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Original Article

Decreased expression of serum miR-647 is associated with poor prognosis in gastric cancer

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Abstract: MicroRNAs (miRNAs) have been demonstrated to be critical players in different types of tumors including gastric cancer (GC). However, the expression level of serum miR-647 in patients with GC and its potential prognostic significance were poorly known. The aim of this study was to investigate the clinical significance of serum miR-647 in GC. A total of 105 patients with GC and 50 healthy volunteers were recruited into this study. Quantitative reverse-transcriptase polymerase chain reaction (qRT-PCR) was used to analyze the expression of serum miR-647. Diagnostic accuracy of serum miR-647 in distinguishing GC patients from healthy controls was assessed by the receiver operating characteristic (ROC) curve analysis. Chi-square was used to evaluate the association between serum miR-647 level and clinicopathologic parameters. Kaplan-Meier method was used to analyze the overall survival (OS) and relapse-free survival (RFS). Multivariate Cox proportional hazards analyses were further used to identify prognostic factors. Our results showed that a significantly downregulated expression of serum miR-647 was found in patients with GC. ROC curve analyses showed that serum miR-647 was highly efficient for discriminating patients with GC from healthy controls. In addition, low serum miR-647 expression was associated with aggressive clinical features and unfavorable survival in GC. Mechanistically downregulation of miR-647 in GC cell lines increased the expression levels of STX6, STX7, and PRKCA. In conclusion, our results demonstrate that serum miR-647 might serve as a novel serum biomarker for monitoring GC progression.

Keywords: Serum miR-647, biomarker, gastric cancer, prognosis, diagnosis

Introduction

Gastric cancer (GC) is one of the most common malignant tumors worldwide and is a serious threat to human life and health, with nearly 1 million new cases diagnosed each year [1]. The risk factors of GC are Helicobacter pylori infection, dietary habits, smoking, obesity, pernicious anemia and chronic atrophic gastritis and are associated with initiation and progression of gastric cancer [2, 3]. The survival outcome of patients diagnosed with early-stage GC have improved with prompt gastrectomy and radical therapy [4, 5]. However, GC usually is clinically silent and most cases are diagnosed at late stage, when effective treatment is very difficult. Therefore, the prognosis of GC remains unfavorable [6]. The initiation and development of GC is a complex multistep process involving the dysregulation of proto-oncogenes and tumor suppressor genes [7]. Exploring the biomarkers that can efficiently diagnose GC at early stage and predict the prognosis is very important for improving the overall survival of GC.

MicroRNAs (miRNAs) are evolutionarily conserved, small non-coding RNA molecules (approximately 18-25 nucleotides in length) that repress protein translation through binding to the 3' untranslated region (3'-UTR) of target mRNAs [8]. MiRNA research has expanded remarkably over the past few years and accumulating evidence suggest that miRNAs participate in essential biologic processes such as cell proliferation, cell cycle, apoptosis, cell differentiation, metastasis, angiogenesis and immune responses [9]. Abnormal miRNA expression has been reported to be involved in the pathogenesis and development of various tumors [10]. For example, miR-30a recently has

Table 1. miR-647 expression and clinical variables

Characteristics	Low serum miR-647 (n=56)	High serum miR-647 (n=49)	*P* value
Median age, years (range)	52 (24-73)	55 (23-78)	0.562
Gender, male/female	26/30	22/27	0.875
Differentiation			0.180
Well differentiated	7	12	
Moderately differentiated	18	10	
Poorly differentiated	31	27	
Tumor location			0.331
Cardia	7	8	
Body	18	21	
Antrum	31	20	
Depth of invasion			0.122
T1/T2	27	31	
T3/T4	29	18	
TNM stage			< 0.001
I/II	11	27	
III/IV	45	22	
Lymph node metastasis			0.008
Negative	22	32	
Positive	34	17	
Distant metastasis			0.015
Negative	43	46	
Positive	13	3	

been reported to be downregulated in gastric cancer cells [11, 12]. Previous studies revealed that miR-223 acted as a tumor suppressor by targeting JAK2 in GC [13, 14]. MiR-93 promotes cell proliferation and metastasis *in vitro* and tumor formation *in vivo* by targeting TIMP2 [15].

