Cancer Biomarkers 41 (2024) 41–54
DOI 10.3233/CBM-230303
IOS Press

Differentially expressed microRNA in prognosis of gastric cancer with Lauren classification

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Received 7 August 2023
Accepted 19 July 2024

Abstract.
BACKGROUND: Gastric cancer (GC) is one of the most common tumors. There were several classifications of GC recently. The value of Lauren classification in evaluating the prognosis after radical gastrectomy was still unclear and the prognosis of gastric cancer remained relatively poor in the absence of prognostic biomarkers. This study aimed to explore microRNA (miRNA) in the prognosis of GC with different Lauren classification.
METHODS: A retrospective study of 1144 patients was performed in this study. Quantificational reverse transcription-PCR (qRT-PCR) was used to examine the expression of miRNAs. Univariate and multivariate analysis were performed to evaluate prognosis value of Lauren classification.
RESULTS: Total 1144 GC patients were recruited in this cohort, including 302 diffuse type (26.4%), 436 intestinal type (38.1%) and 406 mixed type (35.5%) GC. Multivariate analysis showed that Lauren classification, patients' age, tumor size, tumor infiltrating depth, vascular nerve infiltrating and metastatic lymph nodes ration were significantly correlated with GC patients' OS and DFS. The miR-141-3p, miR-200b-3p and miR-133a-5p were significantly down-regulated in diffuse type compared to intestinal type GC tissues, the miR-105-5p had significant lower expression in diffuse type compared with intestinal type and mixed type GC tissues. As a consequence of univariate analysis, low miR-141-3p in diffuse type GC showed significant worse OS and DFS than high miR-141-3p.
CONCLUSIONS: Lauren classification was an independent prognostic factor in GC. MiR-141-3p was an independent prognostic factor and a promising prognostic biomarker in Lauren classification GC.

Keywords: Gastric cancer, Lauren classification, microRNA, prognosis

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1. Introduction

Gastric cancer (GC) is the third leading contributor of cancer mortality worldwide [1]. To date, the anatomical American Joint Committee on Cancer (AJCC) and Union for International Cancer Control (UICC) tumor-node-metastasis (TNM) staging system is the most widely used for describing GC states [2]. However, GC is a multifactorial and multistage disease [3]. It is hard to have better understanding on prognostic value just using TNM classification without reference to its pathology [4]. Since 1965, the Lauren classification has been proposed and become one of effective methods based on the histological structure of GC cells. GC is divided into diffuse type, intestinal type and mixed type according to the Lauren classification [5]. The histopathology of intestinal type is gland like structures and is considered to be associated with chronic inflammation induced by *Helicobacter Pylori* infection, smoking, obesity and other dietary factors [6,7], diffuse type is isolated-cell carcinoma and induced by active inflammation which leads to poor prognosis [8]. Thus, the prognostic relevance of GC using Lauren classification still remains unilluminated.

Primary studies reported that different moleculars played important roles in GC prognosis prediction, such as KRAS, APC, TP53 and so on [9,10]. MicroRNAs (MiRNAs), endogenous non-coding RNAs with 20–22 nucleotides in size, are known as oncogenes or tumor suppressors in various human cancers through regulating target mRNAs [11,12]. MiR-205/miR-338-3p regulated BCL-2 expression to suppress prostate cancer cells apoptosis [13]. Besides, miRNAs become popular biomarkers for cancer diagnosis and prognosis [14]. MiR-16 was confirmed the diagnosis value in common cancer like lung cancer, gastric cancer and endometrioid endometrial cancer [15]. MiR-487a worked as prognostic biomarker in hepatocelluar carcinoma [16]. MiR-194 was verified as favourable prognosis biomarker in GC [17]. It was reported that the down-modulation of miR-375 is specifically linked to Lauren's classification [18]. There is also research that suggests miR-18a-5p plays diagnostic and therapeutic potencies in mixed-type gastric cancer [19]. However, the application of miRNA on predicting prognosis in Lauren classification GC is still underway and needs further investigation.

To the point of controversy, we performed a retrospective study of 1144 patients who received radical gastrectomy and analyzed the clinical characteristics and significance in Lauren classification. In addition, we examined the expression levels and evaluated the prognostic value of related miRNAs from different Lauren classification GC tissues. This study provided the theoretical basis for the potential use of miRNAs in diagnosis and prognosis of gastric cancer with different Lauren classification.

2. Materials and methods

2.1. Study cohort

We collected data on 1144 patients who received radical gastrectomy at First Affiliated Hospital of Nanjing Medical University from January, 2005 to December, 2011. After surgery, GC patients were followed up every 3 months at first 2 years then every 6 months within 5 years, annually after 5 years, the last follow-up was in June, 2017. All patients from the cohort underwent radical gastrectomy and histologically confirmed. Clinical stage and histological classifications of GC were used the WHO classification criteria and the eighth edition of the AJCC TNM classification for GC.

2.2. Sample collection

We collected 145 GC tissues from the study cohort. Inclusion criteria include (1) complete clinical data, (2) Standard D2 lymph node dissection, (3) TNM stage I, to III, (4) no preoperative radiotherapy or chemotherapy, (5) postoperative chemotherapy regimen based on 5-FU and completed at least 4 cycles.

2.3. RNA extraction

Sections (8 μm slices) were prepared from paraffin-embedded specimen. Paraffin was removed by dewaxing reagent to obtain GC tissues after centrifugation. Total RNA was extracted from samples using Trizol (Invitrogen, America) method [20]. The concentration and quality of total RNA were evaluated by the ultraviolet spectrophotometer.

