

Original Article

The prognostic value of miR-34a expression in completely resected gastric cancer: tumor recurrence and overall survival

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Abstract: The prognosis of gastric cancer is mainly linked to tumor recurrence. MicroRNA-34a (miR-34a) is a direct transcriptional target of p53 and links tumor suppressor function and the oncogenic pathways in some cancers. However, the role of miR-34a in predicting prognosis of gastric cancer has not been fully elucidated. In this study, we aimed to investigate the expression level of miR-34a and its prognostic value in gastric cancer. A total of 137 consecutive gastric cancer patients who underwent gastrectomy with D2 lymph node dissection were included in this study. Quantitative real-time polymerase chain reaction (qRT-PCR) was utilized to detect miR-34a expression in gastric cancer tissues and adjacent normal tissues. The results showed that the levels of miR-34a expression were significantly decreased in the tumor tissues compared with the adjacent normal tissues ($P < 0.001$). Low miR-34a expression level was associated with lymph node involvement ($P = 0.004$), advanced TNM stage ($P = 0.006$), poor tumor differentiation ($P = 0.024$), high tumor recurrence rate ($P = 0.008$), and poor five-year survival ($P < 0.001$). The median time to recurrence and median overall survival time were significantly shorter in patients with low miR-34a levels compared with those with high miR-34a levels ($P = 0.028$ and $P = 0.021$, respectively). Furthermore, when analyzed with a multivariate Cox regression model, a low miR-34a level was significantly correlated with high recurrence rate and poor overall survival. Taken together, our results suggest that downregulation of miR-34a in gastric cancer is associated with high recurrence rate and poor overall survival and that miR-34a may be served as a prognostic marker for gastric cancer.

Keywords: microRNA-34a, gastric cancer, tumor recurrence, overall survival

Introduction

Gastric cancer is the fifth leading cause of cancers and the third leading cause of cancer-related death in the world [1]. A total of 950,000 new gastric cancer cases and 723,000 deaths have occurred in 2012, accounting for 7% of the total cancer cases and 9% of total cancer-related deaths, respectively [1]. Although treatment approaches including surgery, chemotherapy, and radiotherapy have been proven to be effective in the past two decades, the prognosis of gastric cancer remains poor with the 5-year survival rate to be less than 10%, due to the fact that gastric cancer has often metastasized by the time of discovery and the fact that most people with this disease condition are elderly at diagnosis [2]. To improve the survival of gastric cancer patients, a better understand-

ing of the underlying molecular mechanisms of gastric cancer, and their application towards helping determine the prognosis and guiding clinicians in designing personalized treatment strategies, is urgently needed.

MicroRNAs (miRNAs) are small non-coding RNA molecules (about 22 nucleotides in length), which function in RNA silencing and post-transcriptional regulation of gene expression [3, 4]. Currently, more than several hundred unique mature human miRNAs are known, and many are involved in tumorigenesis, acting either as oncogenes [5] or tumor suppressors [6, 7]. Aberrant expression of miRNAs has also been linked to gastric cancer, suggesting that miRNAs may serve as prognostic factors for gastric cancer [8]. The microRNA-34 (miR-34) family is consisted of miR-34a, miR-34b, and miR-34c,

Prognostic value of miR-34a expression in gastric cancer

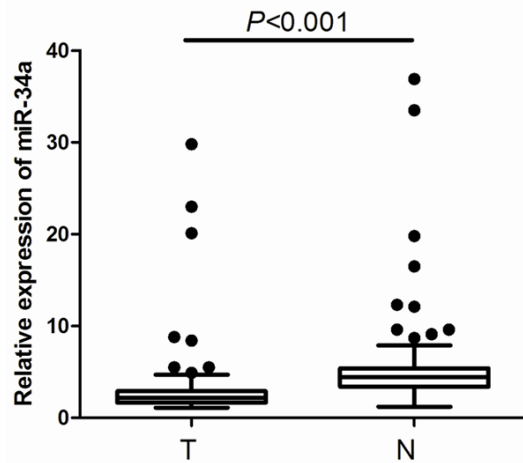


Figure 1. Relative expression of miR-34a in 137 pairs of gastric cancer tissues (T) and adjacent normal tissues (N).