Although miR-647 has been found to be deregulated in different tumors including GC [16-20], the expression pattern and clinical significance of serum miR-647 in GC remain unclear. Therefore, the potential clinical value of serum miR-647 in GC merits further investigation.

Materials and methods

Patients and sample collection

This study was approved by the Ethics Committee of Qingdao Municipal Hospital and written informed consent was collected from each participant. In this study, a total of 105 patients who had a confirmed diagnosis of GC and 60 healthy controls were enrolled. None of the patients had received any chemotherapy or radiotherapy prior to the surgical treatment. Of 105 cases, 38 cases were diagnosed at stage I/II, and 67 cases were diagnosed at stage III/IV. Total gastrectomy was performed in 48 subjects, and partial gastrectomy was performed in the other 57 subjects. The clinicopathologic characteristics of these patients are presented in **Table 1**.

Up to 5 mL of venous blood was collected in EDTA-containing tubes from all the participants. Blood samples were centrifuged at 2000 g for 10 min with half an hour, followed by another centrifugation at 12000 g for 10 min. The supernatant was stored at -80°C for further use.

Cell culture

The gastric cancer cell lines SGC7901 and MKN45 were cultured in RPMI 1640 media supplemented with 10% fetal bovine serum (FBS), penicillin (100 U/mL), and streptomycin (100 μg/mL). The cells were incubated at 37°C in a humidified chamber containing 5% CO_2 and passaged when they reached 90-95% confluence.

Knockdown of miR-647 in GC cells

SGC7901 and MKN45 cells were transfected with miR-647 inhibitor (Sigma-Aldrich, St. Louis, MO, USA) or miRNA control (Sigma-Aldrich) using the RNAiMAX transfection regent (Invitrogen) according to the manufacturer's instructions. The miRNA oligos were incubated for 48 h before the further experiments.

RNA extraction and quantitative reverse-transcriptase polymerase chain reaction (qRT-PCR)

Total RNA was extracted from serum or cell samples by using Trizol reagent (Invitrogen Corp, Carlsbad, CA, USA). RNA concentration was assessed with a NanoDrop ND-1000 spectrophotometer (Thermo Scientific, Wilmington,

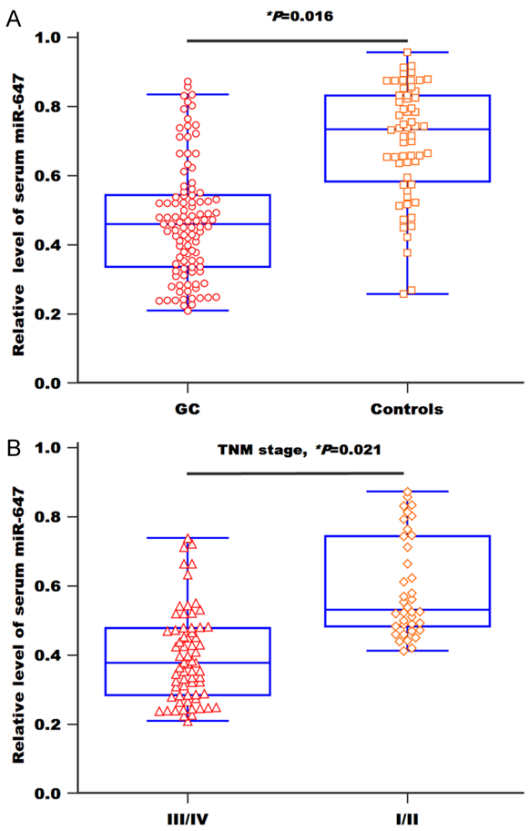

Figure 1. A. Serum miR-647 levels in GC patients were significantly lower than those in controls. B. Serum miR-647 levels in advanced stage GC patients were significantly lower than those in early stage GC.

