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Original Article

Investigation of microRNA-145 as a serum diagnostic and prognostic biomarker for gastric cancer: a Chinese cohort-based study

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Abstract: Background: Despite advances in technology, human gastric cancer (GC) remains a diagnostic challenge mainly because of its variable features and lack of reliable diagnostic method. MicroRNAs (miRNAs) are reported to regulate gene expression through modulating a wide range of pathophysiologic processes. Serum miRNAs are promising biomarkers in various human cancers, including GC. In the present study, we aimed to indentify microRNA-145 (miR-145) as a serum biomarker for GC screening in clinical. Methods: Serum miR-145 expression of 114 GC patients, 68 patients with precancerous lesion (PL) and 95 healthy donors was detected through qRT-PCR. Receiver operator characteristic (ROC) curve analysis was performed to evaluate the diagnostic value of serum miR-145 characterized as the alternative biomarker. Serum miR-145 levels were correlated with the clinicopathological characteristics and follow-up information of the GC patients. Multivariate regression analysis was finally conducted to confirm if serum miR-145 expression could be regarded as an independent risk factor for GC patients' prognosis. Results: Serum miR-145 levels were dramatically lower in the GC patients than in healthy controls and PL patients (all $P<0.001$). ROC curve analysis showed that serum miR-145 was a specific diagnostic biomarker for distinguishing GC patients from healthy individuals and PL patients. Low expression of serum miR-145 was significantly correlated to tumor size ($P=0.018$), distant metastasis ($P=0.039$), histologic differentiation ($P=0.016$) and TNM stage ($P=0.012$). GC patients with relatively lower serum miR-145 levels had remarkably decreased disease-free survival (DFS) rate and overall survival (OS) rate after surgery (all $P<0.01$). Multivariate regression analysis revealed that serum miR-145 level ($P=0.035$) was an independent risk factor for OS in GC. Conclusions: Our findings revealed that serum miR-145 level could be considered as a promising biomarker for GC diagnosis in the future.

Keywords: Circulating miRNA, serum, miR-145, gastric cancer, diagnosis, overall survival

Introduction

As one of the most common malignancies, gastric cancer (GC) is the second leading cause of cancer-related death, causing approximately 800,000 mortalities worldwide annually [1, 2]. In spite of great progress achieved in multiple therapeutic strategies, the morbidity rate of GC remains high in both China and Western countries [3]. The high mortality of GC is mainly due to delayed diagnosis because of the lack of appropriate biomarkers and specific early symptoms. Therefore, identification of novel effective biomarkers with high sensitivity and specificity is of great importance for the progress of diagnostic and prognostic techniques, and for the development of more efficient therapeutic strategies for GC patients.

Currently, microRNAs (miRNAs) have been uncovered to play crucial roles in a wide variety of cellular processes, such as differentiation, progression, apoptosis, and proliferation [4, 5]. MiRNAs are a kind of small, endogenous, noncoding RNAs with about 19-24 nucleotides in length that modulate gene expression through binding with target mRNAs and suppressing the translation or promoting mRNA degradation [6, 7]. Calin et al. reported that nearly half of the known human miRNA genes are located in cancer-associated genomic regions/fragile sites [8]. Aberrant expressions of miRNAs are closely

Table 1. The demographic characteristics of GC patients, PL patients and healthy individuals

Characteristics	GC patients (n=114)	PL patients (n=68)	Healthy individuals (n=95)	*P* value
Age (year)				0.942
<55	49 (43.0%)	31 (45.6%)	42 (44.2%)	
≥55	65 (57.0%)	37 (54.4%)	53 (55.8%)	
Gender				0.779
Male	65 (62.0%)	41 (60.3%)	52 (64.1%)	
Female	49 (38.0%)	27 (39.7%)	43 (35.9%)	
Nation				0.901
Han	106 (94.2%)	62 (91.2%)	88 (92.4%)	
Minority	8 (5.8%)	6 (8.8%)	7 (7.6%)	
Residence				0.650
Urban	60 (52.6%)	37 (%)	56 (58.9%)	
Rural	54 (47.4%)	31 (%)	39 (41.1%)	
Drinking status				0.390
Yes	73 (64.0%)	40 (58.8%)	52 (54.7%)	
No	41(36.0%)	28 (41.2%)	43 (45.3%)	

