Prognostic value of miR-93 overexpression in resectable gastric adenocarcinomas

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Abstract

Background and study aims : **MicroRNAs (miRNAs) have been shown to be aberrantly expressed in many human carcinomas. Emerging evidence indicates that miR-93 plays important oncogenic roles in human carcinogenesis and is often up-regulated. However, its relationship with the clinicopathological features and prognosis of human gastric cancer (GC) has yet to be addressed. In this study, we investigate the expression and clinical significance of miR-93 in human gastric cancer.**

Patients and methods : **158 patients with gastric adenocarcinoma who had undergone gastrectomy were enrolled. Specimens including the tumor and non-neoplastic were detected for the expression of miR-93 by Real-Time reverse transcription-polymerase chain reaction (RT-PCR). Furthermore, the correlation of miR-93 levels with clinicopathologic variables and prognosis was analyzed.**

Results : **miR-93 was significantly up-regulated in 128 cases (81%) of the 158 gastric cancer ($P < 0.05$). Furthermore, the elevated expression of miR-93 was significantly associated with advanced disease stage ($P < 0.001$), deep invasion level ($P < 0.001$) and the presence of nodal metastases ($P < 0.001$). Moreover, gastric cancer patients with high miR-93 expression levels had shorter overall survival ($P = 0.001$) and disease-free survival ($P = 0.006$) than that with low miR-93 expression levels.**

Conclusions : **miR-93 is highly elevated in gastric cancer, especially in advanced and metastasized gastric cancer, suggesting miR-93 may play critical roles in carcinogenesis of gastric cancer. Overexpression of miR-93 can serve as a novel prognostic marker for gastric cancer.** (Acta gastroenterol. belg., **2012**, 75, **22-27**).

Key words : miR-93, miRNA, gastric cancer, clinicopathological features, prognosis.

Introduction

Gastric cancer (GC) ranks as the second leading cause of cancer related mortality worldwide, though the incidence has decreased dramatically in some developed countries over the past decades (1,2). According to the literature, gastric cancer has a higher incidence rate in East Asia, especially in china, with almost 50% of worldwide new cases occur every year (3,4). In the past decades, with advances in chemotherapy and surgical techniques, the outcomes of gastric cancer have been improved dramatically. However, the long-term prognosis of gastric cancer patients remains poor, and the overall 5-year survival rate is lower than 40% (6,7). For patients in advanced stages, the 5-year survival rate is only about 20% (7). Thus, it is still necessary to search novel prognostic markers which accurately represent biological characteristics of tumors, and predict individual risk of recurrence and subsequent prognosis so as to improve the clinical management of gastric cancer. Abundant studies have indicated that disease stage, depth of invasion and lymph node involvement are the most important prognostic factors in gastric cancer (7-9). Moreover, some molecular markers also have been identified and attempted to use clinically (10,11). Nevertheless, other potential prognostic factors related to survival of patients with gastric cancer remain unclear.

MicroRNAs (miRNAs), the small non-coding RNAs (19-25nt in length), can regulate the gene expression either at the transcriptional or at the translational levels, by base pairing interactions with the 3'-untranslated region (UTR) of their target mRNAs, result in the degradation of target mRNAs or repression of their translation (12-14). miRNAs play crucial roles in various physiological and pathological processes, including metabolism, proliferation, differentiation, apoptosis and angiogenesis (12,15). Emerging studies have demonstrated that miRNAs are frequently deregulated in human cancers, including gastric cancer, indicating the involvement of miRNAs in human carcinogenesis (16). Tsukamoto *et al*. found that miR-375 was downregulated in gastric carcinoma and its ectopic expression markedly reduced cell viability by directly target PDK1 (17). Furthermore, miRNAs have shown promise as tissue-based markers for cancer classification and prognostication (18). For example, Li *et al*. identified a seven-miRNA signature by miRNA expression profile, and found that this signature was an independent predictor of overall survival and relapse-free survival (19).