DE). For the serum and cellular miR-647, the reverse transcription reaction was undertaken using a Taqman MicroRNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA). For the STX6, STX7, and PRKCA, first-strand complementary DNA synthesis was performed using the SuperScript III Reverse Transcriptase (Invitrogen, Carlsbad, CA). Quantitative PCR was carried out using Mx3005P qPCR System (Agilent, Santa Clara, CA, USA) according to the manufacturer's protocol. The PCR conditions were as follows: 95°C for 10 min, followed by 40 cycles of 95°C for 15 sec and 60°C for 60 sec. The cer-miR-39 was used as a control for miRNA normalization. Gene expression was normalized against glyceraldehyde 3-phosphate dehydrogenase (GAPDH). The relative expression level of serum miR-647 was calculated with the $2^{-\Delta\Delta Ct}$ method. All procedures were repeated three times independently.

Statistical analysis

The serum miR-647 expression level was compared between GC patients and normal controls using the Mann-Whitney U-test. The receiver operating characteristic (ROC) curve analysis and the area under the ROC curve (AUC) were used to analyze the diagnostic performance of serum miR-647 for GC. Correlations of serum miR-647 expression levels and various clinicopathologic factors were performed with the Chi-square test. The Kaplan-Meier method was used to assess the association between serum miR-647 level and overall survival (OS) as well as relapse-free survival (RFS). Multivariate Cox regression analysis was used to investigate the association between miR-647 expression and GC survival. OS was defined as the amount of time from the day of diagnosis to the date of death or the last follow-up. RFS was defined as the amount of time from the day of diagnosis to the date of relapse or the last follow-up. Statistical analyses were performed using statistical software MedCalc 18.6.0 (MedCalc, Mariakerke, Belgium). $P < 0.05$ was considered significant.

Results

Serum miR-647 expression is significantly decreased in GC patients

Quantitative RT-PCR was applied to detect the levels of serum miR-647 in patients with GC and normal controls. The results revealed that serum miR-647 expression levels were significantly downregulated in GC patients compared with normal controls (**Figure 1A**, P=0.016). In addition, serum miR-647 expression levels in advanced stage GC patients were greatly lower than those in early stage (**Figure 1B**, P=0.021).

Next, we measured the miR-647 expression levels in paired blood samples from all GC patients before and 10 days after surgery. **Figure 2** showed that serum miR-647 expression levels in the post-operative samples were dramatically upregulated compared to those in the pre-operative samples (P=0.037).

The diagnostic significance of serum miR-647 in gastric cancer

The ROC curve was employed to explore the potential diagnostic value of serum miR-647 for GC. Serum miR-647 could well discriminate GC patients from normal controls with high specificity and sensitivity (AUC =0.829; specificity =78.3%, sensitivity =80.0%, **Figure 3**).

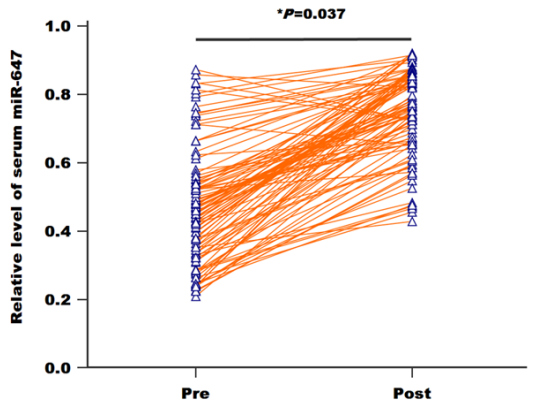

Figure 2. Serum miR-647 levels were significantly elevated after surgical treatment.