2.4. Quantitative RT-PCR

The cDNA was conducted using the specific primers of reverse transcription (RT) (Exiqon, Denmark). The amplification of miRNA was used Bulge-Loop™ miRNA qRT-PCR Primer Set (RiboBio, China) and evaluated the product fluorescence by SYBR Green (TaKaRa, China). RT reaction was carried out at 42°C for 60 min followed by 70°C for 10 min. The quantita-

Table 1
The correlation analysis between Lauren classification and clinicopathological characteristics

Variables	Lauren classification			$\chi 2$	P value
	Diffuse type	Intestinal type	Mixed type		
Gender				24.48	< 0.001
Male	191 (22.6%)	352 (41.6%)	303 (35.8%)		
Female	111 (37.2%)	84 (28.2%)	103 (34.6%)		
Age				71.982	< 0.001
$<$ 45 years	55 (59.1%)	9 (9.7%)	29 (31.2%)		
45–60 years	169 (26.8%)	248 (39.3%)	214 (33.9%)		
$\geqslant$ 60 years	78 (18.6%)	179 (42.6%)	163 (38.8%)		
Tumor site				85.254	< 0.001
Proximal	63 (13.5%)	236 (50.6%)	167 (35.8%)		
Middle	122 (32.4%)	116 (30.9%)	138 (36.7%)		
Distal	117 (38.7%)	84 (27.8%)	101 (33.4%)		
Tumor subtype				58.011	< 0.001
Non-infiltrating type	50 (25.5%)	101 (51.5%)	45 (23.0%)		
Borrmann type	207 (24.0%)	323 (37.4%)	333 (38.6%)		
Infiltrating type	45 (52.9%)	12 (14.1%)	28 (32.9%)		
Pathological classifications				149.681	< 0.001
Adenocarcinoma	217 (22.1%)	413 (42.0%)	354 (36.0%)		
Mucinous carcinoma	35 (32.7%)	23 (21.5%)	49 (45.8%)		
Signet ring carcinoma	50 (94.3%)	0 (0%)	3 (5.7%)		
Tumor size				28.884	< 0.001
$\leqslant$ 3 cm	120 (25.5%)	209 (44.4%)	142 (30.1%)		
3–6 cm	120 (23.8%)	187 (37.1%)	197 (39.1%)		
$>$ 6 cm	62 (36.7%)	40 (23.7%)	67 (39.6%)		
Histopathology classification				487.347	< 0.001
Well differentiated	1 (3.7%)	25 (92.6%)	1 (3.7%)		
Moderately differentiated	4 (1.5%)	247 (91.5%)	19 (7.0%)		
Poor differentiated	297 (35.1%)	164 (19.4%)	386 (45.6%)		
Tumor infiltrating depth				51.995	< 0.001
T_1	59 (29.5%)	95 (47.5%)	46 (23.0%)		
T_2	25 (14.7%)	84 (55.1%)	46 (30.1%)		
T_3	14 (19.4%)	35 (47.8%)	23 (32.8%)		
T_4	204 (28.5%)	222 (31.0%)	291 (40.6%)		
Vascular nerve infiltrating				82.582	< 0.001
Negative	154 (24.5%)	310 (49.4%)	164 (26.1%)		
Positive	148 (28.7%)	126 (24.4%)	242 (46.9%)		
Number of metastatic lymph nodes				110.381	< 0.001
N_0	92 (21.9%)	222 (52.7%)	107 (25.4%)		
N_1	37 (18.0%)	90 (43.7%)	79 (38.3%)		
N_2	63 (27.4%)	79 (34.3%)	88 (38.3%)		
N_3	110 (38.3%)	45 (15.7%)	132 (46.0%)		
Metastatic lymph nodes ration				108.674	< 0.001
0	92 (21.9%)	222 (52.7%)	107 (25.4%)		
$\leqslant$ 0.25	47 (19.5%)	112 (46.5%)	82 (34.0%)		
$\leqslant$ 0.5	66 (32.8%)	50 (24.9%)	85 (42.3%)		
$>$ 0.5	97 (34.5%)	52 (18.5%)	132 (47.0%)		
AJCC 8^{th} TNM stage				71.894	< 0.001
I	64 (23.9%)	144 (53.7%)	60 (22.4%)		
II	54 (20.7%)	124 (47.5%)	83 (31.8%)		
III	184 (29.9%)	168 (27.3%)	263 (42.8%)		

$P < 0.05$, statistical significance.

tive RT-PCR (qRT-PCR) was carried on LightCycler® 480 Real-Time PCR System (Roche Diagnostics, Germany) in 384-well plates at 95°C for 20 s, followed by 40 cycles of 95°C for 10 s, 60°C for 20 s and then 70°C for 10 s, the melting curve program was run immediately. All reactions were performed in triplicate. The *RNU6B* $(U6)$ was used as the standard for miRNA expression normalization [21]. The miRNA relative expression levels were calculated by the $2^{-\Delta\Delta Ct}$ method, $\Delta Ct = Ct\ (miRNA) - Ct\ (U6)$.

