

# Increased expression of microRNA-335 predicts a favorable prognosis in primary gallbladder carcinoma

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**Background:** MicroRNAs (miRNAs) display aberrant expression patterns and functional abnormalities in many types of cancer. However, their roles in primary gallbladder carcinoma (PGC) have not been well documented. miR-335 has been demonstrated to be involved in tumorigenesis of several cancers in the digestive system. The aim of this study was to investigate the clinical significance of miR-335 in PGC.

**Methods:** miR-335 expression in 166 human PGC tissues and matched adjacent nondysplastic gallbladder epithelia was measured by real-time quantitative polymerase chain reaction (RT-PCR) assay.

**Results:** The expression level of miR-335 was significantly lower in PGC tissues than that in nondysplastic gallbladder epithelia ( $P < 0.001$ ). Of 166 PGC patients, 96 (57.83%) had reduced expression of miR-335. Additionally, the expression of miR-335 was significantly lower in PGC tissues with high histologic grade ( $P = 0.02$ ), advanced pathologic T stage ( $P = 0.009$ ) and clinical stage ( $P = 0.008$ ), and with positive lymph node metastasis ( $P = 0.001$ ). In univariate analysis by log-rank test, histologic grade ( $P = 0.03$ ), pathologic T stage ( $P = 0.008$ ), clinical stage ( $P = 0.01$ ), lymph node metastasis ( $P < 0.001$ ), and miR-335 expression ( $P < 0.001$ ) were significant prognostic factors for overall survival of PGC patients. Multivariate analysis further revealed that pathologic T stage ( $P = 0.02$ ), lymph node metastasis ( $P = 0.008$ ), and miR-335 expression ( $P = 0.006$ ) maintained independent prognostic influence on overall survival.

**Conclusion:** This study offers convincing evidence for the first time that miR-335 was downregulated in a majority of PGC patients and may be associated with the aggressive tumor behaviors. Loss of miR-335 expression may be a useful marker for clinical outcome and a therapeutic target for PGC.

**Keywords:** microRNA-335, primary gallbladder carcinoma, real-time quantitative RT-PCR assay, prognosis

## Introduction

Primary gallbladder carcinoma (PGC) represents the most common biliary tract malignancy and the sixth most common cause of cancer-related death.<sup>1</sup> Although the incidence of PGC is lower than other cancers, it is a highly lethal disease. Worldwide, the median survival of PGC patients is less than 1 year, and the 5-year overall survival rate is less than 15%.<sup>2</sup> Because of its nonspecific symptoms, the diagnosis of PGC is usually made postoperatively on tumors at an advanced stage; almost half of patients already have metastatic disease at the time of diagnosis.<sup>3</sup> Complete surgical resection is still the only potentially curative treatment option for PGC patients, but many of the patients treated by curative resection showed poor outcome due to the high recurrence rate because this carcinoma has a great propensity to directly invade the liver, and it

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also frequently metastasizes to the liver and pericholedochal lymph nodes.<sup>4</sup> Despite advances in imaging techniques and aggressive surgical treatment, with consequent disease remission, clinical outcomes vary significantly between patients and can be difficult to predict. Therefore, the goal to attain a more thorough understanding of the molecular biology, genetic causes, and cellular origin of PGC is of great significance in the development of improved therapeutic strategies and in the identification of prognostic markers.

MicroRNAs (miRNAs) are a class of small, single stranded, noncoding RNA molecules of 19–24 nucleotides in length.<sup>5</sup> They control gene expression at the posttranscriptional level through messenger RNA (mRNA) degradation and/or translational repression. It is estimated that up to 60% of genes may be regulated by more than 1,900 human miRNAs thus far identified. The miRNAs bind to the 3' untranslated regions of their target mRNAs, mediating translational repression and/or mRNA degradation.<sup>6,7</sup> Accumulating studies indicate that miRNAs display aberrant expression patterns and functional abnormalities in many types of cancers. They act either as tumor suppressors or oncogenes according to the roles of their target genes. Changes in their expression are able to be used as robust and important biomarkers for cancer risk, diagnosis, and prognosis, and are even considered with great interest as possible miRNA-based therapeutic targets. However, their roles in PGC have not been well documented. There have been few reports on the involvement of miRNAs in PGC. Kono et al<sup>8</sup> found that high miR-155 expression may be correlated with the aggressive behavior of PGCs. Srivastava et al<sup>9</sup> evaluated the effects of genetic polymorphisms in premiRNA genes on the risk of PGC and indicated that common miRNA variants may not contribute to PGC susceptibility in the North Indian population. These reports suggested that miRNAs may control gene expression in PGC and play an important role in carcinogenesis of this disease.