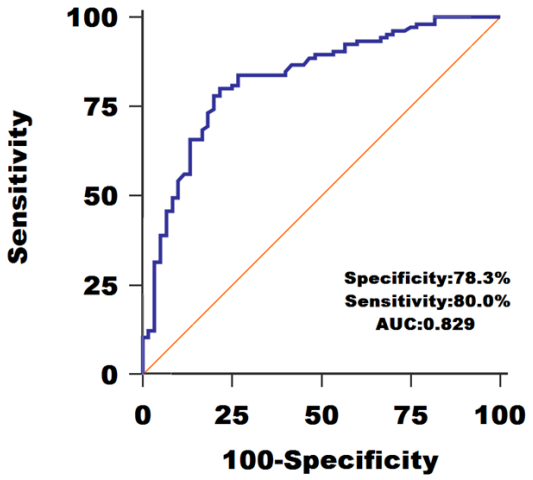

Figure 3. ROC curve plot can discriminate GC patients from controls.

The association between serum miR-647 expression and the clinical features of GC

GC patients expressing miR-647 at levels lower than the median serum miR-647 level were assigned to the low-expression group (n=56), and those cases with expression greater than the median serum miR-647 level were assigned to the high-expression group (n=49). As shown in **Table 1**, reduced serum miR-647 was closely associated with positive lymph node metastasis (P=0.008), advanced TNM stage (P < 0.001), and distant metastasis (P=0.015). However, no significant correlation was observed between serum miR-647 and age, gender, differentiation, tumor location, and depth of invasion (all P>0.05).

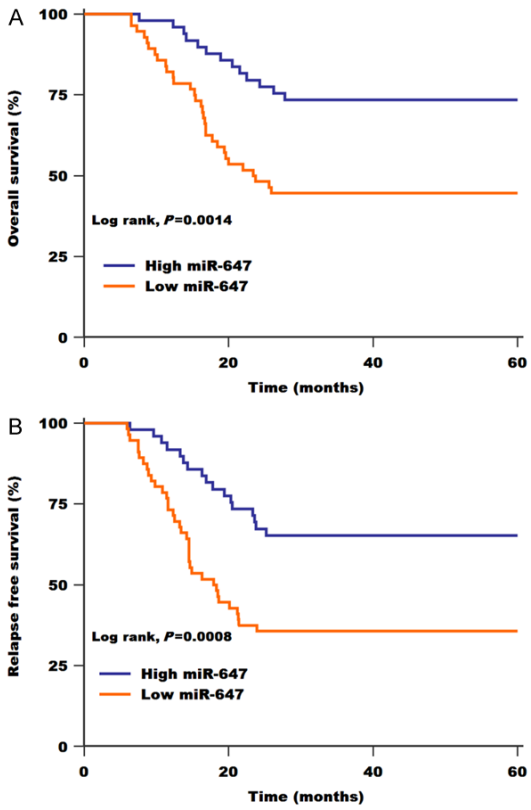

Figure 4. A. Kaplan-Meier survival curves of the overall survival. B. Kaplan-Meier survival curves of the recurrence-free survival.

The association between serum miR-647 expression and the prognosis of GC

Our survival analysis showed that GC patients in the low serum miR-647 expression group displayed shorter OS (**Figure 4A**, P=0.0014) and RFS (**Figure 4B**, P=0.0008) than those in the high serum miR-647 expression group. In a multivariate analysis, TNM stage (HR=5.34, 95% CI=1.47-9.23, P=0.015), lymph node metastasis (HR=3.76, 95% CI=1.13-6.45, P=0.046), distant metastasis (HR=3.42, 95% CI=1.06-5.95, P=0.061) and serum miR-647 expression (HR=4.54, 95% CI=1.38-7.81, P=0.028) were independent prognostic indicators for predicting shorter OS in GC (**Table 2**).