Table 2. The sequences of primers for qRT-PCR of miR-145 and U6

Gene name	Primer sequences
miR-145	
Forward	5'-AGTGCAGGGTCCGAGGTATT-3'
Reverse	5'-CGACGGTCCAGTTTTCCCAG-3'
U6	
Forward	5'-CGAGCACAGAATCGCTTCA-3'
Reverse	5'-CTCGCTTCGGCAGCACATAT-3'

associated withmultiple kinds of cancers, including GC [9, 10]. Intriguingly, several miRNAs, originally investigated in cells and tissues, could be easily detected in extracellular fluids, including plasma, serum and urine [11, 12]. MiRNAs have been considered as reliable biomarkers in various cancer diagnoses [13]. For example, it is previously revealed that down-regulated levels of miR-195 and miR-218 in serum have significant diagnostic value for GC [14, 15].

Overwhelming studies have revealed that miR-145 suppresses cell proliferation, migration and invasion through regulating various downstream targets [16-18], and mounting articles also demonstrated that serum miR-145 might be a useful noninvasive biomarker for the clinical diagnosis of several cancers, including non-small cell lung cancer [19] and ovarian cancer [20]. In the present case-control study, we aimed to measure the serum miR-145 expression levels of GC patients to assess their associations with clinical features and follow-up outcomes. We hypothesized that miR-145 could be used as a promising circulating biomarker of GC in routine clinical diagnosis.

Materials and methods

Study subjects and samples

The informed consents were obtained from all the participants or their relatives, and this study was approved by the ethics committee of the Sichuan Provincial People's hospital and the third People's hospital of Chengdu. 114 GC patients, 68 patients with precancerous lesion (PL) and 95 healthy individuals were recruited from the Sichuan Provincial People's hospital and the third People's hospital of Chengdu, and their serum samples were collected for this study. There were no differences among healthy individuals, PL patients and GC patients on age, gender, nation, residence and drinking status (all $P>0.05$). All patients were diagnosed as GC in histology and pathology and had not received chemotherapy or radiotherapy previously. After surgery, all GC patients were received FAM plan chemotherapy during the whole study. The demographic characteristics of these three groups of participants were recorded in **Table 1**.

Venous blood samples were collected from each participant in the morning. Serum was separated via a centrifugation at 1,600 g for 10 min at 4°C and a second centrifugation at 16,000 g for 10 min at 4°C. Then, supernatant serum was immediately stored at -80°C for further analysis.

RNA extraction from serum and qRT-PCR

Using QIAGEN miRNeasy Mini Kit (Qiagen, Valencia, CA, USA), the RNA was extracted from 100 μl serum. Reverse transcription of miR-145 was conducted through the instructions of the kit (QIAGEN, Valencia, CA, USA). All sequences of primers used in this study were listed in **Table 2**. The qRT-PCR system was 20 μl, con-