miR-335, which is the predicted homologue of a miRNA cloned from rat neuronal tissue and later verified in human, is transcribed from the genomic region on chromosome 7q32.2.<sup>10</sup> It has been demonstrated to function as an oncogenic or a tumor-suppressor miRNA in various human malignancies, and was shown to be upregulated in meningiomas,<sup>11</sup> gliomas,<sup>12</sup> and myeloma,<sup>13</sup> but downregulated in breast cancer,<sup>14</sup> hepatocellular carcinoma,<sup>15</sup> and pancreatic adenocarcinoma.<sup>16</sup> There have been controversial reports on the expression patterns of miR-335 even in the same cancer type. For example, Yan et al<sup>17</sup> indicated that miR-335 was upregulated in gastric cancer, and a high frequency of recurrence

and poor survival was observed in gastric cancer cases with high levels of miR-335. In contrast, Xu et al<sup>18</sup> found that miR-335 was dramatically downregulated in gastric cancer cells, and low expression of miR-335 was significantly associated with lymph node metastasis, poor pT stage, poor pN stage, and invasion of lymphatic vessels, suggesting a metastasis suppressor function in this disease. Since miR-335 has been demonstrated to play important roles in carcinogenesis of the digestive system, and its clinical impacts in PGC have not been previously investigated, in the current study, miR-335 expression levels in resected specimens, including 166 fresh human PGC tissues and matched adjacent nondysplastic gallbladder epithelia were measured by real-time quantitative polymerase chain reaction (RT-PCR) assay. The correlation of miR-335 expression with the clinical behavior and prognosis of PGC was also evaluated.

## Materials and methods

### Patients and tissue samples

This study was approved by the Research Ethics Committee of Xiangya Hospital, Central South University, People's Republic of China. Written informed consent was obtained from all of the patients. All specimens were made anonymous and handled according to the ethical and legal standards.

Fresh PGC tissues and matched adjacent nondysplastic gallbladder epithelia were collected from 166 patients who underwent surgery between April 2005 and March 2009 in Xiangya Hospital. The fresh tissue specimens were immediately frozen in liquid nitrogen until use. No patients had preoperative chemotherapy, radiotherapy, or other treatment history. The pathological stage was classified according to the tumor node metastasis classification of the American Joint Committee on Cancer Staging, 6th edition.<sup>19</sup> Hematoxylin and eosin stained slides were reviewed for histological grade. Invasion and lymph metastases were evaluated according to the standard criteria.<sup>20</sup> The clinicopathologic features of all the patients are summarized in Table 1.

Survival information of 166 patients was successfully obtained, and overall survival of this cohort was analyzed. Overall survival time was calculated from the date of the initial surgical operation to death. Patients who died of diseases not directly related to their PGCs or due to unexpected events were excluded from our enrollment during the case collection in this study. The follow-up period ranged from 1–72 months (median, 29 months; mean, 30 months). Among the 166 patients, 76 patients survived less than 1 year, 90 patients survived over 1 year, and 96 patients died during the follow-up.

**Table 1** Association between miR-335 expression and different clinicopathologic features of primary gallbladder carcinoma

Clinicopathologic features	Number of cases	miR-335 expression (mean $\pm$ SD)	P-value
Age (years)			
<52	80	1.26 $\pm$ 0.41	NS
$\geq$ 52	86	1.45 $\pm$ 0.58	
Sex			
Male	56	1.21 $\pm$ 0.40	NS
Female	110	1.44 $\pm$ 0.56	
Histological grades			
G1–G2	58	1.66 $\pm$ 0.61	0.02
G3–G4	108	1.20 $\pm$ 0.38	
Pathological T stage			
T1–T2	64	1.73 $\pm$ 0.65	0.009
T3–T4	102	1.13 $\pm$ 0.20	
Lymph node metastasis			
No	79	2.02 $\pm$ 0.83	0.001
Yes	87	0.76 $\pm$ 0.12	
Clinical stage			
I–II	72	1.79 $\pm$ 0.50	0.008
III–IV	94	1.03 $\pm$ 0.33	
Invasion of regional tissues			
No	68	1.45 $\pm$ 0.52	NS
Yes	98	1.30 $\pm$ 0.45	