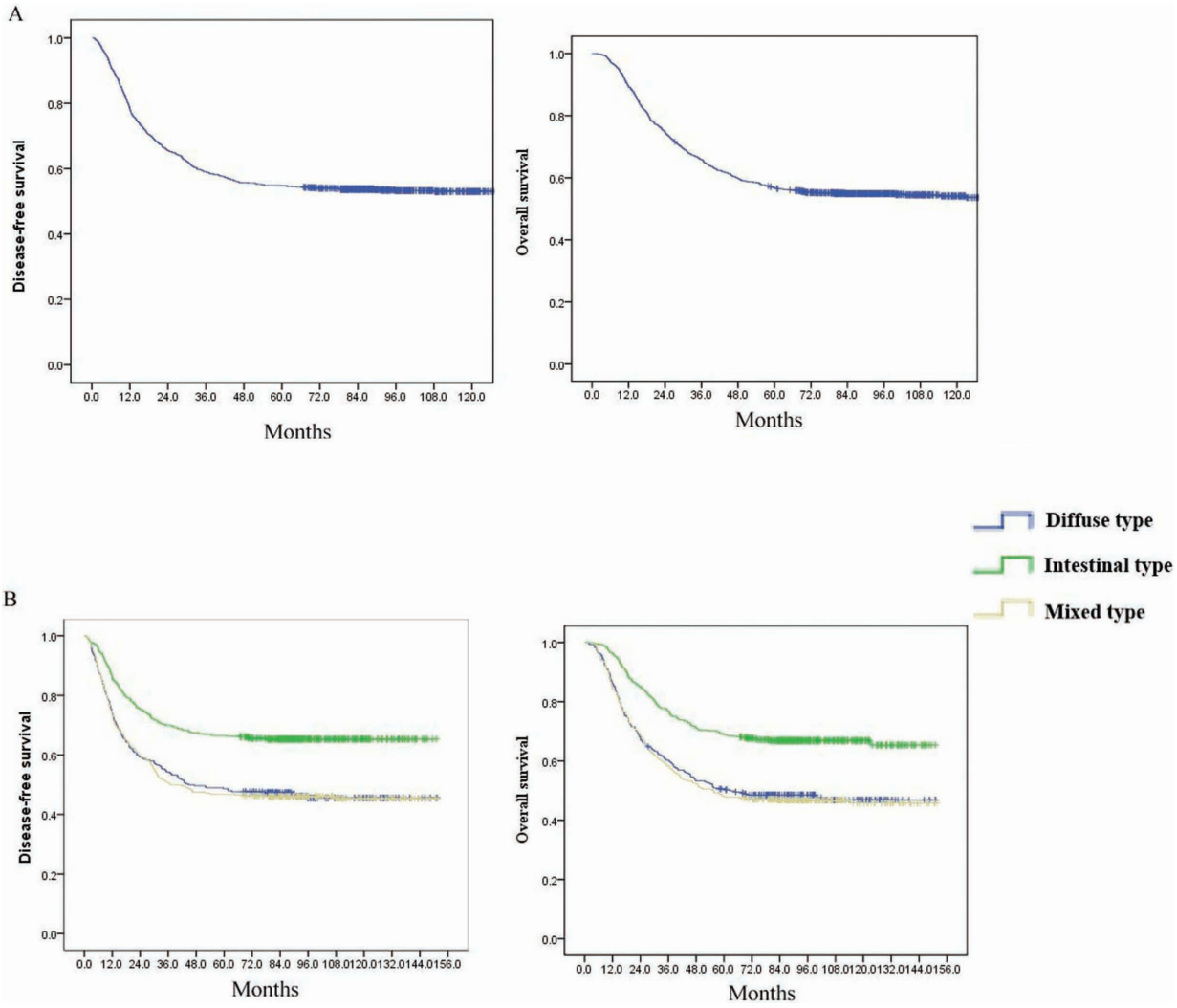


Fig. 1. Overall survival (OS) and disease free survival (DFS) curves plotted by the Kaplan-Meier method for (A) 1144 GC patients (B) GC patients with Lauren classification, diffuse type, intestinal type, mixed type.

2.5. *Statistical analysis*

The differential miRNAs expression levels between GC tissues and control tissues were analyzed by Mann-Whitney test. Clinical characteristics among Lauren classification groups and the relationship with miRNAs were analyzed by one-way ANOVA or $\chi 2$ test. Survival curves and univariate analysis were used the log-rank test. Five-year survival rates were estimated by life-table. Multivariate analysis was performed using Cox's proportional hazards regression model. All the statistical analyses were performed using SPSS software (version 20.0, IBM, USA). P value < 0.05 was defined statistically significant.

3. Results

3.1. *Clinicopathological characteristics of study subjects*

Total 1144 GC patients (846 males and 298 females, mean age, 61 years) were recruited in this cohort, including 302 diffuse type (26.4%), 436 intestinal type (38.1%) and 406 mixed type (35.5%) GC, the demographic information of GC patients were summarized in Table 1. We conducted the correlation analysis between Lauren classification and the differentiated. The results indicated that GC patients with diffuse type were younger ($<$ 45 years) ($P < 0.001$), female predominant ($P < 0.001$), distal stomach predominant ($P < 0.001$),

Table 2
The univariate analysis between disease free survival (DFS) and clinicopathological characteristics

Variables	Numbers	DFS (months)	5-year survival (%)	$\chi 2$	P value
Lauren classification				41.731	< 0.001
Diffuse type	302	44.7	49		
Intestinal type	436	*	67		
mixed type	406	36.933	47		
Gender				0.924	0.336
Male	846	*	54		
Female	298	*	58		
Age				19.242	< 0.001
$<$ 45 years	93	*	58		
45–60 years	631	*	60		
$\geqslant$ 60 years	420	37.267	47		
Tumor site				8.355	0.015
Proximal	466	49.267	49		
Middle	376	*	61		
Distal	302	*	56		
Tumor subtype				84.799	< 0.001
Non-infiltrating type	196	*	83		
Borrmann type	863	70.2	51		
Infiltrating type	85	18.9	32		
Pathological classifications				2.228	0.328
Adenocarcinoma	984	*	55		
Mucinous carcinoma	107	68.633	51		
Signet ring carcinoma	53	43.733	49		
Tumor size				194.55	< 0.001
$\leqslant$ 3 cm	471	*	77		
3–6 cm	504	31.433	43		
$>$ 6 cm	169	16.967	28		
Histopathology classification				53.592	< 0.001
Well differentiated	27	*	96		
Moderately differentiated	270	*	70		
Poor differentiated	847	46.5	49		
Tumor infiltrating depth				258.912	< 0.001
T_1	200	*	97		
T_2	155	*	79		
T_3	72	*	68		
T_4	717	26.167	37		
Number of metastatic lymph nodes				404.815	< 0.001
N_0	421	*	87		
N_1	206		52		
N_2	230	31.533	42		
N_3	287	13.233	20		
Metastatic lymph nodes ration				458.228	< 0.001
0	421	*	87		
$\leqslant 0.25$	241	*	57		
$\leqslant 0.5$	201	27.9	36		
> 0.5	281	12.267	19		
AJCC 8^{th} TNM stage				382.954	< 0.001
I	268	*	96		
II	261	*	72		
III	615	19.7	31		
Vascular nerve infiltrating				144.147	< 0.001
Negative	628	*	70		
Positive	516	26.167	36		

*The median survival time was not reached by follow-up, $P < 0.05$, statistical significance.

more infiltrating type and vascular nerve ($P < 0.001$), higher incidence in signet ring carcinoma and mucinous carcinoma ($P < 0.001$), TNM III stage predominant ($P < 0.001$). The intestinal type GC patients showed that were older ($\geqslant$ 60 years) ($P < 0.001$), less distal stomach predominant ($P < 0.001$), well differentiated

Table 3
The univariate analysis between overall survival (OS) and clinicopathological characteristics

