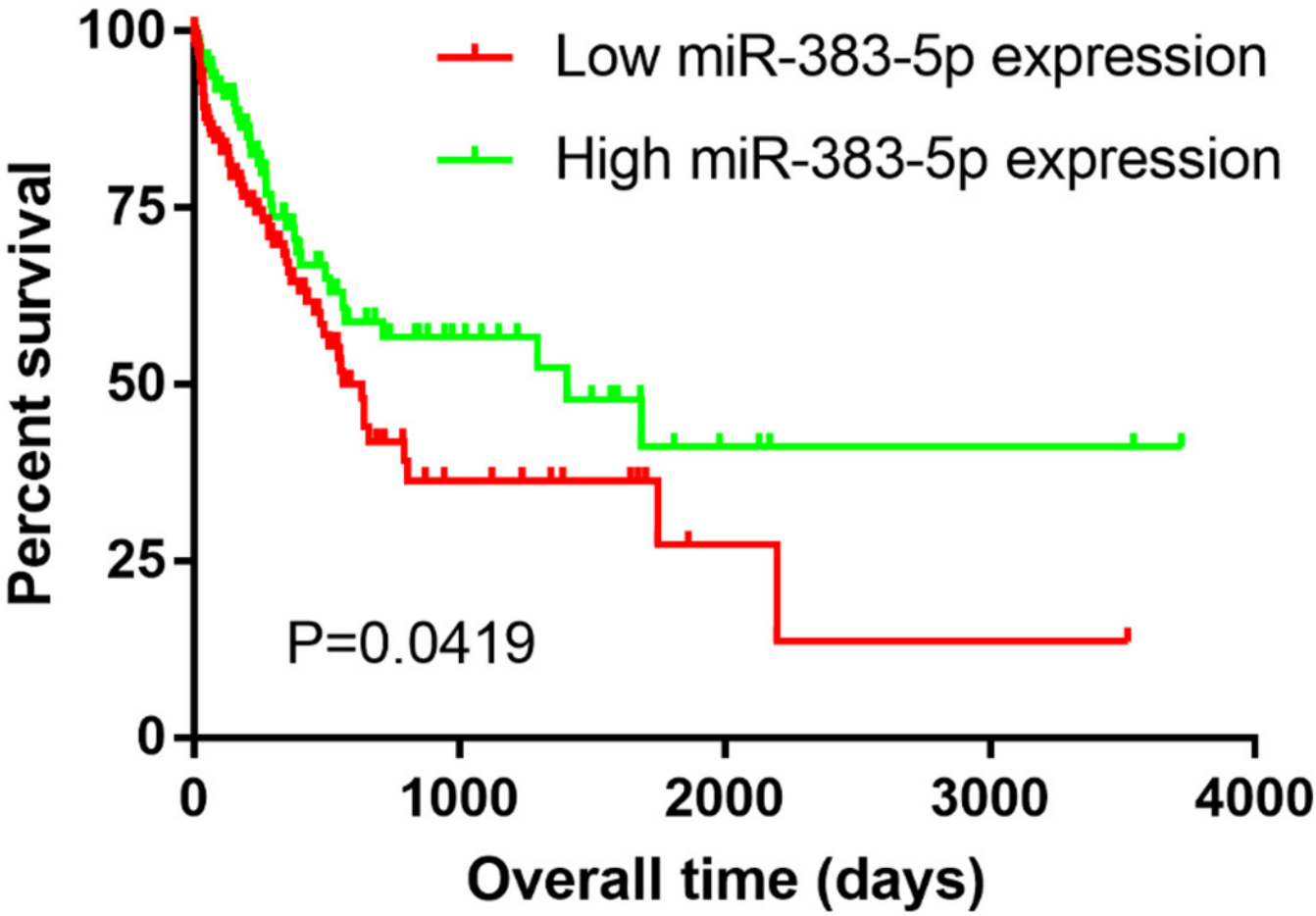

Figure 4

The effect of miR-383-5p on the proliferation and migration of GC cells

A: miR-383-5p mimics inhibited GC cells migration; B: miR-383-5p mimics inhibited GC cells proliferation. * P<0.05 compared with NC group.