**Abbreviations:** miR, microRNA; NS, no statistical significance; SD, standard deviation.

## Real-time quantitative RT-PCR for miRNA

Real-time quantitative RT-PCR for miRNA was performed to detect the expression levels of miR-335 in PGC tissues and matched adjacent nondysplastic gallbladder epithelia. Total RNA was extracted from tissue samples of 166 pairs of PGC tissues and matched adjacent nondysplastic gallbladder epithelia using TRIzol<sup>®</sup> (Life Technologies, Carlsbad, CA, USA) according to the manufacturer's protocol. The specific complementary (c)DNA of miR-335 and RNU6B (Life Technologies), which was used as internal control, were synthesized from total RNA using gene-specific primers according to the TaqMan<sup>®</sup> MicroRNA assays protocol (Life Technologies). The primers for miR-335 were 5'-ACA CTC CAG CTG GGT CAA GAG CAA TAA CGA AA-3' and 5'-CTC AAC TGG TGT CGT GGA-3'; the primer for RNU6B was 5'-ACG CAA ATT CGT GAA GCG TT -3'. Reverse transcriptase reactions contained 10 ng of total RNAs, 50 nmol/L stem-loop RT primer, 1X RT buffer, 0.25 mmol/L each of the four deoxy-nucleotide triphosphates, 3.33 U/ $\mu$ L MultiScribe<sup>™</sup> reverse transcriptase (Life Technologies), and 0.25 U/ $\mu$ L RNase inhibitor. The 10  $\mu$ L reaction volumes were incubated in a Bio-Rad iCycler (Bio-Rad Laboratories, Hercules, CA, USA)

in a 96-well plate for 30 minutes at 15°C, 30 minutes at 40°C, 5 minutes at 86°C, and then held at 4°C. Real-time PCR was performed using an Applied Biosystems 7500 real-time PCR system (Life Technologies). The reaction mixture (10  $\mu$ L total volume per well) included 2 ng cDNA, 1X TaqMan Universal PCR master mix, and 1  $\mu$ L of primers and probe mix from the TaqMan MicroRNA assay kit. Relative quantification of target miRNA expression was evaluated using the comparative cycle threshold method. The raw data were presented as the relative quantity of target miRNA normalized with respect to RNU6B. Each sample was examined in triplicate. Mean normalized gene expression  $\pm$  standard deviation (SD) was calculated from three independent experiments. The median expression level of miR-335 (1.27) was used as a cutoff point to divide all 166 patients into two groups: high miR-335 expression group and low miR-335 expression group.

## Statistical analysis

All computations were carried out using the SPSS software version 13.0 for Windows (IBM Corporation, Armonk, NY, USA). Data were expressed as mean  $\pm$  SD. Paired Student's *t*-test was conducted to compare miR-335 expression in paired clinical samples. The association between miR-335 expression and clinicopathologic characteristics of PGC patients was assessed by Mann–Whitney *U* and Kruskal–Wallis tests. Survival curves were obtained by using the Kaplan–Meier method and compared by using the log-rank test. Multivariate survival analysis was performed using the Cox proportional hazards model. The factors selected from univariate analysis, based on a *P*-value <0.05, were entered into the Cox proportional hazards model. Differences were considered statistically significant when *P*-value <0.05.

## Results

### Reduced expression of miR-335 in human PGC

The expression of miR-335 was detected in 166 human PGC and matched adjacent nondysplastic gallbladder epithelia tissues from 166 PGC patients (56 males and 110 females, median age: 52 years, range: 30–78 years) by real-time quantitative RT-PCR. After normalization to RNU6B, the expression level of miR-335 in PGC tissues (1.36 $\pm$ 0.50) was significantly lower than that in adjacent nondysplastic gallbladder epithelia (3.39 $\pm$ 1.33, *P*<0.001). PGC patients who expressed miR-335 at levels less than the cutoff value were assigned to the low expression group (mean expression value 0.99, *n*=96), and those with expression above the cutoff value were

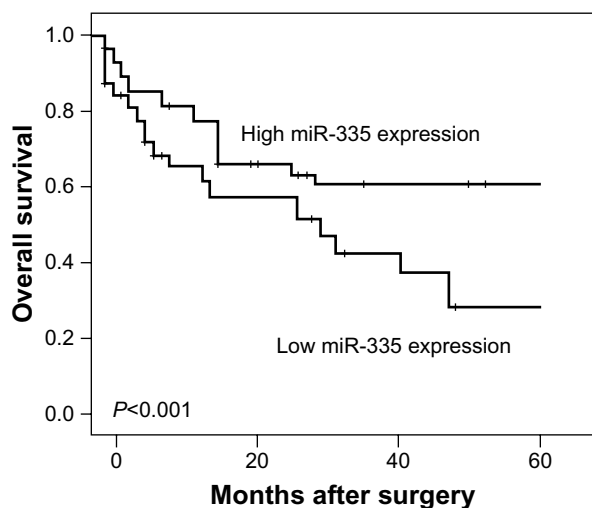
assigned to the high expression group (mean expression value 1.87,  $n=70$ ).

