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Identification of miR-146b-5p in tissues as a novel biomarker for prognosis of gallbladder carcinoma

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Abstract. – OBJECTIVE: Increasing evidence has suggested that dysregulation of microRNAs (miRNAs) could contribute to tumor progression. The aim of present study was to evaluate the feasibility of using miR-146b-5p as a prognostic biomarker in gallbladder cancer (GBC).

PATIENTS AND METHODS: We collected 150 pairs of tissue specimens from patients with GBC and adjacent normal specimens. The expression level of miR-146b-5p was measured in 150 GBC tissues and adjacent normal tissues RT-qPCR. Kaplan-Meier method was used to analyze the overall survival. The univariate and multivariable Cox regression analyses were performed to identify whether miR-146b-5p could serve as an independent prognostic factor for GBC patients.

RESULTS: miR-146b-5p expression was decreased in GBC tissues compared with that in adjacent normal tissues ($p < 0.01$). In addition, miR-146b-5p expression was correlated with TNM stage ($p = 0.009$), liver metastasis ($p = 0.001$) and differentiated degree ($p = 0.022$). Kaplan-Meier survival curves showed that GBC patients with high miR-146b-5p expression showed better overall survival than those with low miR-146b-5p expression ($p = 0.0005$). At last, univariate and multivariate analyses confirmed that increased miR-146b-5p expression was an independent predictive factor of good prognosis for GBC patients.

CONCLUSIONS: Our study showed that miR-146b-5p was a potential prognostic biomarker and higher expression of miR-146b-5p is associated with a poor prognosis in GBC.

Key Words
MiR-146b-5p, Gallbladder cancer, Prognosis.

Introduction

Gallbladder carcinoma (GBC) is the most common malignancy of the biliary tract and represents the fifth most prevalent gastrointestinal cancer worldwide[1]. Early stage GBC is operable with relatively good prognosis[2]. However, because of the nonspecific signs and symptoms, early diagnosis of gallbladder carcinoma is very difficult, the overall survival of GBC remains poor despite improvements in the treatment of GBC[3,4]. In clinical practice, the tumor node metastasis (TNM) staging system is used to predict GBC patients' prognosis[5]. But its results sometimes is not accurate. Therefore, it is important to identify new prognostic biomarkers of GBC.

MicroRNAs (miRNAs) produced by the ribonuclease III-enzyme Dicer are small noncoding RNAs, which could bind target genes to negatively regulate their expression by repressing translation and/or by causing mRNA degradation[6]. miRNA has been reported to be involved in various biological processes such as differentiation, apoptosis, drug resistance, and tumorigenesis[7,8]. Furthermore, there is increasing evidence that miRNAs can function as tumor suppressor genes as well as oncogenes[9]. In cancer patients, differential dysregulation of miRNAs has already been reported to serve as a new tumor biomarker in diagnosis, prognosis, and treatment selection for cancer patients[10-12].

A previous study[13] reported that abnormal expression level of miR-146b-5p was observed in GBC. However, the significance of miR-146b-5 expression in the prognosis of GBC has not been investigated. The purpose of present study was to investigate the clinical significance and prognostic value of miR-146b-5p in GBC.

Patients and Methods

Patients and Tissue Samples

A total of 150 surgical specimens of cancerous tissues and their paired adjacent non-neoplastic tissues were obtained from patients with gallbladder

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Table I. Primers of qRT-PCR.

Primers	Sequence
miR-146b-5p	F: 5'-TGACCCATCCTGGGCCTCAA-3'
	R: 5'-CCAGTGGGCAAGATGTGGGCC-3'.
GAPDH	F: 5'-CTCTGATTTGGTCGTATTGGG-3'
	R:5'-TGGAAGATGGTGATGGGATT-3'

carcinoma who underwent surgery at Cangzhou Central Hospital. None of the patients underwent chemotherapy or radiotherapy before the operation. All specimens had been histologically and clinically diagnosed at Cangzhou Central Hospital. The study was approved by the Ethics Committee of Cangzhou Central Hospital, and informed consent was obtained. The demographic and clinical features of the GBC tissues and the corresponding normal tissues are presented in Table II.