Variables	Numbers	OS (months)	5-year survival (%)	$\chi 2$	P value
Lauren classification				46.781	< 0.001
Diffuse type	302	62.633	51		
Intestinal type	436	*	69		
Mixed type	406	55.367	48		
Gender				1.021	0.312
Male	846	*	55		
Female	298	*	60		
Age				19.161	< 0.001
$<$ 45 years	93	*	63		
45–60 years	631	*	61		
$\geqslant$ 60 years	420	57.6	49		
Tumor site				8.51	0.014
Proximal	466	65.3	51		
Middle	376	*	62		
Distal	302	*	59		
Tumor subtype				82.088	< 0.001
Non-infiltrating type	196	*	86		
Borrmann type	863	114.567	52		
Infiltrating type	85	29.367	35		
Pathological classifications				3.236	0.198
Adenocarcinoma	984	*	58		
Mucinous carcinoma	107	70.133	53		
Signet ring carcinoma	53	59.933	49		
Tumor size				193.159	< 0.001
$\leqslant$ 3 cm	471	*	79		
3–6 cm	504	45.467	45		
$>$ 6 cm	169	25.933	30		
Histopathology classification				56.797	< 0.001
Well differentiated	27	*	96		
Moderately differentiated	270	*	72		
Poor differentiated	847	64.967	50		
Tumor infiltrating depth				264.464	< 0.001
T_1	200	*	98		
T_2	155	*	82		
T_3	72	*	69		
T_4	717	36.733	39		
Vascular nerve infiltrating				145.628	< 0.001
Negative	628	*	72		
Positive	516	36.367	38		
Number of metastatic lymph nodes				412.207	< 0.001
N_0	421	*	88		
N_1	206	*	56		
N_2	230	43.7	44		
N_3	287	20.233	22		
Metastatic lymph nodes ration				462.637	< 0.001
0	421	*	88		
$\leqslant$ 0.25	241	*	60		
$\leqslant$ 0.5	201	37.267	40		
$>$ 0.5	281	20.433	19		
AJCC 8th TNM stage				386.133	< 0.001
I	268	*	96		
II	261	*	75		
III	615	31.133	32		

*The median survival time was not reached by follow-up, $P < 0.05$, statistical significance.

($P < 0.001$), Borrmann type predominant ($P < 0.001$), relative smaller tumor size (diameter $\leqslant$ 3 cm) ($P < 0.001$), less vascular nerve infiltrating ($P < 0.001$), less lymphovascular invasion ($P < 0.001$), TNM I stage predominant ($P < 0.001$).

3.2. Univariate and multivariate analysis for prognosis of gastric cancer

Among 1144 GC patients, the overall survival (OS) of 1-year, 3-year and 5-year were 90%, 75% and 66%,

Table 4
The multivariate COX risk model analysis of overall survival (OS) and between disease free survival (DFS)

Endpoint	Variables	β (regression coefficient)	SE	Wald value	HR (risk ratio) 95% CI	P value
DFS						
	Lauren classification	0.27	0.074	13.13	1.175 (1.041–1.328)	< 0.001
	Tumor size	0.283	0.069	16.777	1.336 (1.156-1.544)	< 0.001
	Tumor infiltrating depth	0.397	0.104	14.621	1.476 (1.187–1.836)	< 0.001
	Vascular nerve	0.359	0.096	13.833	1.433 (1.171–1.754)	< 0.001
	Metastatic lymph nodes ration	0.457	0.092	24.686	1.558 (1.293–1.879)	< 0.001
OS						
	Lauren classification	0.309	0.075	16.723	1.183 (1.047–1.337)	< 0.001
	Tumor size	0.295	0.07	17.836	1.338 (1.156–1.548)	< 0.001
	Tumor infiltrating depth	0.464	0.098	13.579	1.612 (1.284–2.023)	< 0.001
	Vascular nerve	0.36	0.104	10.941	1.412 (1.151–1.732)	< 0.001
	Metastatic lymph nodes ration	0.472	0.092	26.185	1.568 (1.300–1.891)	< 0.001

$P < 0.05$, statistical significance.

while the disease free survival (DFS) of 1-year, 3-year and 5-year were 78%, 66% and 59% (Fig. 1A). The univariate analysis showed that Lauren classification was strongly related to the OS and DFS ($P < 0.05$), the OS and DFS of diffuse type, intestinal type, mixed type GC patients were 51%, 69%, 48% and 49%,67%, 47%, respectively (Fig. 1B). In addition, the univariate analysis also showed that the prognosis of GC patients were strongly related to the patients' age, tumor site and size, tumor subtype, pathological classifications, tumor infiltrating depth, vascular nerve infiltrating, number of metastatic lymph nodes and metastatic lymph nodes ration, AJCC 8th edition TNM classification ($P < 0.05$) (Tables 2–3).

Multivariate analysis were introduced using Cox proportional hazards regression (forward LR stepwise procedure) to analyze independent prognostic predicting factors based on the statistically significant variants in univariate analysis. Multivariate analysis showed that Lauren classification, patients' age, tumor size, tumor infiltrating depth, vascular nerve infiltrating and metastatic lymph nodes ration were significantly correlated with GC patients' OS and DFS ($P < 0.001$) (Table 4). The Lauren classification was an independent prognostic predicting factor in GC and the diffuse type was an independent risk factors for poor prognosis of GC.

3.3. Identification of candidate differentially expressed miRNAs in GC

We searched keywords, gastric cancer, stomach cancer, miRNA and Lauren classification in PubMed website. The latest publication time of reference was January, 2017. Finally, a total of 8 references were in line with our topic idea after intensive reading. There were 22 candidate miRNAs, miR-105-5p, miR-100-5p, miR-199a-5p, miR-99a-5p, miR-133a-5p, miR-373-5p, miR-498, miR-202-5p, miR-32-5p, miR-141-3p, miR-182-5p, miR-125b-5p, miR-143-3p, miR-145-5p, miR-494-3p, miR-21-5p, miR-299-5p, miR-365b, miR-499a-5p, miR-200a-3p, miR-200b-3p and miR-200c-3p [22,23, 24,25,26,27,28,29].

