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Value of Mir-1271 and GPC3 in Prognosis Evaluation of Liver Cancer Patients after Liver Transarterial Chemoembolization

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ABSTRACT

Objective: This research was designed to observe the value of miR-1271 and GPC3 in evaluating the prognosis of liver cancer (LC) patients after liver transarterial chemoembolization (TACE). **Methods:** A total of 80 patients diagnosed as LC in our hospital from January 2018 to April 2019 were included in the LC group (LCG), and then assigned into a survival group (SG) and a death group (DG) based on prognosis. Seventy healthy subjects undergoing physical examination simultaneously were included in the normal group (NG). miR-1271 and GPC3 in serum of two groups of subjects were tested via qRT-PCR. ROC curve was drawn. The prognostic factors were assessed via multivariate Cox regression analysis. **Results:** The expression of miR-1271 in the LCG was notably lower than the NG, while GPC3 expression in the LCG was notably higher ($P < 0.001$). Patients with low miR-1271 expression and high GPC3 expression had a higher incidence of low differentiation, grade III+IV, lymphatic invasion and distant metastasis ($P < 0.05$). Both AUC of miR-1271 and GPC3 single diagnosis were higher than 0.8. The relative expression of serum miR-1271 in the SG was markedly higher than the DG, while that of serum GPC3 in the SG was markedly lower (both $P < 0.001$). ROC was drawn. The AUC of serum miR-1271 and GPC3 in diagnosing prognosis were higher than 0.8, and there was no marked difference in sensitivity and specificity. Cox regression analysis was performed on 5-year survival, and the results manifested that miR-1271 was an independent factor affecting prognosis. **Conclusion:** miR-1271 and GPC3 may be implicated in LC development and metastasis, and can be employed as potential serum biomarkers for post-TACE evaluation.

KEYWORDS

miR-1271; GPC3; hepatocarcinogenesis; short-term prognosis

1 Introduction

Liver Cancer (LC) is a chronic malignant liver tumor. With the influence of people's unhealthy living habit and poor dietary habit in recent years, the population suffering from LC is becoming younger, and the incidence has been increasing, seriously threatening people's life and health [1,2]. Early LC patients lack the first symptoms, so they miss timely treatment. Advanced LC patients have a survival rate less than 5% and an extremely high mortality. The annual mortality worldwide is about 30% to 40% [3,4]. With a deeper knowledge of the pathogenesis, a recent study has found fine biomarkers that help

diagnose and evaluate the disease [5]. At present, transarterial chemoembolization (TACE) is the first choice for LC treatment. TACE can reduce tumor blood supply and cause tumor necrosis [6,7]. Clinical studies discovered that the recurrence of LC after TACE is particularly important, and the search for more effective biomarkers to predict the prognosis has drawn extensive attention from medical research [8].

microRNAs (miRNAs) are endogenous non-coding single-stranded RNAs [9,10]. Current studies have shown that tumor-related miRNAs in the blood circulation are relevant to tumor development and progression and some pathological processes in the body [11,12]. Some researchers have confirmed that Glypican-3 (GPC3) is a transmembrane protein that is over-expressed in LC cells [13]. Over-expressed GPC3 can significantly promote cell invasion and metastasis [14,15], revealing that it is tied to repressor miR-1271 down-regulation [16]. However, the miR-1271 and GPC3 expression in LC patients and their clinical value are vague. Therefore, we conducted a prospective clinical study, so as to observe serum miR-1271 and GPC3 expression, analyze the clinical value and short term prognosis, thereby supplying a novel theoretical basis for diagnosis and treatment in the aspect of molecular biology.

2 Data and Methods

2.1 Data Collection

A total of 80 patients (47 males and 33 females) diagnosed as LC in our hospital from January 2018 to April 2019 were assigned to a LC group (LCG), with a mean age of (55.00 ± 3.19) years. Seventy healthy subjects (40 males and 30 females) undergoing physical examination during the same period were included in the normal group (NG), with an average of (55.12 ± 3.04) years. Inclusion and exclusion criteria were as follows: all LC patients conformed to diagnostic guidelines of LC expert committee [17]; patients with normal hepatic and renal function, without other malignancy. patients received treatment of chemotherapy, immunotherapy or radiotherapy, etc. This study has been approved by the ethical committee. Patients and their families were informed, and they signed the informed consent form.