Real-time Quantitative PCR

For GBC samples, total RNA was isolated using Trizol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instruction. The cDNA was reverse transcribed using All-in-One™ miRNA QPCR Detection Kit (Genecopoeia, Germantown, MD, USA) at 37°C for 60 min followed by 85°C for 5 min. Quantitative RT-PCR was performed in a Bio-Rad CFX96 real-time PCR System (Bio-Rad, Hercules, CA, USA) using TaqMan probes (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. Independent experiments were repeated three times for each sample. The relative expression levels of genes were analyzed using the $2^{-\Delta\Delta Ct}$ method. The primers were shown in Table I.

Statistical Analysis

Statistical evaluation was performed using SPSS v. 17.0 software (SPSS Inc., Chicago, IL, USA). The Chi-square test was applied to the examination of the relationship between miR-146b-5p expression levels and clinicopathologic characteristics. Survival curves were plotted using the Kaplan-Meier method and compared using the log-rank test. One representative experiment is shown in the duplicates or triplicates used for the statistical analysis. The probability of $p < 0.05$ was considered to be statistically significant.

Results

Decreased miR-146b-5p Expression in GBC Tissues

First, we analyzed the miR-146b-5p expression in 150 paired clinical GBC and adjacent noncancerous tissues using qRT-PCR. As shown in Figure 1, the expression of miR-146b-5p was significantly down-regulated in GBC tissues when compared with adjacent normal tissues ($p < 0.01$).

Table II. Analysis of the relationship between miR-146b-5p level and clinicopathological factors of patients with GBC.

Clinicopathological factors	Case number	miR-146b-5p level		*p*-value
		High (N = 76)	Low (N = 74)	
Age (years)				0.423
<60	64	30	34	
≥60	86	46	40	
Gender				0.174
Male	89	41	48	
Female	61	35	26	
TNM stage				0.009
I+II	77	47	30	
III+IV	73	29	44	
Lymph node metastasis				0.314
Yes	43	19	24	
No	107	57	50	
Liver metastasis				0.001
Yes	34	9	25	
No	116	67	49	
Differentiated degree				0.022
Well	108	61	47	
Moderate/poor	42	15	27	

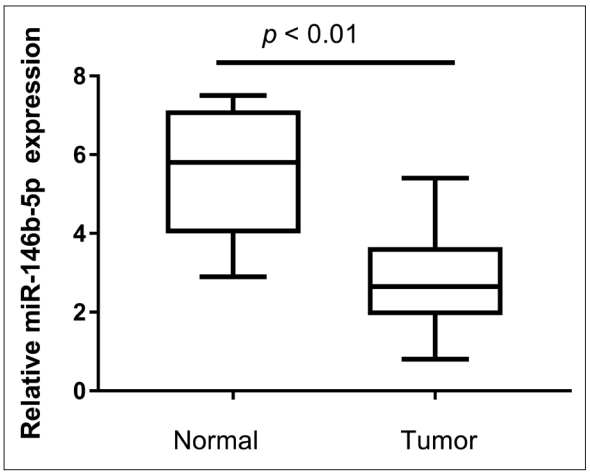

Figure 1. Relative expression of miR-146b-5p in GBC and adjacent non-tumor tissue was examined by qRT-PCR and normalized to GAPDH expression.

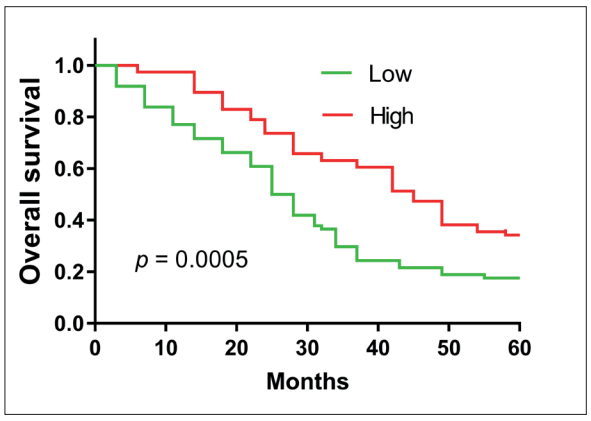

Figure 2. Kaplan-Meier curves for overall survival in patients with GBC: significantly poorer overall survival for patients with low miR-146b-5p expression than for those with high miR-146b-5p expression (p = 0.0005, log-rank test).