Recently, miR-93 has been linked to human cancer. Fang *et al*. found that miR-93 could promote tumor growth and angiogenesis by suppressing, at least in part, integrin-β8 expression (20). Another study showed the expression of miR-93 was necessary for cell proliferation and for anchorage-independent growth (21). These data suggested that miR-93 had important roles in carcinogenesis. Additionally, overexpression of miR-93 has been found in a broad range of cancers, including neuroblastoma, non-small cell lung cancer and hepatocellular carcinoma (22-24). However, the expression of miR-93 in human gastric cancer and its clinical relevance has not been systematically studied yet, and whether miR-93

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expression has influence on prognosis of gastric cancer is still unknown. So, in this study, we investigate the feasibility of miR-93 as a novel prognostic biomarker for HCC.

Patients and Methods

Patients and Tissue Specimens

A total of 158 patients with gastric adenocarcinoma who underwent curative surgery at Xiangya Hospital (Changsha, Hunan, People's Republic of China) from January 2006 to December 2007 were enrolled in this study. Patients with unresectable tumors, history of previous chemotherapy, radiation therapy, or gastric surgery were excluded from the study. All patients enrolled in this study were confirmed pathologically as having gastric adenocarcinoma. There were 110 males and 48 females in this series with a median age of 55 years (range : 26-77). All the patients was given the radical resection and D1+or D2 lymphadenectomy followed by adjuvant chemotherapy with the regimen ECF (Epirubicin, cisplatin and 5-FU), as described before (8). And no preoperative therapy was given for all the patients. Gender, age, tumor location, tumor differentiation, TNM stage, tumor invasion depth and regional lymph nodes metastasis were recorded for each patient in a database. pTNM classification followed the criteria of the 6th edition of the UICC. The clinical and histopathologic data of these patients were summarized in Table 1.

Matched fresh specimens of tumor tissue and non-neoplastic tissue from all patients were collected. Non-neoplastic tissue was defined as the normal gastric tissue at a distance of at least 5 cm from the tumor. All these tissues were immediately frozen in liquid nitrogen and stored at -80°C.

Informed consent was obtained from all subjects and this study was approved by the Review Board of Hospital Ethics Committee.

Table 1. — **Clinical and histopathologic data of the patients**

Variables	Number of cases (%)
Number of patients	158 (100%)
Gender	
Male	110 (69.6%)
Female	48 (30.4%)
Age (years)	
≤ 60	89 (56.3%)
> 60	69 (44.7%)
Tumor location	
Cardia	30 (19.0%)
Gastric body	45 (28.5%)
Gastric fundus	83 (52.5%)
Tumor differentiation	
Undifferentiated	25 (15.8%)
Poorly differentiated	50 (31.6%)
Moderately differentiated	60 (38.0%)
Well differentiated	23 (14.6%)
UICC stage	
Stage I	20 (12.6%)
Stage II	36 (22.8%)
Stage III	60 (38.0%)
Stage IV	42 (26.6%)
Primary tumor	
T1-2	28 (17.7%)
T3-4	130 (82.3%)
Regional lymph nodes	
N0	48 (30.4%)
N1-3	110 (69.6%)

RNA isolation

Total RNA was extracted from frozen tissue specimens (50-100 mg) using mirVana™ miRNA isolation kit (Ambion, Austin, USA) according to the manufacturer's instructions. The quality of RNA was determined by using the agarose gel analysis, and the concentration was measured with a spectrophotometer (Biochrom, Cambridge, England) and then pretreated with RNase-free DNase I (Promega, Madison, USA) to eliminate potential DNA contamination.

Quantitative RT-PCR of mature miRNAs

The mature miR-93 was measured by using TaqMan MicroRNA detection kit (Applied Biosystems, Foster, CA), according to the instructions provided by the manufacturer. RNA samples were reversely transcribed with miRNA-specific primers from TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems). Real-time polymerase chain reaction (PCR) was performed with a TaqMan probe, which ensures discrimination of even one nucleotide difference, by using 7300 sequence detection system (Applied Biosystems). The relative expression levels of miR-93 were calculated and normalized by U6 small nuclear RNA using the comparative ΔCt method and the equation $2^{-\Delta Ct}$ as described previously (25).