Validation of the downstream targets of miR-647 in GC

To explore the potential molecular mechanisms accounting for the tumor suppressive role of miR-647 in GC, we first obtained the validated targets of miR-647 from miRWalk2.0 (http://

Table 2. Multivariate Cox regression analyses for overall survival and relaps- free survival

Factors	Overall survival		
	Hazard Ratio	95% CI	*P*
TNM stage			
III/IV *vs* I/II	5.34	1.47-9.23	0.015
Lymph node metastasis			
Positive *vs* Negative	3.76	1.13-6.45	0.046
Distant metastasis			
Positive *vs* Negative	3.42	1.06-5.95	0.061
Serum miR-647			
Low *vs* High	4.54	1.38-7.81	0.028

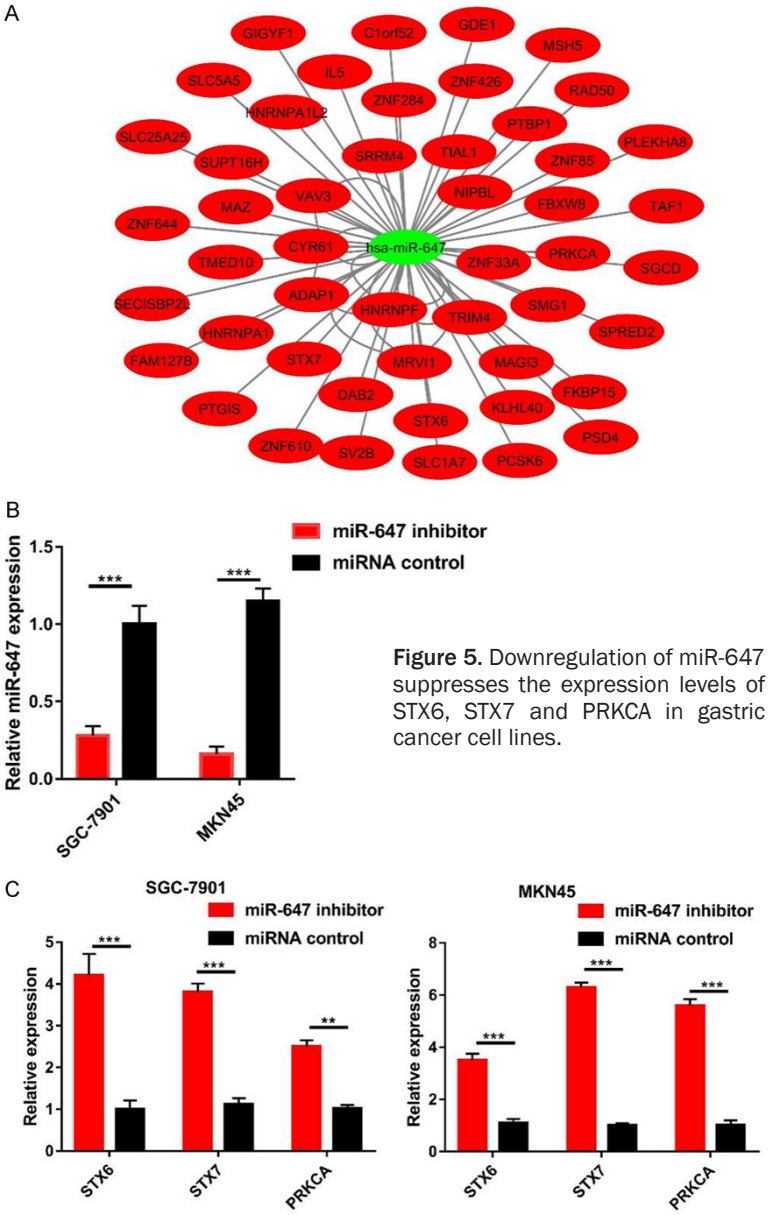

Figure 5. Downregulation of miR-647 suppresses the expression levels of STX6, STX7 and PRKCA in gastric cancer cell lines.

zmf.umm.uni-heidelberg.de/apps/zmf/mirwalk2/). **Figure 5A** showed all the validated targets of miR-647. The expression level of miR-647 was significantly lower in gastric cancer cells transfected with miR-647 inhibitor compared to those transfected with miR-647 control (**Figure 5B**). Our results showed that downregulation of miR-647 suppressed the expression levels of *STX6*, *STX7,* and *PRKCA* in two gastric cancer cell lines (**Figure 5C**).