2.2 Main Reagents, Instruments and Detection Methods

2.2.1 Main Reagents and Instruments

They were: Trizol reagent (Applide Invitrogen, USA), qRT-PCR and minScript reverse transcription kits (TaKaRa, Dalian), HBS-1096A enzyme-labeled analyzer (Nanjing Detie Experimental Equipment Co., Ltd., Nanjing, China), real-time quantitative PCR (BioRad, USA). Primer sequences of miR-1271, GPC3, internal references of U6 and GAPDH, and miRNA negative control were all synthesized and designed by GenePharma Co., Ltd., Shanghai, China (Tab. 1).

Table 1: miR-1271, GPC3 primer sequences and internal references

Groups	Upstream primer	Downstream primer
miR-1271	5'-CAGCACTTGGCACCTAGCA-3'	5'-TATGGTTGTTTTCCCCTCTCTGTCTC-3'
GPC3	5'-ATACCAACTAACAGCACGATT-3'	5'-TTCACCCACAAACTCAAAA-3'
U6	5'-CTCGCTTCGGCAGCACA-3'	5'-AACGCTTCACGAATTTGCGT-3'
GAPDH	5'-ACCACACTGAGAGGGAATC-3'	5'-AGGTCTTIGCGAIGTCCA-3'

2.2.2 Detection of miR-1271 and GPC3

Within 24 h after admission, 5 mL elbow venous blood was drawn from subjects and placed in a vacuum blood collection tube without anticoagulant, and centrifuged at 3500 g for 15 min. The serum was retained in EP tube for later use, and then stored in a low-temperature refrigerator at $-80°C$. The miR-1271 and GPC3 expression in serum were tested by qRT-PCR. Total serum RNA was extracted according to the

Trizol reagent instructions and dissolved in 20 μL DEPC water. The total RNA was then reverse-transcribed using a reverse transcription kit: M-MLV 1 μl, Olig (dT) 1 μl, RNA enzyme inhibitor 0.5 μl, d NTPs 1 μl, with the addition of RNAse free water to 15 μl. It was cultivated at 38°C for 60 min, and 1 μl cDNA was collected at 85°C for 5 s. As a template, the synthesized cDNA was employed for qRT-PCR amplification: 10 × PCR buffer 2.5 μl, d NTPs 1 μl, 1 μl upstream primer, 1 μl downstream primers, Taq DNA Polymerase 0.25 μl, with the addition of dd H_2O to 25 μl. qRT-PCR reactions were then conducted using 5 μL of 1/20 cDNA dilution, 6.25 μL Power SYBR Green master mix, 0.5 μL of both forward and reverse primers, and 0.25 μL of nuclease-free water. Three duplicate wells were constructed for each sample for three reduplicated experiments. The relative quantification of target gene was counted using $2^{-\Delta Ct}$.

3 Statistical Methods

SPSS 10.0 was employed for statistical analysis. The enumeration data was represented by number of cases/percentage [n(%)], and the inter-group comparison was qualified by X^2 test. The measurement data was represented by mean (X ± SD), and the inter-group comparison was qualified by t test or F test. The diagnostic value and 5-year survival of patients were drawn by ROC and K-M survival curve, respectively, and they were analyzed through Log-rank test. The independent risk factors affecting prognosis were assessed by multivariate Cox regression analysis; the comparison between multiple groups was analyzed by one-way analysis of variance (ANOVA) and expressed as F. $P < 0.05$ was statistically significant.

4 Results

4.1 General Clinical Data of Patients

There was no marked difference in age, gender, drinking, education background and other general clinical data between the LCG and the NG ($P > 0.05$) (Tab. 2).