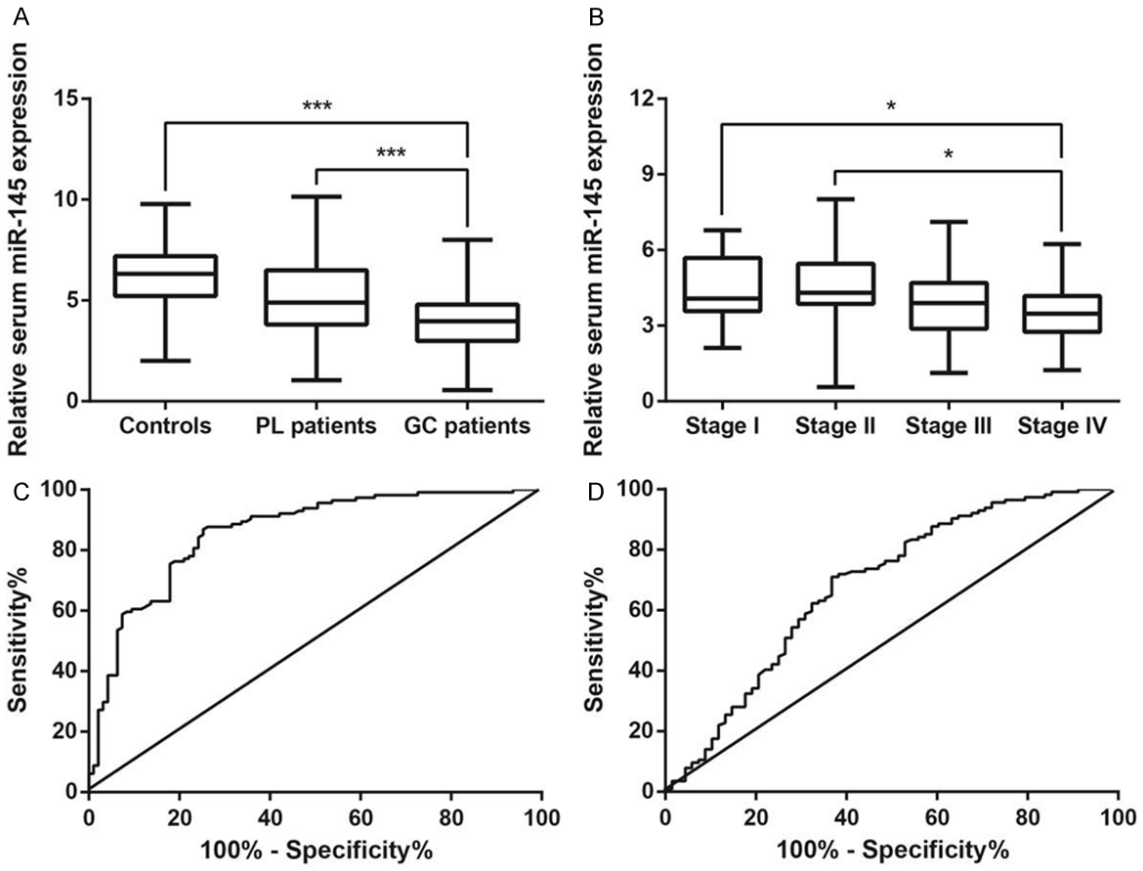

Figure 1. Serum miR-145 expression was markedly decreased in GC patients. A: Serum miR-145 expression was detected and compared among GC patients, PL patients and healthy controls. B: Serum miR-145 expression was detected and compared among GC patients with different TNM stage. C: ROC curve for evaluating the diagnostic value of serum miR-145 when the comparison was made between GC patients compared to healthy individuals (P<0.001; area under the ROC curve, 0.860; cutoff value, 5.28). D: ROC curve for evaluating the diagnostic value of serum miR-145 when the comparison was made between GC patients compared to PL patients (P<0.001; area under the ROC curve, 0.684; cutoff value, 4.61). ***P<0.001, *P<0.05.

taining 0.4 μl Forward Primer, 0.4 μl Reverse Primer, 2 μl cDNA, 0.4 μl ROX Reference Dye II, 10 ul SYBR Premix Ex Taq, and 6.8 ul nuclease-free water. qRT-PCR was performed by a 7300 qRT-PCR system (Applied Biosystems, Foster City, CA, USA) at 95°C for 10 min, followed by 40 cycles at 95°C for 15 s and 60°C for 1 min. Relative expression of miR-145 was calculated with ΔCt method. The housekeeping gene U6 was used as internal control in this study. The qRT-PCR reactions were performed in triplicate.

Receiver operating characteristic (ROC) curve

The expression levels of serum miR-145 of patients and controls were recorded. Then, ROC curve was drawn with the horizontal axis referring to the specificity and the vertical axis to the sensitivity. Diagnosis cut-off points and their specificity and sensitivity were calculated through the ROC curve. The accuracy of miR-145 in the diagnosis of GC was assessed by the area under the curve (AUC).