Discussion

This study investigated the potential clinical utility of serum miR-647 to serve as a noninvasive diagnostic and prognostic biomarker in patients with GC. We found that serum miR-647 expression was significantly lower in GC patients than that in normal individuals. In addition, serum miR-647 levels in post-operative samples were significantly re-elevated compared with the pre-operative samples, indicating that serum miR-647 levels might be useful for monitoring the therapeutic responses of GC. ROC curve analysis revealed that serum miR-647 was able to discriminate between GC cases and normal controls with relatively high accuracy, suggesting that serum miR-647 might be a promising biomarker for the detection of GC. Moreover, the downregulation of miR-647 was strongly correlated with aggressive clinicopathologic features and worse survival. Multivariate analysis demonstrated that low serum miR-647 expression, was an independent prognostic factor for OS in GC. These data

indicated that serum miR-647 might serve as a valuable biomarker for the diagnosis and prognosis of GC. Mechanistically, downregulation of miR-647 in GC cell lines increased the expression levels of *STX6*, *STX7* and *PRKCA*, indicating reduced miR-647 might promote GC progression through upregulating SNARES proteins. STX6 and STX7 are the components of SNARES proteins. SNARES proteins are very important for the cellular communication and substance transportation between cancer cells to cancer cells in the tumor microenvironment [21]. *PRKCA*, which encodes PKC-α, is a known regulator for tumorigenesis [22]. Consistent with our findings, miR-647 was decreased in GC tissues from patients with metastasis and in the vincristine-resistant GC cell line. Enforced miR-647 expression suppressed gastric cancer cell migration and invasion and sensitized tumors to chemotherapy *in vivo*, indicating miR-647 functioned as a tumor suppressor in GC [23]. Similarly, Ye et al reported that miR-647 is downregulated in GC. Restoration of miR-647 by exogenous transfection suppresses cell migration and invasion by targeting SRF/MYH9 axis [24].

MiR-647 has also been shown to function as a tumor suppressor in other types of cancer. For instance, miR-647 is highly expressed in non-small lung cell carcinomas (NSCLC) that have been subjected to argon-helium cryoablation treatment, compared with those that underwent other treatments. In addition, overexpression of miR-647 inhibited the proliferation of NSCLC cells by delaying G1/S phase transition through targeting TNF receptor-associated factor 2 and the NF-κB pathway, suggesting miR-647 might play a tumor suppressive role in tumorigenesis of NSCLC [25]. MiR-647 was overexpressed in Taxol-resistant ovarian cancer cells compared with Taxol-sensitive ovarian cancer cells, suggesting that miR-647 level might be associated with chemoresistance of ovarian cancer cells. Interestingly, for the Taxol sensitive ovarian cancer patients, upregulated miR-647 expression was associated with favorable overall survival [17]. A panel of twelve biomarkers including miR-647 could discriminate prostate patients with and without biochemical recurrence with high accuracy. Interestingly, miR-647 was negatively correlated with the recurrence of prostate cancer following surgery in the Cox proportional hazards model, suggesting that reduced miR-647 might be associated with prostate cancer recurrence [16]. However, in Patnaik's study no significant difference was found in the miR-647 expression between localized stage I non-small cell lung cancer patients with recurrence and those without recurrence [20].

The current study has several limitations which must be pointed out. First, our sample size was relatively small. Further studies are required with a larger cohort, which may help validate our findings. Secondly, although our evidence has shown that serum miR-647 was reduced in patients with GC, the potential mechanisms accounting for its downregulation in the circulation need further investigation. In addition, the underlying molecular mechanisms responsible for the tumor suppressive role of miR-647 in GC also warrant exploration.

In conclusion, our study has demonstrated that serum miR-647 is downregulated in patients with GC. In addition, low serum miR-647 is significantly correlated with various unfavorable clinicopathologic parameters. Therefore, serum miR-647 might be a promising biomarker for GC diagnosis and prognosis.

Disclosure of conflict of interest

None.

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