Table 2: General clinical data of patients

Groups	LCG (n = 80)	NG (n = 70)	$t/F/X^2$	P
Age (years)	55.00 ± 3.19	55.12 ± 3.04	0.235	0.815
Gender				
Male	47 (58.75)	40 (57.14)	0.040	0.842
Female	33 (41.25)	30 (42.86)		
BMI (kg/m^2)	18.62 ± 1.12	18.73 ± 1.13	0.598	0.551
Education background			0.001	0.970
Bachelor or above	42 (53.16)	37 (52.86)		
Below bachelor	37 (46.84)	33 (47.14)		
Smoking			5.362	0.021
With	59 (73.75)	39 (55.71)		
Without	21 (26.25)	31 (44.29)		
Drinking			0.196	0.658
With	60 (75.00)	43 (71.67)		
Without	20 (25.00)	17 (28.33)		

(Continued)

Table 2 (continued).

Groups	LCG (n = 80)	NG (n = 70)	t/F/X^2	P
Differentiation			–	–
Poor differentiation	44 (55.00)	–		
Middle + high differentiation	36 (45.00)	–		
TMN staging			–	–
Grade I + II	43 (53.75)	–		
Grade III + IV	37 (46.25)	–		
Lymphatic invasion			–	–
Yes	45 (56.25)	–		
No	35 (43.75)	–		

4.2 miR-1271 and GPC3 Expression in LCG and NG before TACE

miR-1271 expression in the LCG and NG were (0.20 ± 0.10) and (0.41 ± 0.10), respectively, while GPC3 expression in the LCG and NG were (0.8 ± 0.10) and (0.60 ± 0.10), respectively. miR-1271 expression in serum of the LCG was markedly lower than that of the NG ($P < 0.001$), while GPC3 expression of the LCG was dramatically higher than that of the NG ($P < 0.001$) (Fig. 1).
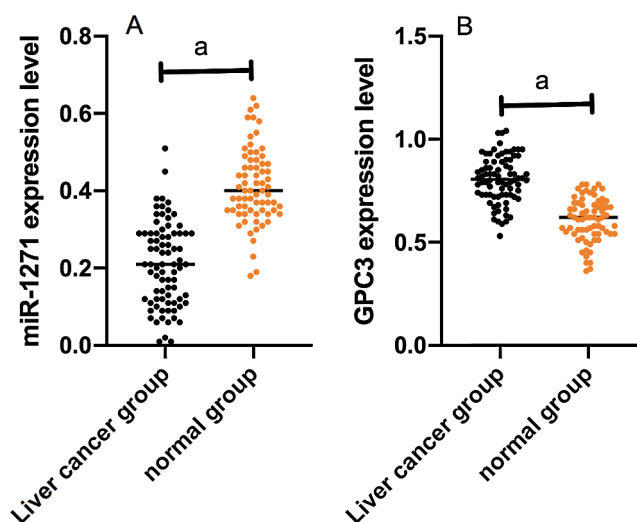
Figure 1: Comparison of miR-1271 and GPC3 relative expression in the LCG and the NG. (A) miR-1271 relative expression in serum of the LCG is markedly higher than that of the NG ($P < 0.001$). (B) GPC3 relative expression of the LCG is dramatically lower than that of the NG ($P < 0.001$). Note: A denotes $P < 0.001$

4.3 miR-1271 and GPC3 Expression and Clinicopathologic Features of LC Patients before TACE

4.3.1 Relationship between miR-1271 and Pathological Data of LC Patients

LC patients were divided into a miR-1271 high-expression group and a low-expression group according to the median level of miR-1271. By analyzing the pathological data, we found that patients with low

expression were more likely to have higher incidence of low differentiation, Grades III + IV, lymphatic invasion and distant metastasis ($P < 0.05$) (Tab. 3).