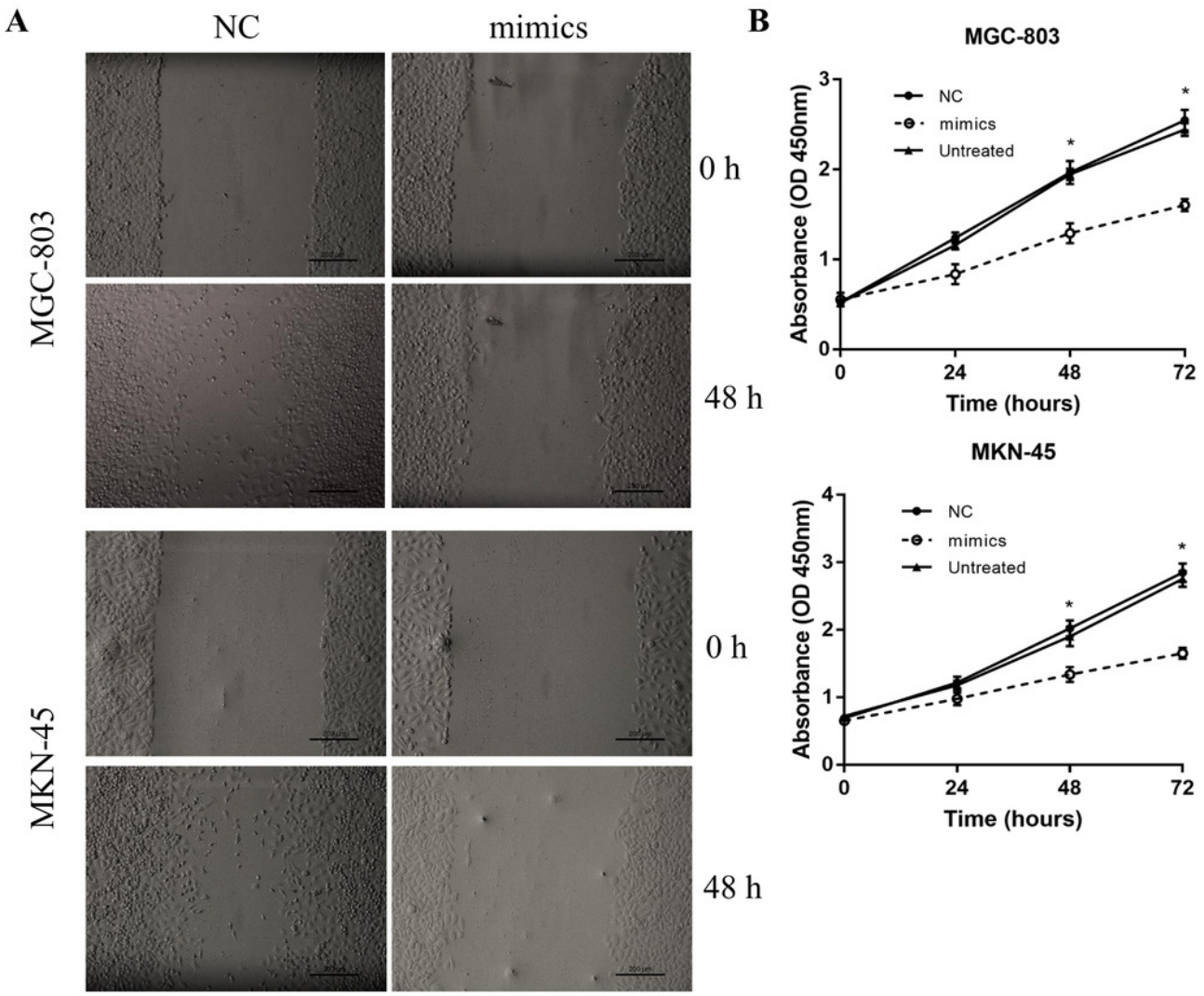

Figure 5

The venn plot of target genes

A total of 49 consensus genes were obtained from TargetScan, miRDB, and starBase websites.