The expression levels of 22 miRNAs were verified in 145 GC tissues, 47 diffuse type (32.4%), 50 intestinal type (33.1%), 48 mixed type (34.5%), the clinical features of patients were listed in Table 5. The expressed of miR-202-5p, miR-299-5p, miR-32-5p, miR-365b, miR-373-5p, miR-494-3p, miR-498 and miR-499a-5p were weakly expressed in GC tissues. As shown in Fig. 2, there were no significant difference in miR-100-5p, miR-199a-5p, miR-99a-5p, miR-182-5p, miR-125b-5p, miR-143-3p, miR-145-5p, miR-21-5p, miR-200a-3p, and miR-200c-3p among diffuse type, intestinal type and mixed type GC tissues. Compared to intestinal type GC tissues, the miR-141-3p, miR-200b-3p and miR-133a-5p were significantly down-regulated in diffuse type. Meanwhile, the miR-105-5p had significant lower expression in diffuse type compared with intestinal type and mixed type GC tissues.

3.4. Diagnostic and prognostic value of miRNAs in different Lauren classification GC

During the followed up, the median DFS and OS of 145 GC patients was 47.9 $\pm$ 17.9 and 50.1 $\pm$ 14.8 months, 114 (78.6%) were still alive. To further explore the prognostic value of miRNAs, log-rank test was introduced to analyze the OS and DFS between high miRNA expression and low expression of 14 miRNAs (miR-100-5p, miR-199a-5p, miR-99a-5p, miR-182-5p, miR-125b-5p, miR-143-3p, miR-145-5p, miR-21-5p, miR-200a-3p, miR-200c-3p, miR-141-3p, miR-200b-3p, miR-133a-5p and miR-105-5p) (Table 6). The

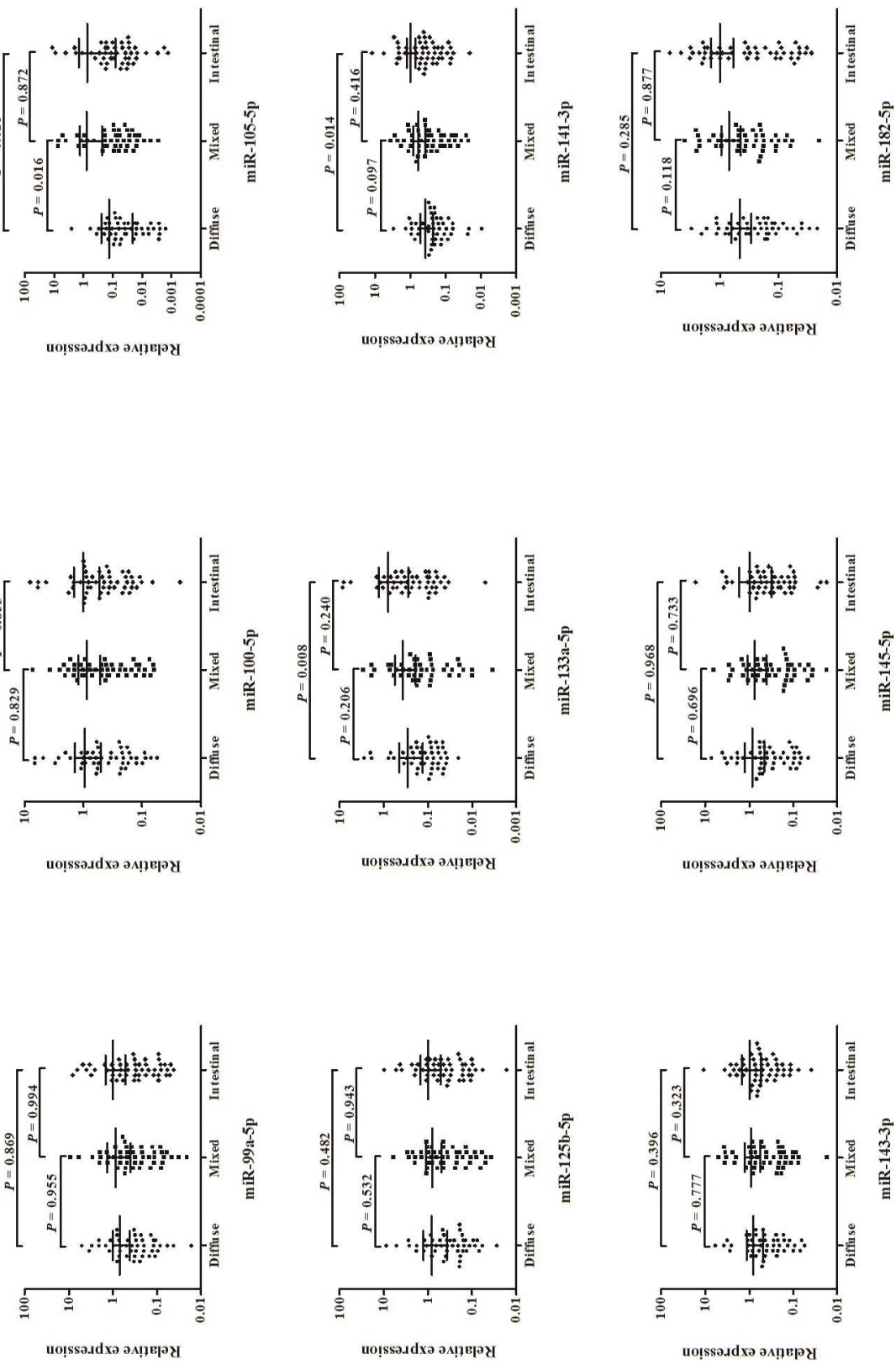
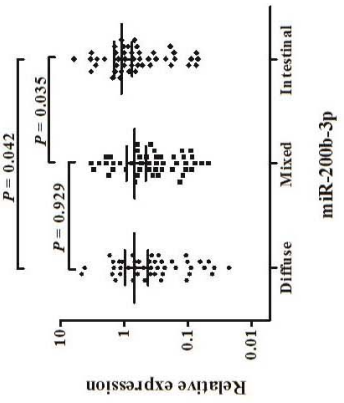
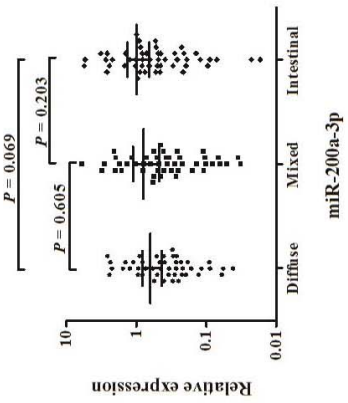
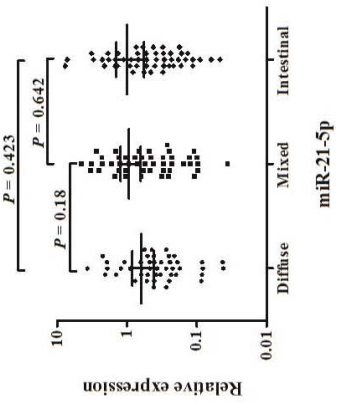
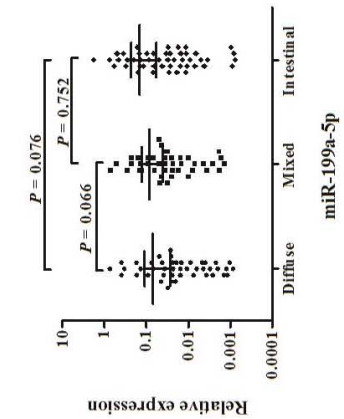