Table 3: Relationship between miR-1271 and pathological data of LC

Parameters		miR-1271 expression		χ^2 value	P value
		Low expression	High expression		
Gender				0.052	0.820
	Male (n = 47)	24 (60.00)	23 (57.50)		
	Female (n = 33)	16 (40.00)	17 (42.50)		
Age (years)				0.201	0.654
	≥55 years old (n = 42)	20 (50.00)	22 (55.00)		
	<55 years old (n = 38)	20 (50.00)	18 (45.00)		
Position of lesion				1.270	0.260
	Right/left lobe of liver (n = 45)	20 (50.00)	25 (62.50)		
	Other (n = 35)	20 (50.00)	15 (37.50)		
Differentiation				15.500	<0.001
	Low differentiation (n = 44)	13 (32.50)	37 (74.00)		
	Middle + high differentiation (n = 36)	27 (67.50)	13 (26.00)		
TMN staging				18.061	<0.001
	Grades I + II (n = 43)	30 (75.00)	11 (27.50)		
	Grades III + VI (n = 37)	10 (25.00)	29 (72.50)		
Lymphatic invasion				12.930	<0.001
	Present (n = 45)	10 (25.00)	26 (65.00)		
	Absent (n = 35)	30 (75.00)	14 (35.00)		
Vascular invasion				0.050	0.823
	Present (n = 43)	21 (52.50)	22 (55.00)		
	Absent (n = 37)	19 (47.50)	18 (45.00)		
Distant metastasis				8.498	0.004
	Present (n = 37)	12 (30.00)	25 (62.50)		
	Absent (n = 43)	28 (70.00)	15 (37.50)		

4.3.2 Analysis of GPC3 Expression and Clinicopathologic Features of LC Patients

LC patients were divided into a GPC3 high-expression group and a low-expression group in the light of the median level of GPC3. By analyzing the pathological data, we found that patients with high expression were more likely to have a higher incidence of low differentiation, Grades III + IV, lymphatic invasion and distant metastasis ($P < 0.05$) (Tab. 4).

4.4 miR-1271 and GPC3's Value in LC Prognosis

The sensitivity, specificity and AUC of miR-1271 alone in diagnosing LC patients were 84.29%, 86.25% and 0.9227, respectively, while those of GPC3 alone in diagnosis were 75.71%, 85.00%, and 0.9006, respectively (Tab. 5, Fig. 2).

Table 4: GPC3 expression and clinicopathologic features of LC patients before TACE

Parameters		GPC3 expression		χ^2 value	P value
		Low expression	High expression		
Gender				0.487	0.485
	Male (n = 47)	23 (60.00)	24 (67.50)		
	Female (n = 33)	17 (40.00)	16 (32.50)		
Age (years)				0.201	0.448
	≥55 years old (n = 42)	22 (55.00)	20 (50.00)		
	<55 years old (n = 38)	18 (45.00)	20 (50.00)		
Position of lesion				0.457	0.499
	Right/left lobe of liver (n = 45)	21 (52.50)	24 (60.00)		
	Other (n = 35)	19 (47.50)	16 (40.00)		
Differentiation				5.051	0.025
	Low differentiation (n = 44)	17 (42.50)	27 (67.50)		
	Middle + high differentiation (n = 36)	23 (57.50)	13 (32.50)		
TMN staging				18.150	<0.001
	Grades I + II (n = 43)	12 (30.00)	31 (77.50)		
	Grades III + VI (n = 37)	28 (70.00)	9 (22.50)		
Lymphatic invasion				11.430	<0.001
	Present (n = 45)	15 (37.50)	30 (75.00)		
	Absent (n = 35)	25 (62.50)	10 (25.00)		
Vascular invasion				0.050	0.823
	Present (n = 43)	21 (52.50)	22 (55.00)		
	Absent (n = 37)	19 (47.50)	18 (45.00)		
Distant metastasis				48.320	<0.001
	Present (n = 37)	3 (7.50)	34 (85.00)		
	Absent (n = 43)	37 (92.50)	6 (15.00)		

**Table 5:** Serum miR-1271 and GPC3's diagnostic value on LC before treatment

Index	miR-1271	GPC3
AUC	0.9227	0.9006
95%CI	0.8799–0.9655	0.8538–0.9474
Std. Error	0.0218	0.0239
Cut-off value	0.335	0.685
Sensitivity (%)	84.29	75.71
Specificity (%)	86.25	85.00