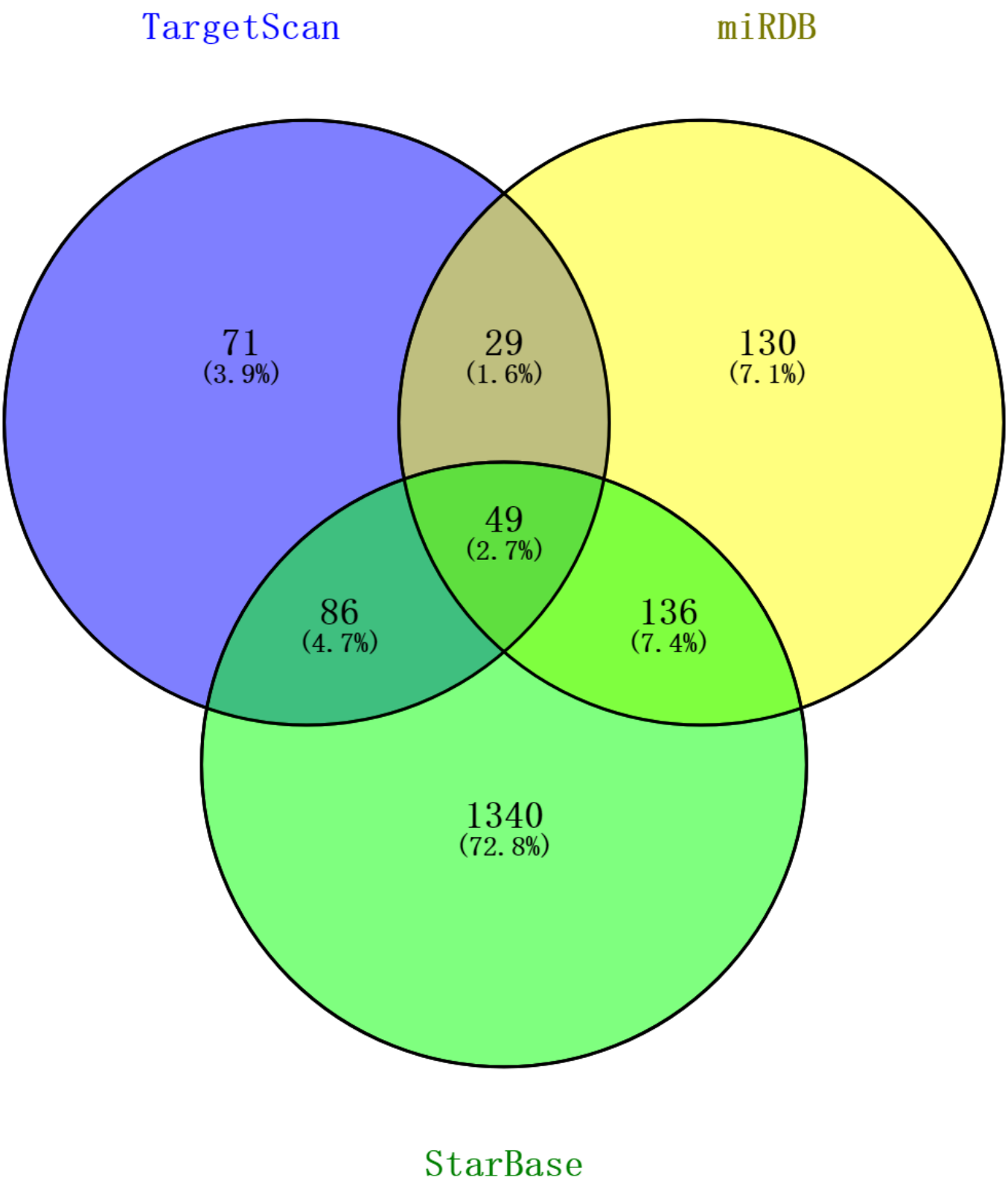
TargetScan
miRDB
71
(3.9%)
29
(1.6%)
130
(7.1%)
49
(2.7%)
86
(4.7%)
136
(7.4%)
1340
(72.8%)
StarBase