## Reduced expression of miR-335 associates with aggressive clinicopathologic features of human PGC

Table 1 summarizes the association between miR-335 expression and clinicopathologic features in PGCs. miR-335 expression was significantly lower in the cancerous tissues of PGC patients with high histologic grade than those with low histologic grade ( $P=0.02$ ) (Table 1). In addition, tumors with advanced pathologic T stage ( $P=0.009$ ) and clinical stage ( $P=0.008$ ) expressed lower miR-335 (Table 1). Moreover, miR-335 was expressed at significantly lower levels in lymph node metastasis-positive patients than in lymph node metastasis-negative patients ( $P=0.001$ ) (Table 1). However, miR-335 expression was not significantly related to sex, age, or invasion of regional tissues (all  $P>0.05$ ) (Table 1).

## Reduced expression of miR-335 associates with poor prognosis in patients with human PGC

The association between miR-335 expression and overall survival of PGC patients was investigated by Kaplan–Meier analysis and the log-rank test. As shown in Figure 1, PGC patients with low miR-335 expression tend to have shorter overall survival than those with high miR-335 expression (log-rank test:  $P<0.001$ ). Univariate analysis of factors related to overall survival in PGC is summarized in Table 2. Histologic grade ( $P=0.03$ ), pathologic T stage ( $P=0.008$ ),



**Figure 1** Kaplan–Meier curves for survival time in patients with primary gallbladder carcinoma divided according to miR-335 expression.

**Abbreviation:** miR, microRNA.

**Table 2** Univariate analysis of prognostic parameters in patients with primary gallbladder carcinoma

Variables	Patients alive at 5 years %	Univariate log-rank test (P-value)
Age at diagnosis (years)		
<52 versus $\geq 52$	38.73 versus 39.53	0.33
Sex		
Male versus female	48.18 versus 34.55	0.61
Histological grades		
G1–G2 versus G3–G4	51.52 versus 35.19	0.03
Pathological T stage		
T1–T2 versus T3–T4	51.53 versus 31.37	0.008
Lymph node metastasis		
No versus yes	56.94 versus 22.99	<0.001
Clinical stage		
I–II versus III–IV	51.36 versus 29.79	0.01
Invasion of regional tissues		
No versus yes	42.65 versus 36.71	0.06
miR-335 expression		
High versus low	61.43 versus 22.92	<0.001

**Abbreviation:** miR, microRNA.

clinical stage ( $P=0.01$ ), lymph node metastasis ( $P<0.001$ ), and miR-335 expression ( $P<0.001$ ) were significant prognostic factors for overall survival of PGC patients. As shown in Table 3, multivariate analysis further revealed that pathologic T stage ( $P=0.02$ ), lymph node metastasis ( $P=0.008$ ), and miR-335 expression ( $P=0.006$ ) maintained independent prognostic influence on overall survival.

## Discussion

PGC remains a highly lethal disease with a poor prognosis. Although several clinicopathologic features have been the standard for determining the clinical outcome of PGC patients, this classification scheme is probably an imprecise predictor of the prognosis of an individual patient.<sup>21</sup> Thus, it is necessary to identify novel and effective biologic markers which are associated with advanced tumor progression for the early diagnosis and the discovery of a therapeutic target. In the current study, there are three main findings according to our data. First, real-time quantitative RT-PCR of a large cohort revealed that 57.83% of PGC patients had reduced expression of miR-335. Notably, the expression level of miR-335 in PGC tissues was significantly lower than that in nondysplastic gallbladder epithelia ( $P<0.001$ ). Second, loss of miR-335 expression in PGC tissues was significantly correlated with advanced tumor progression and aggressive clinicopathologic features. Third, the impact of miR-335 expression on clinical outcome was assessed by Kaplan–Meier analysis, and the results showed that PGC patients



**Table 3** Multivariate analysis of prognostic parameters in patients with primary gallbladder carcinoma

Variables	Characteristics		Hazard ratio	95% CI	P-value
	Favorable	Unfavorable			
Histological grades	G1–G2	G3–G4	2.36	1.01–4.82	0.11
Pathological T stage	T1–T2	T3–T4	3.52	1.12–7.88	0.02
Lymph node metastasis	No	Yes	6.06	1.86–13.53	0.008
miR-335 expression	High	Low	6.63	1.91–13.87	0.006