4.5 Correlation of miR-1271 and GPC3 with Survival of LC Patients

All patients were followed up for 5 years. The 5-year survival rate was 8%. The rate of the miR-1271 high-expression group was dramatically higher than that of the low-expression group. The rate of the GPC3 high-expression group was markedly lower than that of the low-expression group ($P < 0.05$) (Fig. 3).
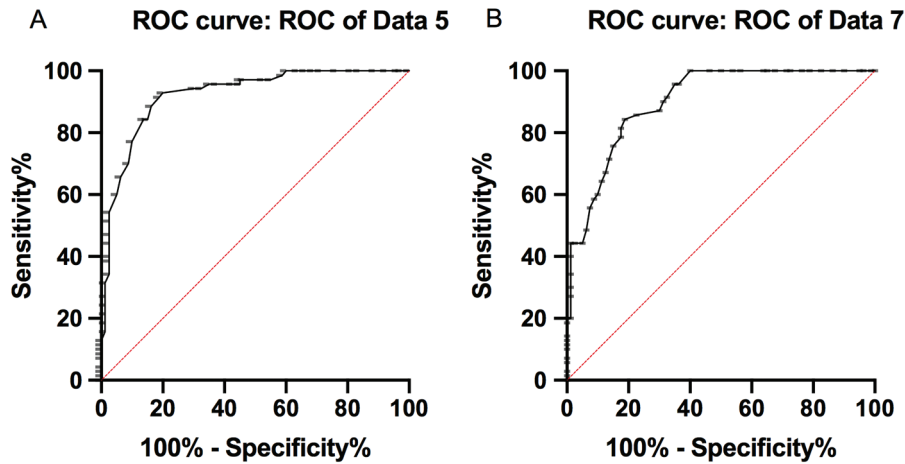
Figure 2: ROC of serum miR-1271 and GPC3 in diagnosing LC. (A) The sensitivity, specificity and AUC of miR-1271 alone in diagnosing LC patients were 84.29%, 86.25%, and 0.9227, respectively. (B) The sensitivity, specificity and AUC of GPC3 alone in diagnosing LC patients were 75.71% , 85.00%, and 0.9006, respectively
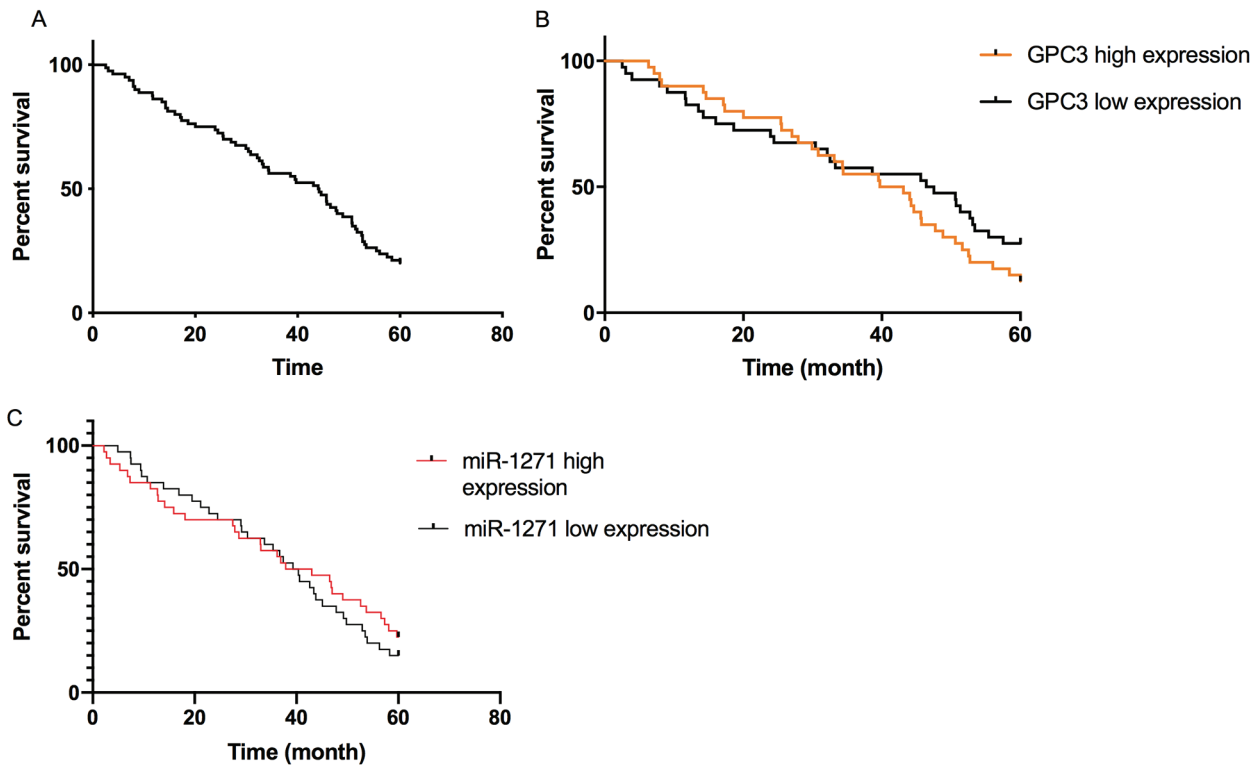
Figure 3: Correlation of miR-1271 and GPC3 with 5-year survival of LC patients. (A) Overall survival rate of LC patients. (B) The 5-year survival rate of the GPC3 high-expression group is lower than the low-expression group ($P < 0.05$). (C) The 5-year survival rate of the miR-1271 high-expression group is higher than the low-expression group ($P < 0.05$)