Figure 6

Gene functional enrichment analysis

Biological pathway analysis revealed that these genes participate in PI3K, mTOR, c-MYC, TGF-beta receptor, VEGF/VEGFR and E-cadherin signaling pathways.

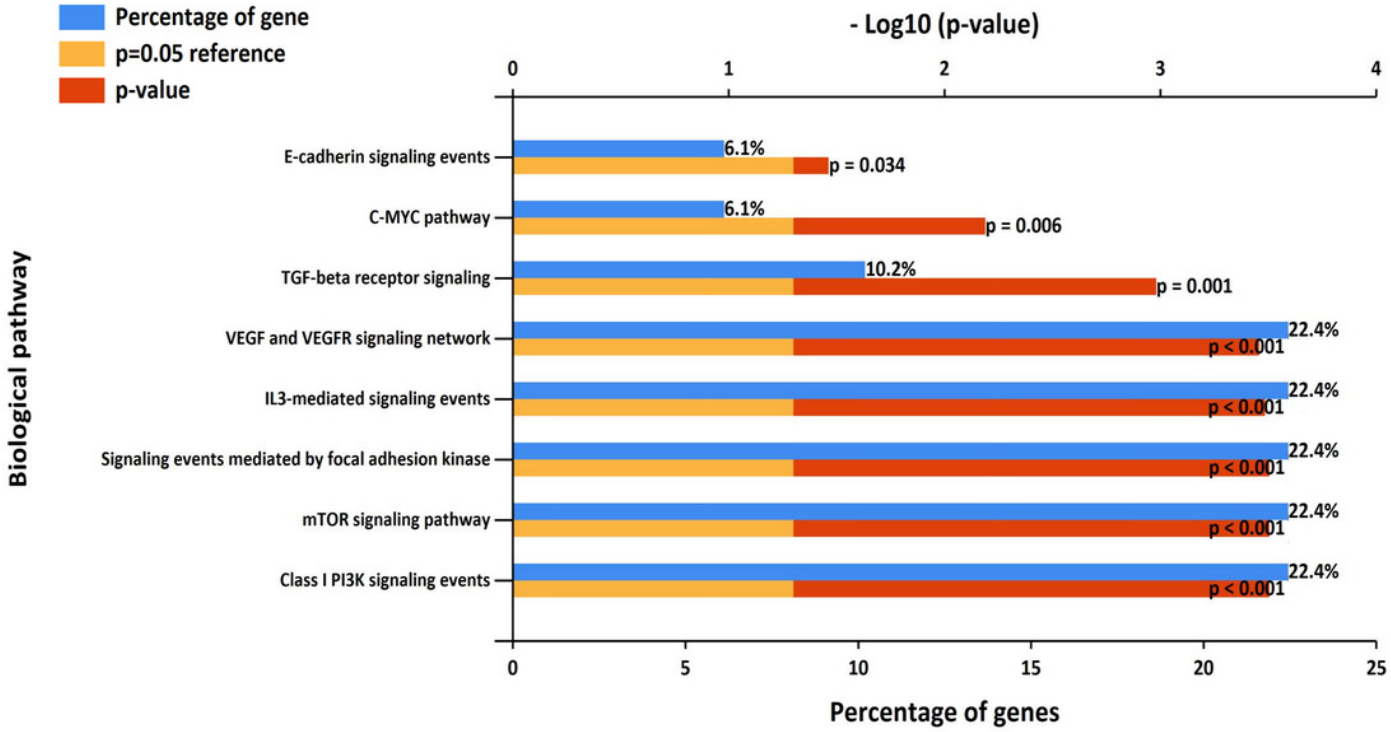

Table 1(on next page)

Sequence of primers used for PCR.