Fig. 2. Expression levels of 14 candidate miRNAs in the diffuse type, intestinal type and mixed type GC tissues. Horizontal line: mean with 95% CI. Each P value was calculated by the Mann–Whitney test. $P < 0.05$ was defined statistically significant.









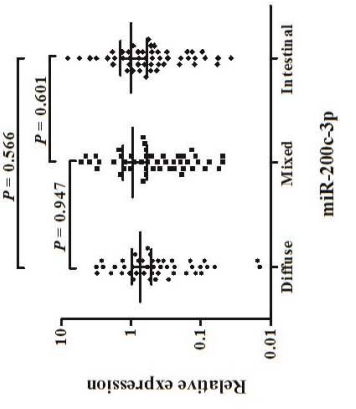


Fig. 2. continued.

Table 5
Clinicopathological characteristics used in miRNA expression detection

Age	
$<$ 45 years	13 (9.0%)
45–60 years	80 (55.1%)
$\geqslant$ 60 years	52 (35.9%)
Gender	
Male	101 (69.7%)
Female	44 (30.3%)
Tumor site	
Proximal	62 (42.8%)
Middle	44 (30.3%)
Distal	39 (26.9%)
Tumor subtype	
Non-infiltrating type	29 (20.0%)
Borrmann type	101 (69.7%)
Infiltrating type	15 (10.3%)
Pathological classifications	
Adenocarcinoma	118 (81.4%)
Mucinous carcinoma	10 (6.9%)
Signet ring carcinoma	17 (11.7%)
Tumor size	
$\leqslant$ 3 cm	62 (42.8%)
3–6 cm	57 (39.3%)
$>$ 6 cm	26 (17.9%)
Histopathology classification	
Well differentiated	3 (2.1%)
Moderately differentiated	30 (20.7%)
Poor differentiated	112 (77.2%)
Tumor infiltrating depth	
T_1	40 (27.6%)
T_2	15 (10.3%)
T_3	30 (20.7%)
T_4	60 (41.4%)
Number of metastatic lymph nodes	
N_0	65 (44.8%)
N_1	18 (12.4%)
N_2	31 (21.4%)
N_3	31 (21.4%)
Metastatic lymph nodes ration	
0	65 (44.8%)
$\leqslant$ 0.25	30 (20.7%)
$\leqslant$ 0.5	20 (13.8%)
$>$ 0.5	30 (20.7%)
AJCC 8^{th} TNM stage	
I	47 (32.4%)
II	28 (19.3%)
III	70 (48.3%)
Vascular nerve infiltrating	
Negative	94 (64.8%)
Positive	51 (35.2%)
Lauren classification	
Diffuse type	47 (32.4%)
Intestinal type	50 (33.1%)
Mixed type	48 (34.5%)

results showed that only low expression of miR-141-3p lead to worse OS and DFS in contrast to high miR-141-3p ($P < 0.05$). There was no significant difference of OS and DFS between low miRNA expression and high miRNA expression of remaining miRNAs.

The univariate analysis indicated that the prognostic value of GC patients was related to tumor size, tumor infiltrating depth, vascular nerve infiltrating, number of metastatic lymph nodes and metastatic lymph nodes ration, TNM stage, Lauren classification and miR-141-3p. Multivariate analysis of OS and DFS revealed that metastatic lymph nodes ration, Lauren classification and miR-141-3p were independent prognostic factors (Table 7).

To explore the prognostic value of miR-141-3p in Lauren classification GC, we conducted univariate analysis of miR-141-3p among diffuse type, intestinal type and mixed type GC. As a consequence of univariate analysis, low miR-141-3p in diffuse type GC showed significant worse OS and DFS than high miR-141-3p ($P < 0.05$). There was no significant difference of miR-141-3p in intestinal type and mixed type GC (Fig. 3).

4. Discussion

Currently, there are several classifications of GC, including histologically and molecular [30]. It is hard to say which classification is the best one due to the morphological characteristics of GC. Choosing one classification system is insufficient to provide precise prognosis for individual treatment. Lauren classification is the most-used one, however, there is still controversial whether Lauren classification plays better roles on prognosis performance of GC. In this study, we performed a retrospective study of 1144 patients who received radical gastrectomy. The results indicated that intestinal type GC (38.1%) had higher incidence than diffuse type GC (26.4%) which was line with previous study [31]. The difference with the study was that mixed type GC had higher incidence due to regional diversity. In diffuse type, the incidence was higher in younger ones than elder ones and females were predominant which may be related with oestrogen receptor [32]. The signet ring carcinoma, mucinous carcinoma, poor differentiated, deeper infiltration and higher metastatic lymph nodes ration were found in diffuse type GC than intestinal type GC, resulting in poor prognosis in diffuse type GC. The OS, DFS and 5-year survival of intestinal type GC had obvious survival advantages than diffuse type GC. According to multivariate analysis, Lauren classification was an independent prognostic factor and diffuse type GC was an independent risk factor for poor prognosis. In addition, our study showed that diffuse type GC was predominant in younger population in our country, the late TNM stage, large tumor size, lymphatic metastasis.