Follow-up

All patients in this series entered a follow-up program. The follow-up was performed as described previously (26). Within the first 2 years after surgery, the routine examination, including blood tests, abdominal ultrasonography or CT scan was done every 3 months. And endoscopy was performed every 6 months. Whereas, for the next several years, patients were followed up every 6 months and underwent endoscopy every 12 months. The recurrence was defined as local relapse or metastasis at distant sites. The follow-up end point was defined as the recurrence or metastasis of the cancer. The median follow-up time was 20 months (range, 2-48 months).

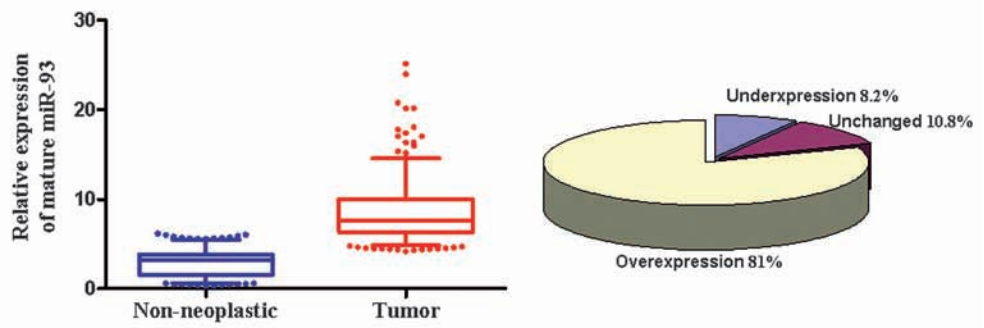

Fig. 1. — miR-93 is frequently up-regulated in gastric cancer. The expression level of mature miR-93 in gastric cancer tissues and corresponding non- non-neoplastic tissues was detected by using real-time RT-PCR. Box-plot lines represent medians and interquartile ranges of the normalized threshold values ; whiskers and spots indicate 10-90 percentiles and the remaining data points. Pie chart represents the percentage of the gastric cancer cases with different miR-93 expression type.

Statistical analysis

SPSS13.0 for Windows (Chicago, Ill, USA) was used for statistical analysis. The Mann-Whitney test was performed to determine the significance of miR-93 levels. Spearman rank-correlation analysis was used to analyze the correlation between miR-93 expression levels and clinicopathologic characteristics in patients with gastric cancer. Survival curves were plotted using the Kaplan-Meier method and were analyzed using the log-rank test. All tests were 2-tailed, and $P < 0.05$ was considered statistically significant.

Results

miR-93 is frequently upregulated in gastric cancer

We first determined the expression of miR-93 in 158 cases of gastric cancer tissues and corresponding non-neoplastic tissues by using real-time quantitative RT-PCR. The results showed that the expression of miR-93 is significantly upregulated in gastric cancer tissues (median expression level : 7.25, range 5.28-25.13) when compared with non-neoplastic tissues (median relative expression level : 3.09, range 0.20-6.19 ; $P < 0.05$, Fig. 1). Furthermore, upregulation of miR-93 was obtained in 81% (128/158) of the cases, whereas, 8.2% (13/158) of the cases showed underexpression of miR-93 and 10.8% (17/158) of the cases showed no difference (Fig. 1). These results suggested that miR-93 may play an important role in the pathological process of gastric cancer.

miR-93 expression levels correlate with clinicopathological features of gastric cancer

For better understanding of the clinical relevance of miR-93 expression in gastric cancer, we divided the 158 patients with gastric cancer into miR-93 high expression group (n = 90) and miR-93 low expression group (n = 68), according to the median expression level of miR-93 (7.25). Then, we analyzed the relationship of miR-93 expression with various clinical features of gastric cancer. As summarized in Table 1, the results showed that high level of miR-93 expression was associated with advanced disease stage ($P < 0.001$), deep invasion level ($P < 0.001$) and the presence of nodal metastases ($P < 0.001$). However, there was no significantly correlation of miR-93 expression with other clinical features, such as gender, age, tumor location and tumor differentiation ($P > 0.05$, Table 2).