Sequence of primers used for PCR.

1 **Table 1. Sequence of primers used for PCR.**

Name	Sequence (5′-3′)
mR-383-5p (RT)	GTCGTATCCAGTGCGTGTCGTGGAGTCGGCAATTGCACTGG ATACGACAGCCAC
mR-383-5p (forward)	GGGAGATCAGAAGGTGATTGTGGCT
mR-383-5p (reverse)	CAGTGCGTGTCGTGGAGT
U6 (forward)	CTCGCTTCGGCAGCACA
U6 (reverse)	AACGCTTCACGAATTTGCGT

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Table 2(on next page)

42 DEMs identified between GC and adjacent normal tissues.

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Table 1 42 DEMs identified between GC and adjacent normal tissues.

Down-regulated	logFC	P value	Up-regulated	logFC	P value
miR-1-3p	-3.13	1.56E-19	miR-196a-5p	5.55	3.01E-39
miR-133a-3p	-3.13	9.66E-20	miR-196b-5p	4.94	3.33E-28
miR-133b	-3.03	9.52E-20	miR-767-5p	4.05	3.28E-06
miR-802	-2.46	0.000768	miR-552-3p	3.55	0.000579
miR-490-3p	-2.46	2.63E-08	miR-105-5p	3.49	7.65E-06
miR-1265	-2.43	0.014022	miR-135b-5p	3.46	2.25E-21
miR-204-5p	-2.10	3.67E-11	miR-767-3p	3.44	0.003757
miR-145-5p	-1.87	3.04E-17	miR-194-5p	2.97	2.20E-22
miR-139-3p	-1.76	2.84E-21	miR-200a-5p	2.94	2.40E-23
miR-145-3p	-1.75	2.37E-19	miR-192-5p	2.87	1.71E-18
miR-129-5p	-1.70	4.41E-08	miR-200a-3p	2.81	2.26E-19
miR-139-5p	-1.59	2.49E-21	miR-200b-3p	2.80	1.08E-22
miR-30a-3p	-1.41	4.25E-13	miR-141-5p	2.79	5.05E-23
miR-490-5p	-1.40	6.09E-07	miR-1269a	2.78	0.003274
miR-551b-3p	-1.32	1.73E-06	miR-183-5p	2.71	4.27E-21
miR-143-3p	-1.27	1.05E-11	miR-194-3p	2.64	1.03E-15
miR-486-5p	-1.22	2.70E-07	miR-429	2.58	2.15E-14
miR-29c-3p	-1.14	7.86E-12	miR-141-3p	2.57	5.25E-17
miR-383-5p	-1.12	1.13E-05	miR-675-5p	2.55	0.011142
miR-195-3p	-1.06	1.83E-09	miR-146b-5p	2.50	4.41E-38

Table 3(on next page)

Association between the genes and clinical features.

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Table 3. Association between the genes and clinical features.

Variables	MiR-383-5p expression		Total samples	P value
	Low (n,%)	High (n,%)		
Age				
<60	11 (17.1)	12 (13.4)	23	0.783
≥60	16 (28.0)	15 (41.5)	31	
Gender				
Male	17 (32.9)	13 (28.1)	30	0.273
Female	10 (25.6)	14 (13.4)	24	
Tumour size				
≤5 cm	5 (9.8)	14 (15.9)	19	0.010*
>5 cm	22 (48.8)	13 (25.6)	35	
Lymph node metastasis				
Negative	12 (13.4)	18 (19.5)	30	0.100
Positive	15 (45.1)	9 (22.0)	24	
TNM stage				
I + II	13 (28.0)	20 (15.9)	33	0.051
III + IV	14 (30.5)	7 (25.6)	21	
Differentiation grade				
Well and moderate	6 (17.1)	16 (18.3)	29	0.006*
Poor	21 (41.5)	11 (23.2)	53	

* $P < 0.05$, statistically significant.