Table 6
Log-rank test analysis of overall survival (OS) and disease-free survival (DFS) of candidate miRNA

miRNA	Expression status	Overall survival (OS)		Disease-free survival (DFS)	
		Mean ± SD (months)	P-value	Mean ± SD (months)	P-value
miR-99a-5p	Low	48.8 ± 14.1	0.201	46.2 ± 17.9	0.47
	High	51.5 ± 15.2		49.7 ± 17.8	
miR-100-5p	Low	50.3 ± 13.7	0.956	48.4 ± 16.6	0.602
	High	49.9 ± 15.8		47.4 ± 19.3	
miR-105-5p	Low	50.3 ± 13.5	0.396	48.6 ± 16.0	0.287
	High	50.3 ± 15.8		47.6 ± 19.4	
miR-125b-5p	Low	50.6 ± 12.7	0.71	48.7 ± 15.7	0.346
	High	49.6 ± 16.7		47.1 ± 19.9	
miR-133a-5p	Low	48.9 ± 14.3	0.205	46.2 ± 18.3	0.457
	High	51.3 ± 15.2		49.7 ± 17.4	
miR-141-3p	Low	47.7 ± 16.0	**0.007**	44.8 ± 19.8	**0.036**
	High	52.8 ± 12.8		51.3 ± 15.0	
miR-143-3p	Low	50.1 ± 13.6	0.653	47.5 ± 17.8	0.953
	High	50.1 ± 15.9		48.3 ± 18.3	
miR-145-5p	Low	50.1 ± 13.9	0.654	48.0 ± 17.1	0.896
	High	50.1 ± 15.6		47.8 ± 18.7	
miR-182-5p	Low	50.5 ± 15.1	0.396	48.1 ± 18.7	0.903
	High	49.3 ± 14.7		47.3 ± 17.4	
miR-199a-5p	Low	48.2 ± 15.3	0.083	45.7 ± 18.8	0.251
	High	52.0 ± 13.9		50.2 ± 16.8	
miR-200a-3p	Low	48.9 ± 14.6	0.203	46.2 ± 18.4	0.457
	High	51.4 ± 14.9		49.6 ± 17.3	
miR-200b-3p	Low	48.8 ± 14.6	0.577	46.4 ± 17.6	0.48
	High	51.5 ± 14.9		49.4 ± 18.1	
miR-200c-3p	Low	51.7 ± 11.6	0.236	50.0 ± 14.9	0.086
	High	48.5 ± 17.3		45.8 ± 20.4	
miR-21-5p	Low	52.1 ± 13.1	0.146	50.6 ± 15.7	0.089
	High	48.1 ± 16.0		45.1 ± 19.6	

$P < 0.05$, statistical significance.

Table 7
The COX risk model analysis of overall survival (OS) and disease-free survival (DFS) of miR-141-3p

Variables	Overall survival (OS)				Disease free survival (DFS)			
	Univariate analysis	Multivariate analysis			Univariate analysis	Multivariate analysis		
	P value	HR	95%CI	P value	P value	HR	95%CI	P value
Age	0.288				0.689			
Gender	0.106				0.108			
Tumor site	0.858				0.768			
Tumor subtype	0.063				0.057			
Pathological classifications	0.054				0.106			
Tumor size	0.002	1.421	0.665–3.036	0.364	0.002	1.589	0.770–3.276	0.21
Histopathology classification	0.0503				0.062			
Tumor infiltrating depth	< 0.001	1.95	0.709–5.364	0.196	< 0.001	1.974	0.770–5.061	0.157
Number of metastatic lymph nodes	< 0.001	1.488	0.867–2.555	0.149	< 0.001	1.508	0.877–2.592	0.138
Metastatic lymph nodes ration	< 0.001	0.217	0.065–0.729	0.013	< 0.001	0.244	0.084–0.705	0.009
AJCC 8^{th} TNM stage	< 0.001	0.757	0.131–4.384	0.756	< 0.001	0.941	0.194–4.571	0.94
Vascular nerve infiltrating	0.007	0.971	0.448–2.106	0.942	0.009	0.899	0.430–1.878	0.776
adjuvant chemotherapy	0.346				0.196			
Lauren classification	0.012	0.562	0.347–0.911	0.019	0.004	0.502	0.314–0.804	0.004
miR-141-3p	0.007	0.408	0.169–0.988	0.047	0.036	0.563	0.258–1.228	0.149

$P < 0.05$, statistical significance.

It may be strongly correlated with heredity and may help to further understand the tumorigenesis of diffuse type GC.

Previous researches have confirmed that miRNAs have clinical implications in pathogenesis, diagnosis and prognosis of human cancers [11,12,33]. To have better understanding on targeted therapies in GC, we analyzed the expression levels of miRNAs in Lauren