miR-93 expression levels correlate with prognosis of patients with gastric cancer

To confirm the prognostic value of miR-93 overexpresson, Kaplan-Meier method was used to analyze the relationship of miR-93 expression level and the prognosis of patients with gastric cancer. The results indicated that patients who had high miR-93 expression had a shorter overall survival and disease-free survival than patients who had low miR-93 expression (median overall survival, 29 months vs. 38 months, $P = 0.001$, Fig. 2A ; median disease-free survival, 23 months vs. 36 months, $P = 0.006$, Fig. 2B).

Discussion

The occurrence and development of GC correlated with various molecular and genetic incidents. To investigate the significance of the molecular expression in GC may help us to identify potential treatment target and (or) predictive marker of prognosis and treatment response. Analyses of miRNA expression profiles have shown that many microRNAs are expressed aberrantly in various tumors, including gastric cancer. By using miRNA microarray, Ueda *et al*. found that in 160 paired samples of non-tumour mucosa and cancer, 22 miRNAs were upregulated and 13 miRNAs were downregulated in gastric cancer (27). miRNAs play important roles in gastric cancer as oncogenes or tumor suppressor genes,

Table 2. — **Expression of miR-93 in correlation with clinicopathologic variables**

Clinicopathologic variables	miR-93 expression level		*P Value*
	Low expression Number of cases (%)	High expression Number of cases (%)	
Gender			
Male	48 (43.6%)	62 (56.4%)	0.819
Female	20 (41.7%)	28 (58.3%)	
Age (years)			
≤ 60	37 (41.6%)	52 (58.4%)	0.674
> 60	31 (44.9%)	38 (55.1%)	
Tumor location			
Cardia	12 (40.0%)	18 (60.0%)	0.841
Gastric body	20 (44.4%)	25 (55.6%)	
Gastric fundus	36 (43.4%)	47 (56.6%)	
Tumor differentiation			
Undifferentiated	10 (40.0%)	15 (60.0%)	0.859
Poorly differentiated	22 (44.0%)	28 (56.0%)	
Moderately differentiated	26 (43.3%)	34 (56.7%)	
Well differentiated	10 (43.5%)	13 (56.5%)	
UICC stage			
Stage I	14 (70.0%)	6 (30.0%)	< 0.001
Stage II	20 (55.6%)	16 (44.4%)	
Stage III	23 (38.3%)	37 (61.7%)	
Stage IV	11 (26.2%)	31 (73.8%)	
Primary tumor			
T1-2	21 (75.0%)	7 (25.0%)	< 0.001
T3-4	47 (36.2%)	83 (63.8%)	
Regional lymph nodes			
N0	31 (64.6%)	17 (35.4%)	< 0.001
N1-3	37 (34.6%)	73 (66.4%)	

and correlate with tumorigenesis, progression, and prognosis of gastric cancer. Let-7f which act as tumor suppressors in many cancers, was decreased in metastatic gastric cancer tissues, and overexpression of let-7f could inhibit invasion and migration of gastric cancer cells through directly targeting the tumor metastasis-associated gene MYH9 (28). Whereas, miR-126 was elevated in gastric cancer, and overexpression of miR-126 contribute to gastric carcinogenesis by targeting SOX2, suggesting its oncogenic activity (29). Considering the fact that miRNAs can posttranscriptionally regulate almost 30% of human genes (30), miRNAs had becoming a hotspot of cancer research as biomarkers therapy targets.

miR-93 is a member within the miR-106b□25 cluster (31). Amplification and overexpression of the miR-93 has been reported, with pointers to functional involvement in the development of many human cancers. Kim *et al*. found that miR-93 can target p21 and ectopic expression of miR-93 resulted in activation of Cdk2 and facilitation of G1/S phase transition of gastric cancer cells (32). These data indicated that miR-93 possessed oncogenic activities in malignancies (20). However, until now, studies about the expression and clinical relevance of miR-93 in human cancer tissues are limited (33). Furthermore, the relationship of miR-93 expression with the clinicopathological features and prognosis of gastric cancer remains unknown.