with miR-34a being a part of the p53 tumor suppressor network [9]. It has been demonstrated that the miR-34 family directly links tumor suppressor function and the oncogenic pathways in some cancers [10]. In p53-deficient human gastric cancer cells, restoration of functional miR-34 inhibits cell growth and induces chemosensitization and apoptosis, indicating that miR-34 may restore p53 function [11]. Previous studies have demonstrated that miR-34a expression was downregulated in gastric cancer cells and tissues [12-14]. However, the study by Osawa et al. examining miRNA expression profile from formalin-fixed, paraffin embedded gastric cancer samples showed an upregulated miR-34a level in gastric cancers compared with normal gastric tissues [8]. Furthermore, in that study, miR34a was the only independent prognostic factor for overall survival. In another study examining miRNA profile of gastric cancer, 22 miRNAs including miR-34a were significantly upregulated [15]. Therefore, the expression level of miR-34a in gastric cancer remains controversial. Thus, aiming to investigate the exact expression level of miR-34a and its prognostic value in gastric cancer, we assessed the expression of miR-34a in gastric cancer tissue samples from operative resection.

Materials and methods

Patients and tissue specimens

A total of 137 consecutive gastric cancer patients who underwent gastrectomy with D2

lymph node dissection at Yantai Yuhuangding Hospital between January 2007 and December 2019 were included in this study. None of the patients had received chemotherapy or radiotherapy prior to surgery. Fresh tissues including gastric cancer tissues and adjacent normal tissues were collected and immediately snap-frozen in liquid nitrogen after surgery and were stored at -196°C until used. Patient preoperative demographic and clinical data, including age, gender, details of pathological diagnosis, serum carcino-embryonic antigen (CEA) and carbohydrate antigen (CA 72-4) levels, follow-up period, tumor recurrence, and overall survival were collected prospectively. Patients with stage II, IIIA, or IIIB gastric cancer were given postoperative adjuvant chemotherapy every three weeks for six months (Oxaliplatin 130 mg/m²d1 + Capecitabine 1000 mg/m² d1-d14). The study has been conducted in accordance with the ethical standards and according to the principles of the Declaration of Helsinki and has been approved by the Institutional Review Board of Yuhuangding Hospital. Written informed consent was obtained from all of the patients.

RNA isolation and qRT-PCR

Quantitative real-time polymerase chain reaction (qRT-PCR) was utilized to detect miR-34a expression in gastric cancer tissues. Briefly, RNA was extracted using the TaqMan miRNA Isolation kit (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. U6 small nuclear RNA (snRNA) was used as an internal control. The reverse transcriptase (RT) reaction contained 10ng of total RNAs, 50 nmol/l stem-loop RT primer, 1 \times RT buffer, 0.25 mmol/l each of deoxynucleotide triphosphate (dNTP), 3.33 U/ μ l MultiScribe reverse transcriptase, and 0.25 U/ μ l RNase Inhibitor. The 20 μ l reaction volumes were incubated at 16 $^{\circ}\text{C}$ for 30 min, 40 $^{\circ}\text{C}$ for 30 min, and 85 $^{\circ}\text{C}$ for 5 min. Real-time PCR was then performed on a StepOnePlus real-time PCR system (Applied Biosystems, Foster City, CA, USA). The following PCR parameters were used: 95 $^{\circ}\text{C}$ for 30 sec, followed by 40 cycles of 95 $^{\circ}\text{C}$ for 10 sec and 60 $^{\circ}\text{C}$ for 30 sec. All reactions were performed in triplicate and the cycle threshold (CT) value in each reaction well was recorded. The relative quantification method was used to determine the changes in the expression of miR-34a. The change in amplification was normalized to the expression of the U6 snRNA. The fold change in expression was calculated using