Statistical analysis

The data were presented as mean ± standard deviation (SD). Statistical analyses were conducted via SPSS 17.0 (Chicago, USA) and Graph PAD prism 6.0 (GraphPad Software, Inc., US). The optimal cut-off value for serum miR-145 was determined through receiver operating characteristics (ROC) analysis. The difference in qualitative variable was tested using Chi-square test, and Student's *t*-test was used to compare quantitative variable. Survival durations in the patient groups after surgery were

Table 3. Correlations between serum miR-145 levels and clinicopathological characteristics in 114 GC patients

Characteristics	Total number	Serum miR-145 expression		*P* value
		Low (n=56)	High (n=58)	
Age, years				0.725
<55	49	25 (44.6%)	24 (41.4%)	
≥55	65	31 (55.4%)	34 (48.6%)	
Gender				0.468
Male	69	32 (57.1%)	37 (63.8%)	
Female	45	24 (42.9%)	21 (36.2%)	
Drinking status				0.403
Yes	73	38 (67.9%)	35 (60.3%)	
No	41	18 (32.1%)	23 (39.7%)	
Tumor size, cm				0.018
<5	43	15 (26.8%)	28 (48.3%)	
≥5	71	41 (73.2%)	30 (51.7%)	
Distant metastasis				0.039
Yes	58	34 (60.7%)	24 (41.4%)	
No	56	22 (39.3%)	34 (58.6%)	
Lymph node invasion				0.227
Yes	71	38 (67.9%)	33 (56.9%)	
No	43	18 (32.1%)	25 (43.1%)	
Histologic differentiation				0.016
Well	14	7 (12.5%)	7 (12.1%)	
Moderate	40	15 (26.8%)	25 (43.1%)	
Poor	50	34 (60.7%)	16 (44.8%)	
TNM stage				0.012
I-II	35	11 (19.0%)	24 (41.4%)	
III-IV	79	45 (81.0%)	34 (58.6%)	

calculated with Kaplan-Meier survival curve and Long-rank test. Overall survival (OS) was assessed as the time from cancer diagnosis to death or date of last follow-up, and disease-free survival (DFS) was measured as the time from complete remission to treatment failure including relapse, death, or date at last follow-up. The joint effect of covariates was investigated with multivariate regression to verify whether serum miR-145 expression is an independent prognostic factor for OS of GC patients. A *P*-value of <0.05 was considered statistically significant.

Results

Expression level of serum miR-145 in GC and its effectiveness in diagnosis

The serum miR-145 levels were measured in an independent cohort with 114 GC patients, 68 PL patients and 95 healthy individuals. As exhibited in **Figure 1A**, compared with healthy individuals and PL patients, GC patients had markedly lower serum miR-145 expression (all *P*< 0.001), indicating that serum miR-145 expression was significantly reduced in GC. Next, to identify whether the expression of serum miR-145 was correlated with TNM stage of GC patients, the levels of serum miR-145 from the same 114 GC patients were analyzed based on their TNM stage. Our data revealed that the levels of serum miR-145 were dramatically decreased as the TNM stage elevated, and serum miR-145 expression was significantly lower in TNM IV stage patients than stage I, or II patients (all *P*<0.05) (**Figure 1B**).

Subsequently, ROC curve analysis was performed to determine the diagnostic value of serum miR-145 in GC screening. When a comparison was made between GC patients compared to healthy individuals, the AUC of ROC curve of serum miR-145 was 0.860 (95% CI: 0.809-0.911, *P*<0.001), with diagnostic threshold of 5.28, specificity of 73.68% and sensitivity of 87.72%, respectively (**Figure 1C**). When a comparison was made between GC patients compared to PL patients, the AUC of ROC curve of serum miR-145 was 0.684 (95% CI: 0.600-0.768, *P*<0.001), with diagnostic threshold of 4.61, specificity of 63.24% and sensitivity of 71.05%, respectively (**Figure 1D**). These data suggested that serum miR-145 might serve as a promising biomarker for distinguishing GC patients from healthy individuals and PL patients.

Relationship between clinicopathological characteristic and serum miR-145 level in GC

The average level of serum miR-145 was used as a cut-off point to allocate all the 114 patients into two groups: high serum miR-145 expression group (n=58; serum miR-145 level ≥ medi-