In the present study, we firstly measured miR-93 expression levels in 158 paired gastric cancer tissues and corresponding non-neoplastic tissues. Our results revealed that miR-93 was significantly up-regulated in 128 cases (81%) of the 158 gastric cancer tissues. These results were consistent with the previous studies in other cancer tissues (22-24,33). However, the mechanism by which miR-93 was upregulated in gastric cancer is still unclear. One study showed that miR-106b-25 cluster was activated by E2F1 in parallel with its host gene – Mcm7, suggesting miR-93 maybe transcriptional regulated by E2F (34). Further studies were needed to investigate the detailed mechanism of miR-93 overexpression in human cancers.

Then, we analyzed the clinical relevance of miR-93 overexpression in gastric cancer. We observed that increased expression of miR-93 was correlated with

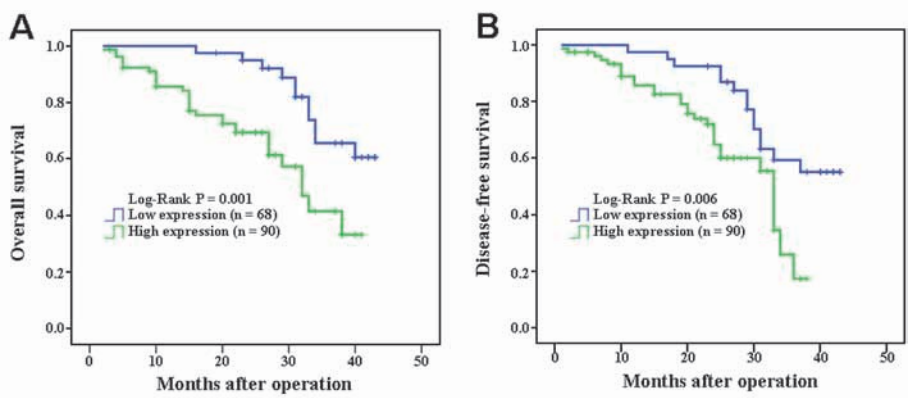

Fig. 2. — miR-93 associates with prognosis of patients with gastric cancer. According to the median expression level of miR-93, the patients with gastric cancer were divided into low miR-93 expression group and high miR-93 expression group. Survival curves of overall survival (A) and disease-free survival (B) were constructed using the Kaplan-Meier method and evaluated using the log-rank test.

advanced disease stage, deep invasion level, the presence of nodal metastases, shorter overall survival and disease-free survival of gastric cancer, suggesting miR-93 could serve as a novel prognostic marker for gastric cancer. As everyone knows, the prediction of recurrence, metastasis, and prognosis of patients with gastric cancer after resection is an important clinical issue that could determine the surgical therapeutic regimen. The disease stage, depth of invasion and lymph node involvement have been proved as the most important prognostic factors in gastric cancer (7-9). Besides these clinicopathological features, recent findings have exposed miRNAs expression profiles as potential biomarkers for gastric cancer. Katada *et al*. found that the probability of survival was significantly lower in gastric cancer patients with high expression levels of mir-20b or 150 (36). Furthermore, down-regulation of miR-451 was associated with worse prognosis of patients with gastric cancer (37). Recently, advances in circulating miRNAs research have generated the concept that circulating miRNAs are stably present and reproducible among individuals (38), suggesting circulating miRNAs can serve as useful biomarkers for diagnosis and prognosis of cancer.

In summary, this study demonstrated that miR-93 was frequently upregulated in gastric cancer, and overexpression of miR-93 was correlated with poor prognosis of patients with gastric cancer, suggesting that miR-93 may serve as a novel prognostic marker for gastric cancer.

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