4.6 ROC Analysis of miR-1271 and GPC3 in Predicting Prognosis of LC Patients

On the basis of 5-year follow-up, LC patients were assigned into a survival group (SG, n = 15) and a death group (DG, n = 65) according to survival. The serum miR-1271 and GPC3 relative expression in the SG were (0.40 ± 0.10) and (0.70 ± 0.10), respectively, and those in the DG were (0.30 ± 0.10) and (0.86 ± 0.10), respectively. The relative expression of serum miR-1271 was markedly higher in the SG than the DG, while that of serum GPC3 was markedly lower in the SG (both $P < 0.001$). ROC of serum miR-1271 and GPC3 in diagnosing the prognosis of LC patients were drawn. The AUC, sensitivity, specificity, and cut-off value of serum miR-127 were 0.845, 83.08%, 73.33%, and 0.395, respectively, while the four of serum GPC3 were 0.893, 87.69%, 80.00%, and 0.755, respectively (Tab. 6, Fig. 4).

Table 6: Prognostic value of serum miR-1271 and GPC3 in LC patients before treatment

Index	miR-1271	GPC3
AUC	0.845	0.893
95%CI	0.7427–0.9475	0.7971–0.9895
Std. Error	0.0522	0.0491
Cut-off value	0.395	0.755
Sensitivity (%)	83.08	87.69
Specificity (%)	73.33	80.00

4.7 Cox Regression Analysis of 5-Year Survival of LC Patients

The 5-year survival of LC patients was assessed by Cox regression analysis, so as to observe independent factors affecting prognosis. The results showed that TMN staging, lymphatic invasion, distant metastasis and miR-1271 were independent factors affecting prognosis (Tab. 7).

5 Discussion

Currently, we are continuously looking for more sensitive and exact prognosis assessment methods for LC patients after TACE treatment [18,19]. Due to the lack of certain specificity of clinical manifestations of LC patients, conventional diagnostic methods can not provide a comprehensive and complete assessment of the prognosis of LC patients after TACE, resulting in poor prognosis. Therefore, it is critical to explore biomarkers relevant to diagnosis and prognosis [20].