Prognostic value of miR-34a expression in gastric cancer

Table 1. Patient characteristics according to the expression of miR-34a

Characteristics	All patients (n=137)	High miR-34a level (n=64)	Low miR-34a level (n=73)	P-value
Age (years)	58.3±12.4	56.3±11.9	59.6±14.1	0.538
Gender				0.681
Male	75 (54.7%)	36 (56.3%)	39 (53.4%)	
Female	62 (45.3%)	28 (43.7%)	34 (46.6%)	
Tumor size (cm)				0.097
≤5	81 (59.1%)	39 (60.9%)	42 (57.5%)	
>5	56 (40.9%)	25 (39.1%)	31 (42.5%)	
Depth of wall invasion				0.065
T1~T2	79 (57.7%)	33 (51.6%)	46 (63.0%)	
T3~T4	58 (42.3%)	31 (48.4%)	27 (37.0%)	
Lymph node involvement				0.004
Negative	67 (48.9%)	40 (62.5%)	27 (37.0%)	
Positive	70 (51.1%)	24 (37.5%)	46 (63.0%)	
Distant metastasis				0.079
No	134 (97.9%)	63(98.4%)	71 (97.3%)	
Yes	3 (2.1%)	1 (1.6%)	2 (2.7%)	
TNM stage ^a				0.006
IA~IB	22 (16.1%)	14 (21.9%)	8 (11.0%)	
IIA~IIB	59 (43.1%)	27 (42.2%)	32 (43.8%)	
IIIA~IIIB	51 (37.2%)	22 (34.4%)	29 (39.7%)	
IIIC~IV	5 (3.6%)	1 (1.5%)	4 (5.5%)	
Histologic types				0.285
Adenocarcinoma	104 (75.9%)	49 (76.6%)	55 (75.3%)	
Others ^b	33 (24.1%)	15 (23.4%)	18 (24.7%)	
Differentiation				0.024
Well	38 (27.7%)	23 (35.9%)	15 (20.6%)	
Moderate	42 (30.7%)	18 (28.1%)	24 (32.9%)	
Poor	49 (35.8%)	20 (31.3%)	29 (39.7%)	
Undifferentiate	8 (5.8%)	3 (4.7%)	5 (6.8%)	
Adjuvant chemotherapy				0.077
No	24 (17.5%)	10 (15.6%)	14 (19.2%)	
Yes	113 (82.5%)	54 (84.4%)	59 (80.8%)	
Tumor recurrence				0.008
No	77 (56.2%)	42 (65.6%)	35 (47.9%)	
Yes	60 (43.8%)	22 (34.4%)	38 (52.1%)	
Five-year survival				<0.001
No	66 (48.2%)	24 (37.5%)	42 (57.5%)	
Yes	71 (51.8%)	40 (62.5%)	31 (42.5%)	

^aTNM staging was classified according to the 7th edition of the AJCC cancer staging manual. ^bOther cell types included signet ring cell carcinoma (n=15), adenosquamous carcinoma (n=11), and squamous cell carcinoma (n=7).

$$2^{-\Delta\Delta CT}, \text{ where } \Delta\Delta CT = (CT_{\text{miR-34a}} - CT_{U6})_{\text{gastric cancer tissue}} - (CT_{\text{miR-34a}} - CT_{U6})_{\text{adjacent normal tissue}}$$

Statistical analysis

The main end points were tumor recurrence and overall survival. Tumor recurrence was

defined as evidence of a new lesion in the remaining stomach or elsewhere outside the stomach and was classified as local recurrence and distant recurrence. Time to recurrence was defined as the interval between the time of surgery and the discovery of the recurrence. Overall survival was measured from the time of

Prognostic value of miR-34a expression in gastric cancer

Table 2. Univariate and multivariate Cox analyses of clinical variables for time to recurrence