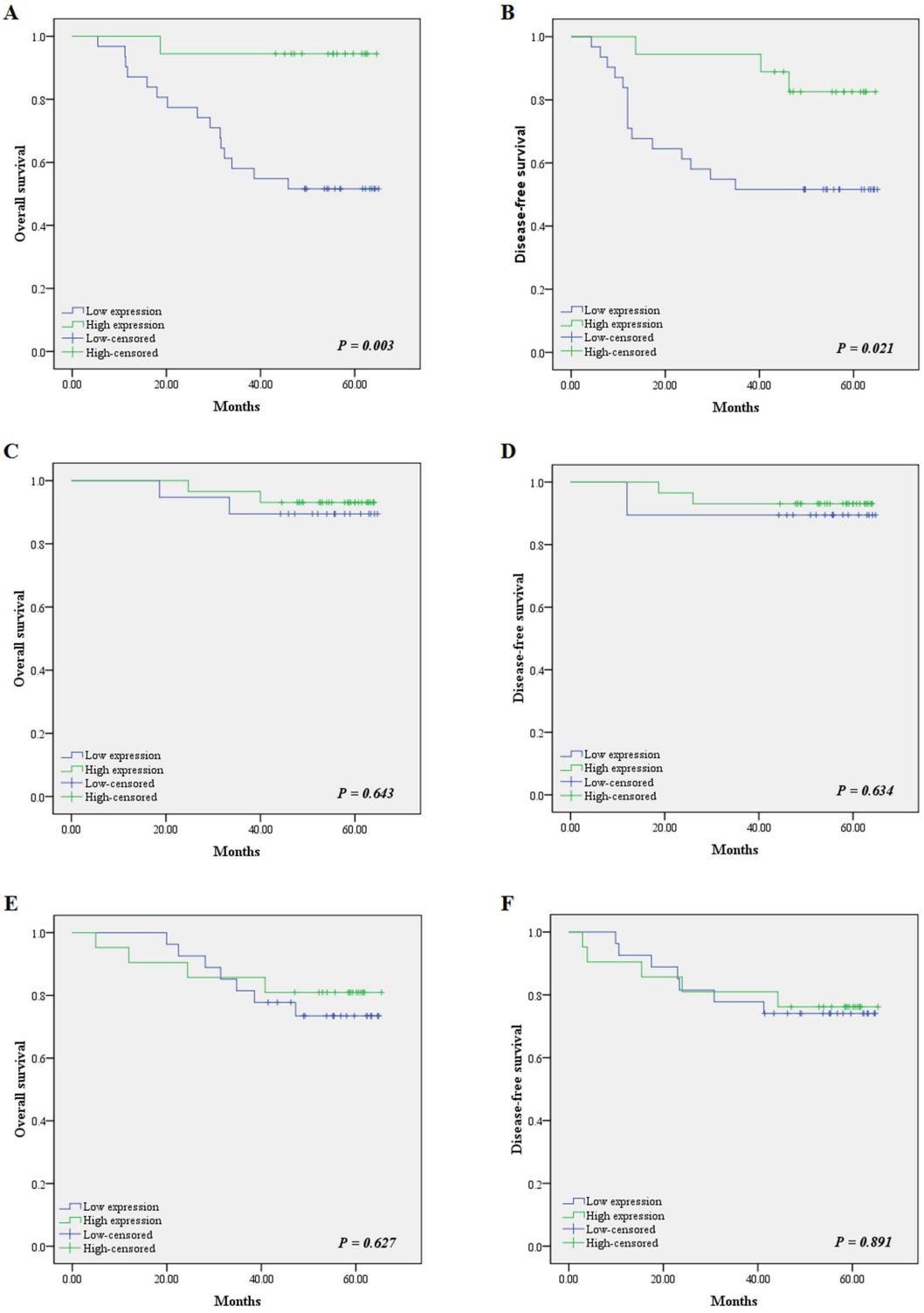


Fig. 3. Overall survival (OS) and disease free survival (DFS) curves of low miR-141-3p and high miR-141-3p group plotted by the Kaplan-Meier method in (A–B) diffuse type, (C–D) intestinal type, (E–F) mixed type GC. Each P value was calculated by the log-rank test. $P < 0.05$ was defined statistically significant.

classification GC to seek for a prognostic biomarker. In our study, the expression levels of miR-141-3p were significantly down-regulated in diffuse type compared to intestinal type GC tissues using qRT-PCR. Multivariate analysis of OS and DFS revealed that metastatic lymph nodes ration, Lauren classification and miR-141-3p were independent prognostic factors. The low expression of miR-141-3p lead to worse OS and DFS in contrast to high miR-141-3p in diffuse type GC. MiR-141 was demonstrated as tumor suppressor through regulating target gene TAZ in GC. Inhibition of miR-141 resulted in promoting GC cells proliferation, invasion and migration *in vitro* [34]. MiR-141-3p, a member of miR-200 family, and its target gene ZEB1 and ZEB2 (E-cadherin transcriptional repressors) were associated with epithelial to mesenchymal transition (EMT). Enhanced expression of miR-141-3p suppressed EMT, while inhibition of miR-141-3p induced EMT. Down-regulation of miR-141-3p played important roles on tumor progression [35]. The results were consistent with the low expression of miR-141-3p lead to worse OS and DFS in diffuse type GC. Using DIANA-miRPath v3.0 online software to assess miR-141-3p regulatory roles and the identification of controlled pathways [36]. As shown in Table S1, KEGG molecular pathways showed that miR-141-3p was associated with P53 signaling pathway. It was confirmed that overexpression P53 indicated the poor prognosis in GC, especially in diffuse type [37]. GO pathway analysis showed that miR-141-3p was associated with epidermal growth factor receptor signaling pathway, which was confirmed to be an independent prognostic factor influenced prognosis of GC [38].

There were some limitations in our study, the number of candidate miRNAs in predicting prognosis of GC and subtype of GC were relatively small. Also, neoadjuvant chemotherapy, *Helicobacter Pylori* infection and HER-2 gene amplification were not included in the prognostic factors.

5. Conclusions

Taken together, the present study suggested that Lauren classification was an independent prognostic factor in GC and the diffuse type was an independent risk factors for poor prognosis of GC. Lauren classification may help clinical doctors provide a reasonable plan for individual treatment combining with other clinicopathological characteristic. MiR-141-3p was an independent prognostic factor and may become a promising prognostic biomarker in Lauren classification GC. However, the mechanism of miR-141-3p in prognosis of Lauren classification still need further investigation.

Ethics statement

The medical ethical committee of First Affiliated Hospital of Nanjing Medical University. Written informed consent was obtained from all GC patients included in the study.

Author contributions

Conception: WC, ZH, MZ and SQ.

Interpretation or analysis of data: WC, QG, ZH, and ZH. WC, QG, YD and ZH performed the experiments.

Preparation of the manuscript: WC, QG and ZH.

Revision for important intellectual content: YZ, MZ and SQ.

Supervision: MZ and SQ.

Acknowledgments

The staff authors are sincerely grateful to all volunteers who participated in this follow-up study. The work was supported by the Jiangsu Provincial Medical Key Discipline (Grant number: ZDXK202235).

Conflict of interest

The authors have declared that no potential conflict of interest exists.

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