Association of miR-146b-5p Expression with Clinicopathologic Features of GBC Patients

To elucidate the underlying function of miR-146b-5p in the development of GBC, We analyzed the relationship between the miR-146b-5p expression level and the clinicopathological factors of the GBC patients. As shown in Table II, The results revealed that a low level of miR-146b-5p expression was correlated with high TNM stage (p = 0.009), liver metastasis (p = 0.001) and differentiated degree (p = 0.022). However, there was no significant association with age, gender and lymph node metastasis ($p > 0.05$, respectively).

Significance of miR-146b-5p Expression in GBC Prognosis

To further investigate the correlation between miR-146b-5p expression and survival of cervical cancer patients, we used Kaplan-Meier survival and log-rank analysis. It became clear that shorter overall survival was significantly related to low expression (in the log-rank test, p = 0.0005; Figure 2). Finally, we performed Cox proportional hazards regression analysis to explore the effects o miR-146b-5p and clinicopathological factors on patient survival. The univariate analysis demonstrated that TNM stage (p = 0.013), liver metastasis (p = 0.005), differentiated degree (p = 0.009) and miR-146b-5p expression (p = 0.003) were significantly associated with overall survival of GBC patients (Table III). Multivariate Cox regression analysis showed that lowly expressed miR-146b-5p (p = 0.005) were independent prognostic factors for overall survival of GBC prognosis (Table III).

Discussion

GBC is a relatively rare neoplasm associated with poor prognosis[14]. The development of new therapies has progressed little and improvement of survival has made little in the last 20 years[15].

Table III. Univariate and multivariate Cox regression analyses for overall survival.

Variable	Univariate analysis		Multivariate analysis	
	HR (95% CI)	p	HR (95% CI)	p
Age (years)	1.365 (0.513-2.774)	0.287	—	—
Gender	1.785 (0.457-3.352)	0.381	—	—
TNM stage	2.146 (0.631-5.772)	0.013	1.893 (0.498-3.773)	0.017
Lymph node metastasis	2.541 (0.931-3.945)	0.138	—	—
Liver metastasis	2.638 (1.782-6.481)	0.005	2.158 (1.237-5.791)	0.008
Differentiated degree	2.931 (1.314-4.229)	0.009	2.318 (0.933-4.013)	0.011
miR-146b-5p expression	3.127 (1.349-6.744)	0.003	2.898 (1.034-6.114)	0.005

Therefore, clarification of the molecular pathogenesis of GBC is crucial for developing effective intervention and therapeutic strategies. However, identification of molecular biomarkers with clinical value is a huge challenge.

Subsequent studies have shown that high expression of miR-146b-5p was observed in many malignancies. For instance, Deng et al[16] showed that ectopic expression of miR-146b-5p could suppress the proliferation, migration and invasion capacity of thyroid cancer cells. Also, ZNRF3 was identified as a direct target of miR-146b-5p. Xu et al[17] found that miR-146b-5p overexpression promoted migration and invasiveness in osteosarcoma by targeting zinc and ring finger 3. On the other hand, miR-146b-5p function as a tumor suppressor in some other tumors. For example, Liu et al[18] observed that overexpression of miR-146b-5p in the glioma cell lines markedly reduced cell growth and induced cell apoptosis by targeting TRAF6. They also identify miR-146b-5p as a novel prognostic biomarker of gliomas. Recently, Cai et al[13] found that miR-146b-5p expression was down-regulated in GBC tissue and cell lines, and overexpression of miR-146b-5p suppresses tumor growth *in vivo* by targeting epidermal growth factor receptor. Those results indicated that miR-146b-5p served as a tumor suppressor in GBC. However, the clinical significance of miR-146b-5p has not been investigated.

In the present study, the qRT-PCR analysis showed that the expression of miRNA-146b-5p in GBC tissues was significantly lower than that in normal tissues, which was consistent with the previous report[13]. We further found that the level of miRNA-146b-5p in GBC was strongly correlated with TNM stage, liver metastasis, and differentiated degree. Kaplan-Meier analysis revealed that GBC patients with high 146b-5p expression had poorer overall survival. Moreover, univariate and multivariate survival analysis showed that miR-146b-5p might be involved in GBC and could be used as a potential prognostic biomarker for GBC. To our knowledge, this is the first study to investigate the impact of miR-146b-5p expression on prognosis using a large number of clinical samples.

Conclusions

In this examination, we evidenced that miR-146b-5p was downregulated in GBC. Its levels are associated with overall survival. These new findings indicated that miR-146b-5p might be used as a potential marker for the prognosis of GBC.

Conflicts of interest

The Authors declare that they have no conflict of interests.

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