miRNAs are endogenous, non-coding single-stranded RNAs that promote or suppress cancer [21,22]. Relevant studies on LC progression showed that miR-1271, as a new tumor suppressor, was significantly down-regulated in endometrial cancer, LC, prostate cancer and other diseases [23]. Furthermore, miR-1271 inhibits LC cell growth and induces apoptosis by targeting FOXQ1. It is reported that the GPC3 over-expression is related to miR-1271 down-regulation [24]. At present, there are few studies on the significance of miR-1271 and GPC3 expression changes on patient condition change and prognosis after TACE. We analyzed the differences of miR-1271 and GPC3 expression in serum of LC patients and healthy subjects firstly, and found down-regulated miR-1271. In contrast, GPC3 was up-regulated in LC. By analyzing the correlation of miR-1271 and GPC3 expression with clinicopathologic features of patients, we found that the expression was not associated with gender, age and position of lesion, but was related to vascular invasion, differentiation, TMN staging, lymphatic invasion and distant metastasis. Recently, researchers have focused on the biological therapy via regulating miRNAs. Current studies have shown that GPC3 is significantly abnormally expressed in both white blood cells and plasma of hepatocellular carcinoma. With the aggravation of TNM staging, serum GPC3 over-expression is more obvious [25]. However, miR-1271 is hardly expressed in adult normal liver cells and is expressed in LC

cells, negatively correlated with the disease condition [26]. Combined with ROC data, we believed that serum miR-1271 and GPC3 could been used in LC patients in evaluating the severity of the disease and the subsequent health monitoring. Then, we analyzed the expression of serum miR-1271 and GPC3 according to different prognosis, and found that the serum miR-1271 in the surviving patients was higher than that in the dead patients, while the relative expression of serum GPC3 was dramatically lower. In the end, we analyzed the prognostic factors affecting the 5-year survival of patients. It was found that TMN staging, lymphatic invasion, distant metastasis, miR-1271 and GPC3 were independent factor affecting 5-year survival prognosis. A previous research has revealed that TNM staging, lymphatic invasion, distant metastasis are independent prognostic factors affecting prognosis [27], but it is the first time that GPC3 and miR-1271 can be employed as independent prognostic factors. So, we preliminarily determined the clinical value of miR-1271 and GPC3, which two were expected to be potential diagnostic and prognostic indicators. Both miR-1271 and GPC3 were critical in clinical diagnosis and prognosis.
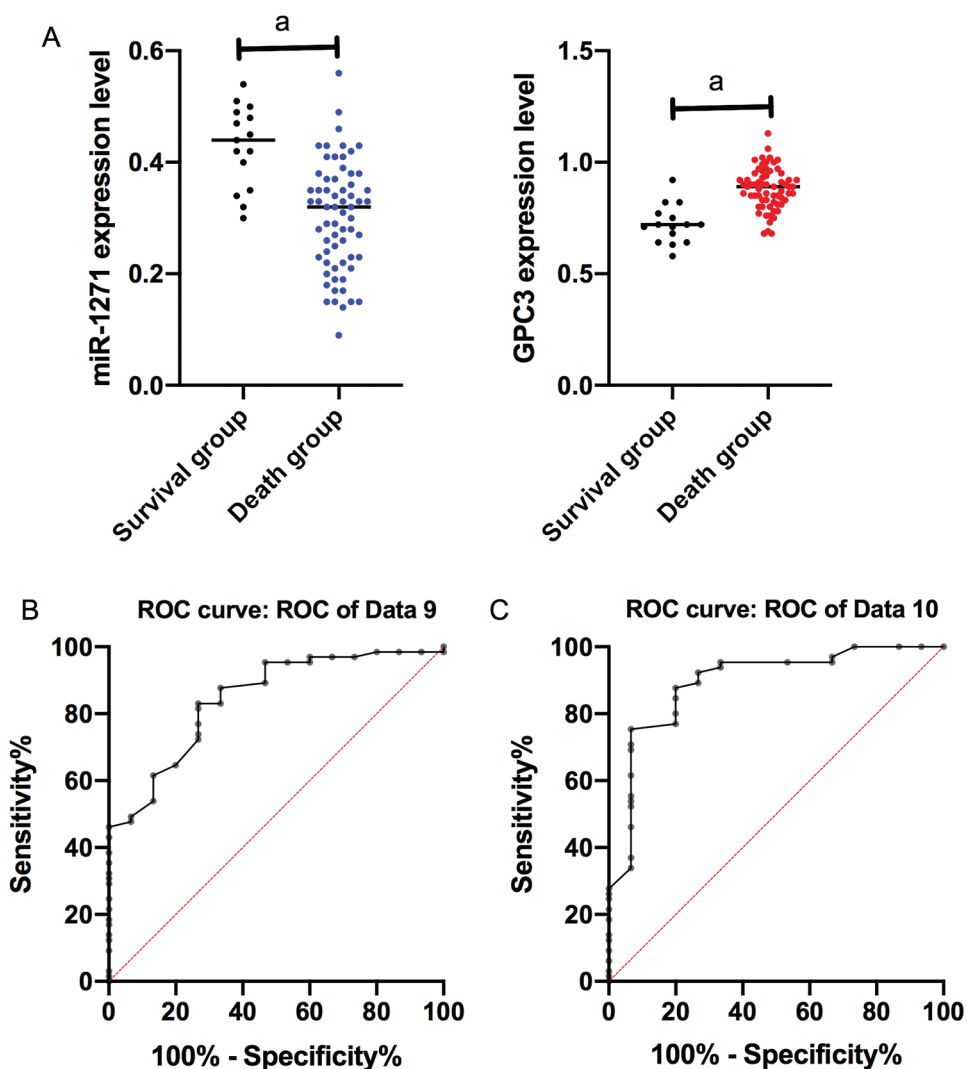
Figure 4: ROC of serum miR-1271 and GPC3 in diagnosing prognosis of LC patients. (A) Relative expression of serum miR-1271 and GPC3 in both groups (a represents $P < 0.001$). (B) The AUC, sensitivity, specificity and cut-off value of serum miR-1271 in diagnosing prognosis are 0.845, 83.08%, 73.33% and 0.755, respectively. (C) The AUC, sensitivity, specificity, and cut-off value of serum GPC3 in diagnosing prognosis are 0.893, 87.69%, 80.00% and 0.755, respectively