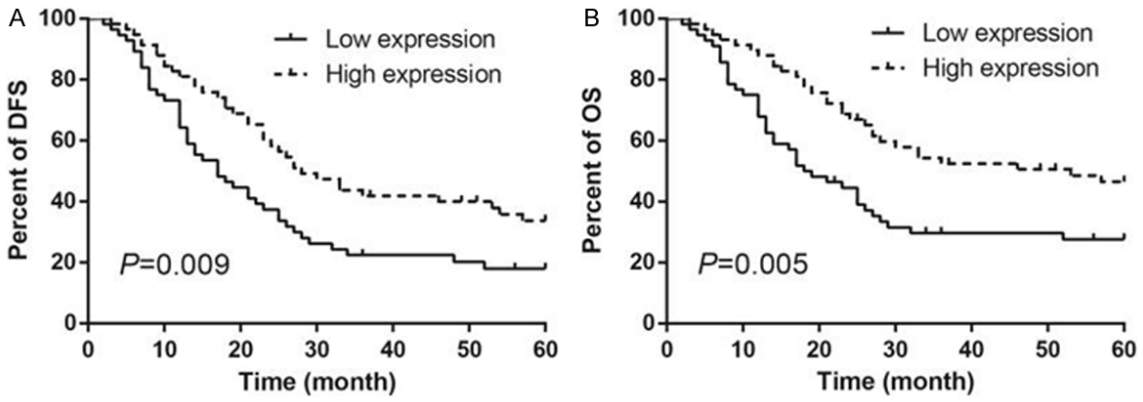

Figure 2. Kaplan-Meier survival curves of 5-year survival rates in GC patients subdivided by serum miR-145 levels. A: Correlation between serum miR-145 expression and DFS after surgery (*P*=0.009). B: Correlation between serum miR-145 expression and OS after surgery (*P*=0.005).

Table 4. Univariate regression analysis of clinicopathological features of 114 GC patients in relation to their OS

Characteristics	Category	*P* value	Exp (B)	95% CI for Exp (B)
Serum miR-145 level	Low vs. High	0.015	0.381	0.175-0.830
Age, years	<55 vs. ≥55	0.114	1.853	0.863-3.983
Gender	Male vs. Female	0.804	0.907	0.420-1.959
Drinking status	Yes vs. No	0.205	0.603	0.276-1.317
Tumor size, cm	<5 vs. ≥5	0.079	0.490	0.221-1.086
Distant metastasis	Present vs. Absent	0.032	0.429	0.197-0.931
Lymph node invasion	Present vs. Absent	0.082	0.502	0.231-1.092
Histologic differentiation	Poor vs. Well and Moderate	0.098	0.517	0.237-1.128
TNM stage	III-IV vs. I-II	0.008	0.327	0.144-0.746

Note: Exp (B), index of the regression coefficients; CI, confidence interval.

an level) and low serum miR-145 expression group (n=56; serum miR-145 level ≤ median level). The correlations between clinicopathological variables and serum miR-145 levels of 114 GC patients were recorded in **Table 3**. The data showed that reduced expression of serum miR-145 was closely correlated to tumor size (*P*=0.018), distant metastasis (*P*=0.039), histologic differentiation (*P*=0.016) and TNM stage (*P*=0.016), whereas no significantly associated with age (*P*=0.725), gender (*P*=0.468), drinking status (*P*=0.403) and lymph node invasion (*P*=0.227).

Low serum miR-145 levels decreased 5-year survival rates in GC patients

We further assessed whether serum miR-145 expression level correlated with prognosis of GC patients after surgery. DFS and OS curves were plotted according to serum miR-145 expression level by the Kaplan-Meier analysis and log-rank test, respectively. The results showed that, in comparison to those with higher serum miR-145 levels, GC patients with lower serum miR-145 levels had markedly shorter DFS (*P*=0.009; **Figure 2A**) and OS (*P*=0.005; **Figure 2B**) after surgery, which indicated that serum miR-145 might be regarded as a promising prognostic indicator for DFS and OS of GC patients.

Multivariate regression analysis of the prognostic powers of parameters in GC patients

To evaluate the role of clinicopathological characteristics as probable risk factors for prognosis, univariate analysis was performed to analyze nine clinicopathological variables in the 114 GC patients. As present in **Table 4**, the results indicated that distant metastasis (*P*=0.032), TNM stage (*P*=0.008) and serum miR-

Table 5. Multivariate regression analysis of clinicopathological features of 114 GC patients in relation to their OS

Characteristics	Category	*P* value	Exp (B)	95% CI for Exp (B)
Serum miR-145 level	Low vs. High	0.035	0.417	0.184-0.942
Distant metastasis	Present vs. Absent	0.211	0.589	0.257-1.349
TNM stage	III-IV vs. I-II	0.024	0.370	0.156-0.878

Note: Exp (B), index of the regression coefficients; CI, confidence interval.