Variables	Univariate analysis		Multivariate analysis	
	HR (95% CI)	P-value	HR (95% CI)	P-value
Age (>50 vs. ≤50 years)	1.14 (0.83-1.45)	0.547		
Gender (Male vs. Female)	0.97 (0.78-1.21)	0.821		
Tumor size (>5 vs. ≤5 cm)	1.34 (1.12-1.56)	0.003	1.17 (0.99-1.35)	0.052
Depth of wall invasion	1.19 (0.98-1.43)	0.058		
Lymph node involvement	1.29 (1.09-1.51)	0.017	1.20 (1.02-1.37)	0.044
Distant metastasis	1.05 (0.66-1.49)	0.732		
TNM stage				
IA~IB	1.0		1.0	
IIA~IIB	1.16 (1.08-1.25)	0.022	1.09 (1.01-1.22)	0.047
IIIA~IIIB	1.37 (1.12-1.64)	0.006	1.28 (1.07-1.46)	0.018
IIIC~IV	1.39 (1.11-1.72)	0.004	1.31 (1.13-1.52)	0.009
Differentiation				
Well/Moderate	1.0		1.0	
Poor/Undifferentiate	1.35 (1.08-1.56)	0.009	1.28 (1.04-1.56)	0.016
Adjuvant chemotherapy (No vs. Yes)	1.09 (0.80-1.44)	0.652		
Serum CEA level (high vs. low)	1.14 (0.92-1.36)	0.216		
Serum CA 72-4 level (high vs. low)	1.06 (0.85-1.29)	0.579		
MiR-34a level (low vs. high)	1.23 (1.11-1.34)	0.003	1.17 (1.05-1.30)	0.015

HR hazard ratio, CI confidence interval.

Table 3. Univariate and multivariate Cox analyses of clinical variables for overall survival

Variables	Univariate analysis		Multivariate analysis	
	HR (95% CI)	P-value	HR (95% CI)	P-value
Age (>50 vs. ≤50 years)	1.21 (0.78-1.65)	0.692		
Gender (Male vs. Female)	1.01 (0.85-1.19)	0.546		
Tumor size (>5 vs. ≤5 cm)	1.17 (0.98-1.38)	0.054		
Depth of wall invasion	1.26 (0.93-1.57)	0.069		
Lymph node involvement	1.33 (1.18-1.51)	0.022	1.19 (1.00-1.40)	0.050
Distant metastasis	1.07 (0.76-1.38)	0.439		
TNM stage				
IA~IB	1.0		1.0	
IIA~IIB	1.28 (1.14-1.45)	0.008	1.19 (1.10-1.28)	0.010
IIIA~IIIB	1.44 (1.20-1.71)	0.002	1.35 (1.16-1.65)	0.007
IIIC~IV	1.42 (1.09-1.68)	0.014	1.29 (1.05-1.54)	0.019
Differentiation				
Well/Moderate	1.0		1.0	
Poor/Undifferentiate	1.29 (1.05-1.54)	0.023	1.18 (0.99-1.44)	0.053
Adjuvant chemotherapy (No vs. Yes)	1.02 (0.77-1.41)	0.548		
Serum CEA level (high vs. low)	1.23 (0.97-1.47)	0.068		
Serum CA 72-4 level (high vs. low)	1.12 (0.89-1.38)	0.431		
MiR-34a level (low vs. high)	1.46 (1.23-1.70)	<0.001	1.33 (1.14-1.61)	0.008

HR hazard ratio, CI confidence interval.

surgery to death. Continuous variables were presented as means ± standard deviations (SD). Categorical variables were expressed as

frequencies (percentages). Point-biserial correlation coefficients were calculated to estimate the correlation between tumor recurrence and