**Abbreviations:** CI, confidence interval; miR, microRNA.

with low miR-335 expression had shorter overall survival than those with high miR-335 expression. Moreover, both univariate and multivariate analyses clearly demonstrated that miR-335 expression was a statistically significant risk factor affecting overall survival of patients with PGC. Interestingly, the statistically significant impact of miR-335 expression was more significant than pathological T stage and status of lymph node metastasis, which is widely used in clinics at present, indicating that miR-335 expression may be a useful marker to predict patient survival. To the best of our knowledge this is the first report demonstrating an association between expression and clinical relevance of miR-335 in PGC.

miRNAs regulate the expression of roughly 10%–30% of all human genes through posttranscriptional mechanisms, and their abnormal expression has been linked with many human diseases including cancer.<sup>22</sup> Accumulating studies have explored the clinical significance of miRNAs in cancers including gliomas, papillary thyroid carcinoma, breast cancer, non-small-cell lung cancer, hepatocellular carcinoma, colon cancer, prostate cancer, and bladder cancer.<sup>23–28</sup> However, the role of miRNAs in PGC is yet to be fully elucidated. Among human miRNAs, miR-335 as an evolutionarily conserved miRNA has been demonstrated to have a close correlation with cancer development. In particular, it plays different roles in different tumor types, depending on the cell type involved. For example, Dohi et al<sup>15</sup> reported that the expression levels of miR-335 were significantly lower in primary hepatocellular carcinoma tissues compared to their nontumor tissue counterparts, and were significantly lower in tumor tissues with distant metastasis compared to those without distant metastasis; the expression of miR-335 may be lost in the majority of primary breast tumors from patients who relapse, and the loss of miR-335 expression may be associated with poor distal metastasis-free survival.<sup>14</sup> In contrast, Shu et al<sup>12</sup> indicated that miR-335 may act as a tumor promoter in conferring tumorigenic features such as growth and invasion on malignant astrocytomas. Shi et al<sup>11</sup> also showed that miR-335 may be a typical microRNA overexpressed in human meningiomas, and elevated levels

of miR-335 may promote proliferation of tumor cells. Moreover, miR-335 also represents a potential therapeutic target for the differentiation therapy of highly aggressive and therapy-refractory tumor. It regulates a set of genes, which are *SOX4*, *RUNX2*, *Rb1*, *PTPRN2*, *BRCA1*, *MERTK* and *SP1*, the collective expression of which in a large cohort of human tumors is associated with the risk of aggressive tumor progression.<sup>29,30</sup> Since there have been no reports on the clinical relevance of miR-335 to PGC, we in the present study, performed a real-time quantitative RT-PCR assay to explore the expression pattern of this miRNA as well as to investigate its association with clinicopathologic features of PGC patients. The feature of miR-335 downregulation, mostly in PGC tissue but rarely in normal tissue, in addition to its association with high pathological T stage and clinical stage, low histological grade, and positive lymph node metastasis, makes miR-335 a possible useful marker in the diagnosis of patients with advanced stages of PGC and a potential candidate for therapeutic intervention. These findings were similar with those made in other digestive tumors, such as hepatocellular carcinoma,<sup>15</sup> gastric cancer,<sup>18</sup> and pancreatic adenocarcinoma.<sup>16</sup>

Furthermore, this study demonstrated that the reduced miR-335 expression was significantly correlated with shorter overall survival in PGC patients. Interestingly, this relationship was maintained after adjusting for other prognostic parameters in the multivariate analysis, indicating that reduced miR-335 expression was an independent predictor of poor prognosis for overall survival in PGC patients. In contrast, Jiang et al<sup>31</sup> found that glioma patients with high miR-335 expression tumors had significantly shorter survival times than those with low miR-335 expression tumors. Yan et al<sup>17</sup> indicated that a high frequency of recurrence and poor survival were observed in gastric cancer patients with high levels of miR-335. Although confirmation requires larger and more long-term studies, the variable interpretations of the significance of miR-335 expression between PGC and other types of cancers suggest that the actions and roles of miR-335 may depend on the cell type of tumor origin.

The limitation of the current study is the number of cases with relatively small subgroups. Further investigations with a larger number of cases would help us better understand the clinical value of miR-335 dysregulation in cancer progression. Additionally, such information may direct us toward novel therapeutic and prognostic possibilities for treating PGC and improving patient outcomes.

## Conclusion

This study offers convincing evidence for the first time that miR-335 was downregulated in a majority of PGC patients and may be associated with the aggressive tumor behaviors. Loss of miR-335 expression may be a useful marker for clinical outcome and a therapeutic target for PGC.

## Disclosure

The authors report no conflicts of interest in this work.

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