145 level (*P*=0.015) were prognostic factors with statistical significance. Then, these three risk factors were further analyzed by multivariate analysis to determine independent factors for prognosis. As shown in **Table 5**, TNM stage (*P*=0.024) and serum miR-145 level (*P*=0.035) served as independent biomarkers of OS in GC patients.

Discussion

It is extensively acknowledged that cancers are usually diagnosed at a late stage with concomitant poor prognosis. The improvement of minimally invasive methods for the early detection of common tumors could dramatically decrease the serious health burden of cancers [21]. With the rapid development of microarray technology, a large number of miRNAs have been investigated and applied for cancer identification and classification, which definitely accelerate the exploration of novel miRNAs for GC diagnose.

Although tissue miRNAs can provide a relatively accurate diagnosis for various cancers, the difficulty in acquiring tissue specimens limits its wide application as cancer biomarkers. Collection of tissue specimens is an invasive process and mainly relays on surgical sections after initial clinical classification [22]. MiRNAs derived from epithelial tumors are rapidly released into the blood streamin forms of protein-bound complex [23] or membrane-bound vesicles, including microparticles exosomes [24]. Accumulating studies have demonstrated that in comparison to DNA and mRNA, circulating miRNAs, not only abundant in human bodily fluids, but also very stable, might be ideal biomarkers due to the convenience and the noninvasiveness of their detection in patients [25, 26]. Serum specimens could be easily acquired at different time points during the disease course and the circulating miRNAs could be detected via qRT-PCR analysis [27]. Accordingly, circulating miRNAs as potential minimally invasive indicators have crucial applications in cancer detection.

Previous studies have suggested that miR-145 expression is extremely down-regulated in various cancers, including lung cancer [28], gallbladder cancer [29], hepatocellular carcinoma [30] and GC. Takaqi et al. analyzed the tissues from 43 patients with GC showed that the expression of miR-145 were significantly down-regulated in most GC samples [31]. Since expression of serum miRNAs might mirror the clinicopathological characteristics of GC patients and associate with the aberrant pattern of the parental tumor, in the present study we compared the expression profile of miR-145 in sera from 114 GC patients with 68 PL patients and 95 healthy controls, and demonstrated that serum expression of miR-145 strongly differentiated the GC patients from healthy controls and PL patients. A highly significant decrease was detected in miR-145 in serum of GC patients compared with that of healthy individuals. Moreover, ROC analysis indicated that serum miR-145 could be a promising diagnostic biomarker for distinguishing GC patients from healthy individuals and PL patients. The down-regulation of serum miR-145 expression might indicate the probability of diagnosing GC; thus, serum miR-145 may be a candidate biomarker for GC screening in clinical.

We uncovered remarkable correlation between serum miR-145 expression and the clinicopathological status of GC patients, which are consistent with an inhibitory effect of miR-145 on cell proliferation, metastasis and invasion revealed by previous studies [32-34]. Another intriguing point of our research is that miR-145 expression in serum also functions as a potential prognostic biomarker for GC patients. Our findings revealed that lower serum miR-145 level was associated with unfavorable prognostic outcomes in GC patients. Furthermore, mul-

tivariable Cox proportional hazards model illustrated that decreased expression of serum miR-145 was an independent prognostic variable for OS of GC patients.

Our results are quite promising and compelling; however, the current study still has some limitations. A leading weakness of our research is the relative small size of samples in a single center; therefore, our data need to be further verified through a multi-center study in a larger cohort of patients before clinical application. Moreover, it is necessary to confirm the contribution of GC to serum miRNAs via *in vitro* assays. Particularly, miRNA release mechanisms, including secretion and necrosis, should be clarified in the near future.

In conclusion, our data revealed the level of serum miR-145 has potential predictive value as a novel GC biomarker and closely associates with tumor size, distant metastasis, histologic differentiation, TNM stage, prognosis of GC patients. The research of circulating miRNAs might help us find their potential values in clinical detection of various human diseases.

Disclosure of conflict of interest

None.

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