Prognostic value of miR-34a expression in gastric cancer

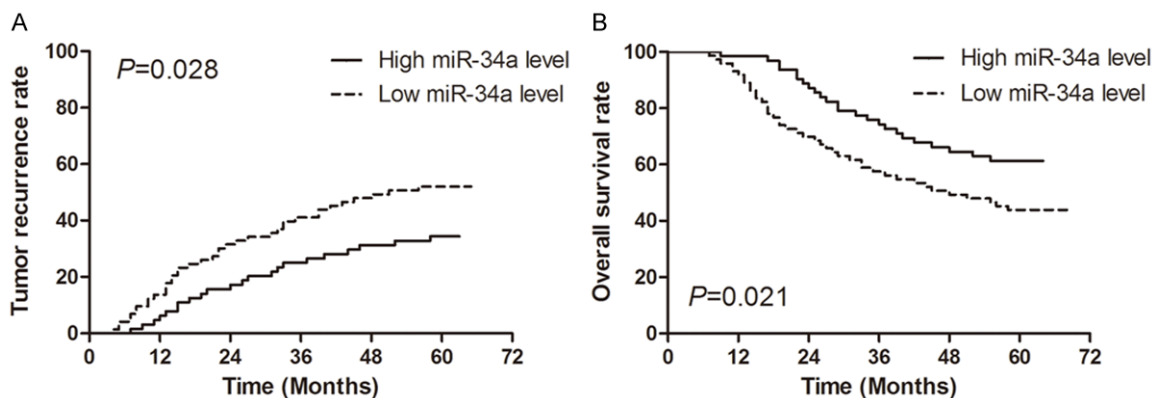


Figure 2. Kaplan-Meier estimates of tumor recurrence (A) and overall survival (B) for low miR-34a expression level versus high miR-34a expression level in patients with gastric cancer.

the miR-34a level. The Kaplan-Meier method was used to estimate recurrence and survival rates, and the log-rank test was used to assess the differences between groups. The Cox proportional hazards model for multivariate survival analysis was used to assess predictors related to overall survival. All statistical analyses were performed using SPSS software (SPSS 19.0, Chicago, IL, USA) and the difference was considered statistically significant when the P value was less than 0.05.

Results

One hundred and thirty-seven consecutive patients with gastric cancer treated with surgical resection were enrolled in this study. The baseline characteristics of patients are summarized in **Table 1**. To explore the roles of miR-34a in human gastric cancer development, we detected its expression levels in these cases of gastric cancer tissues and adjacent normal tissues. According to the qRT-PCR analysis, the levels of miR-34a expression were significantly decreased in the tumor tissues compared with the adjacent normal tissues ($P < 0.001$, **Figure 1**). The median miR-34a expression level in tumor tissue was 2.44 (range 0.12-29.83). By adopting cut-off value according to the median miR-34a level, patients were sorted into two categories: 64 patients with high miR-34a levels and 73 patients with low miR-34a levels. To determine the clinical relevance of miR-34a expression, we further assessed its correlation with patient clinicopathological factors. As shown in **Table 1**, low miR-34a expression level was associated with lymph node involvement ($P = 0.004$), advanced

TNM stage ($P = 0.006$), poor tumor differentiation ($P = 0.024$), high tumor recurrence rate ($P = 0.008$), and poor five-year survival ($P < 0.001$).

Univariate analysis was carried out to identify those factors significantly associated with tumor recurrence. As shown in **Table 2**, tumor size, lymph node involvement, TNM stage, and tumor differentiation were associated with recurrence. In addition, low miR-34a level was associated with high recurrence rate, while high CEA and CA 72-4 levels were not. Multivariate analysis indicated that lymph node involvement, TNM stage, tumor differentiation, and miR-34a expression were independent prognostic factors of tumor recurrence (**Table 2**). In the univariate analysis of overall survival, the significant factors were lymph node involvement, TNM stage, and miR-34a expression. In the multivariate analysis including all the variables from the univariate analysis, only advanced TNM stage and low miR-34a level were independent prognostic factors of poor overall survival (**Table 3**).