Table 7: Univariate and multivariate Cox regression analysis of 5-year survival of serum miR-1271 and GPC3 in LC

Factors	5-year univariate Cox		5-year multivariate Cox	
	P value	HR (95CI%)	P value	HR (95CI%)
Gender	0.732	0.819 (0.145–2.042)		
Age (years)	0.563	1.030 (0.482–4.208)		
Position of lesion	0.821	0.745 (0.326–1.998)		
Differentiation	0.003	0.318 (0.112–0.845)	0.800	0.834 (0.395–2.466)
TMN staging	0.001	3.599 (1.840–6.369)	0.001	8.371 (2.200–18.364)
Lymphatic invasion	0.000	0.140 (0.017–0.384)	0.000	0.246 (0.124–0.579)
Vascular invasion	0.366	1.408 (0.706–2.119)		
Distant metastasis	0.002	0.145 (0.040–0.601)	0.001	0.093 (0.014–0.290)
miR-1271	0.004	0.399 (0.247–0.860)	0.005	2.484 (1.205–9.366)
GPC3	0.005	0.401 (0.233–0.564)	0.005	2.462 (1.231–8.992)

We confirmed the expression and prognostic value of miR-1271 and GPC3 in LC patients, but there are still some limitations. For example, there was no specific analysis on the regulatory effect of miR-1271 and GPC3 expression on relevant white blood cells, and no further explanation of biological functions was given. Moreover, miR-1271, GPC3, and conventional clinical inflammatory factors were not analyzed, all of which had certain influence on the refinement of the study design. Hence, we will refer to the latest research in real time at the later stage to add corresponding research solutions, and make up for design flaws, so as to optimize the research.

All in all, miR-1271 was down-regulated in LC, while GPC3 expression was up-regulated. miR-1271 and GPC3 may be implicated in disease development and progression, and can be employed as potential serum biomarkers for TACE evaluation.

Conflicts of Interest: The authors declare that they have no conflicts of interest to report regarding the present study.

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