The Kaplan-Meier curves for the effect of miR-34a expression on tumor recurrence and overall survival are shown in **Figure 2**. The median times to recurrence were 41.3 months and 29.7 months for the patients with high miR-34a levels and those with low miR-34a levels, respectively, and there was a significant difference between the two groups ($P = 0.028$; **Figure 2A**). The median survival times were 56.9 months and 37.4 months for the patients with high miR-34a levels and those with low miR-34a levels, respectively, and there was also a

Prognostic value of miR-34a expression in gastric cancer

significant difference between the two groups ($P=0.021$; **Figure 2B**).

Discussion

MiR-34a is a direct proapoptotic transcriptional target of p53 that can mediate some of p53's biological effects [16]. It is commonly deleted in various types of cancers, such as breast cancer, lung cancer, pancreatic cancer, and bladder cancer [17]. The downregulated expression of miR-34a genes may occur due to mutations that inactivate p53 in tumor cells [18]. In previous studies [8, 12-15], there was a dispute about the expression level of miR-34a in gastric cancer. Therefore, we conducted this study to investigate the exact expression level of miR-34a. We found that the expression level of miR-34a was significantly downregulated in gastric cancer. We also demonstrate herein that miR-34a has an independent predictive value of tumor recurrence and overall survival of gastric cancer. These results could improve decision-making in the gastric cancer therapeutic area, as stratification according to miR-34a expression level would allow a better selection of patients for adjuvant therapy after tumor resection, help determine the prognosis, and guide clinicians in designing personalized treatment strategies.

In our current study, we found that miR-34a expression level was significantly associated with lymph node involvement, TNM stage, tumor differentiation, tumor recurrence, and five-year survival. This is in line with previous reports [19], indicating that miR-34a expression may be complementary to other clinical relevant prognostic indicators in patients with gastric cancer. A previous study has also found that miR-34a overexpression could improve the sensitivity of gastric cancer cells against cisplatin-based chemotherapies [13]. However, we didn't identify any significant correlation between miR-34a expression level and adjuvant chemotherapy. This may be due to the different chemotherapy regimens used and the fact that the patients in our study were given chemotherapy after tumor resection. In addition, low miR-34a level predicted high tumor recurrence rate and poor overall survival in patients with gastric cancer. As miR-34a expression level is measured by qRT-PCR using tumor tissue specimens, it may be widely used

in clinical practice and may prove beneficial in prognostic stratification of patients with gastric cancer in clinical trials and improve the outcomes of patients with gastric cancer.

The already identified targets of miR-34a include Bcl-2, E2F3, Met, SATB2, CD44, AXIN2, and PDGFR [20]. Besides, miR-34a can inhibit breast cancer proliferation by targeting LMTK3 (lemur tyrosine kinase 3), inhibit colonic cancer cell proliferation by targeting PAR2 (proteinase activated receptor 2), inhibit pancreatic cancer cell proliferation by targeting Notch1 [21-23]. In gastric cancer, miR-34a can inhibit gastric cancer tumorigenesis by targeting PDGFR and Met through the PI3K/Akt pathway [13]. In addition, Cao et al. has found miR-34a can negatively regulate the expression of the survivin protein and inhibit gastric cancer cell proliferation and invasion [12]. Furthermore, a recent study has also found that miR-34a-YY1 (Yin Yang 1) axis plays an important role in the control of gastric carcinogenesis [9]. These results indicate that the effect of miR-34a on the development and progression of gastric cancer may be based on multiple targets and pathways. Figuring out the exact mechanisms of miR-34a may contribute to the development of new therapeutic strategies for gastric cancer.

In conclusion, we have reported the differential expression of miR-34a in gastric cancer and adjacent normal tissues. Our results suggest that downregulation of miR-34a in gastric cancer is associated with high recurrence rate and poor overall survival and that miR-34a may serve as a prognostic marker for gastric cancer.

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Disclosure of conflict of interest

None.

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