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ORIGINAL ARTICLE

Prognostic Implications of miR-302a/b/c/d in Human Gastric Cancer

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Abstract Background: The microRNA (miR)-302 family consisting four members, miR-302a, miR-302b, miR-302c and miR-302d, plays an important role in diverse biological processes, and regulates many pathological changes, including cancer. However, the involvement of the miR-302 family into human gastric cancer (GC) remains unclear. The aim of this study was to investigate the expression patterns of miR-302a/b/c/d and determine their clinical significance in GC. Materials and methods: Expression levels of miR-302a/b/c/d in 160 pairs of human GC and matched normal mucosa tissues were detected by quantitative real-time polymerase chain reaction. Then, the associations of miR-302a/b/c/d expression with various clinicopathological characteristics and patients' prognosis were statistically evaluated. Results: The expression levels of miR-302a, miR-302b and miR-302c in GC tissues were all significantly lower than those in matched normal mucosa (all $P < 0.001$), but miR-302d expression had no significant differences between cancer and normal groups. Additionally, GC patients with low miR-302a, miR-302b and miR-302c expression more frequently had positive lymph node metastasis (all $P < 0.05$), advanced TNM stage (all $P < 0.05$) and great depth of invasion (all $P < 0.05$). More importantly, low miR-302a, miR-302b and miR-302c expression in GC tissues were significantly associated with shorter disease-free and overall survivals of GC patients (all $P < 0.05$). Further multivariate analysis identified miR-302a, miR-302b and miR-302c as independent prognostic markers for GC patients. Conclusions: GC patients with the decreased expression of miR-302a, miR-302b and miR-302c may had aggressive cancer progression and unfavorable prognosis. Further rigorous validation based on a large cohort of clinical cases should be performed.

Keywords Gastric cancer · miR-302a/b/c/d · Clinicopathological feature · Disease-free survival · Overall survival

Introduction

As the fourth most prevalent type of cancer, gastric cancer (GC) represents the second leading cause of cancer-related death worldwide [1]. According to the Lauren classification, the histological subtypes of GC patients are divided into the intestinal type and the diffuse type, which are both associated with Helicobacter pylori infection [2]. Although the overall five-year survival rate of GC patients with early stage can be above 90% following surgical treatment, about two thirds of GC patients may occur tumor metastasis or local invasion at diagnosis [3]. The median survival time of GC patients with late stage is only six to nine months after surgical treatment [4]. It is extremely necessary to develop early detection methods and novel treatment strategies. In line with other human cancers, it is a multiple-stage process during the development of GC and the accumulation of molecular changes may lead to malignant phenotypes with the aggressive progression properties [5]. However, the precise molecular mechanisms underlying GC carcinogenesis and progression have

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not been fully elucidated. Therefore, it is of clinical significance to identify novel molecular markers to improve diagnosis and prognosis, and to develop efficient therapeutic strategies for GC patients.

MicroRNAs (miRNAs), endogenous, small non-coding RNAs with 18–25 nucleotides in length, function as crucial post-transcriptional regulators of gene expression [6]. In humans, more than 900 miRNAs have been identified and are estimated to regulate more than 30 % of genes in the genome [7]. Growing evidence show that miRNAs are involved into multiple biological processes, such as cell growth, differentiation and apoptosis, as well as various pathological changes, such as tumorigenesis, tumor development, metastasis and angiogenesis [8]. The miR-302 family, containing four members, miR-302a, miR-302b, miR-302c and miR-302d, has the same seed sequence (5′-aagugcu-3′) and is ubiquitously distributed in vertebrates and occupies an intragenic cluster located in the gene La-related protein 7 (LARP7) [9]. miR-302 s play crucial roles in self-renewal and stemness maintenance in stem cells, and regulate diverse biological processes, such as early embryo development and sex determination [10]. Pathologically, accumulating studies have reported the involvement of miR-302 s in various human cancers. For example, Zhao et al. [11] indicated that miR-302 s might cooperatively sensitize breast cancer cells to adriamycin via suppressing P-glycoprotein by targeting MEKK1 of ERK pathway, suggesting miR-302 s might be a potential target for reversing P-gp-mediated chemoresistance in breast cancer; Guo et al. [12] provided evidence that miR-302a might play a key role in inhibition of the ovarian cancer cell proliferation, and enhancing cell apoptosis through targeting SDC1, and strongly suggested that exogenous miR-302a might have therapeutic value in treating ovarian cancer; Wei et al. [13] indicated that miR-302a overexpression could inhibit proliferation and invasion of colorectal cancer cells by reducing the expressions of related proteins through suppressing the MAPK and PI3K/Akt signaling pathways; Cai et al. [14] showed that miR-302b could enhance the sensitivity to 5-FU in HCC cell lines and verified its two putative targeted genes responsible for its 5-FU sensitivity; Ge et al. [15] reported that miR-302b might act as a tumor suppressor in epithelial ovarian cancer by targeting RUNX1 and modulating the activity of the STAT3 signaling pathway; Wang et al. [16] found that miR-302b suppressed hepatocellular carcinoma growth via targeting the EGFR/AKT2/CCND1 pathway. These findings highlight the crucial roles of the miR-302 family in tumorigenesis, tumor progression and tumor prevention.

Especially in human GC, a previous study of Chen et al. [17] performed miRNA array based on clinical tissue samples and identified that the expression of miR-302 s might be modulated by RACK1, which regulated the IL8 expression and tumor invasion of GC through miR-302c; Khalili et al. [18] indicated that miR-302b was downregulated in gastric adenocarcinoma, and played a potential tumor-suppressor role in tumorigenesis of gastric tissues. These observations led us to investigate the expression profiles of miR-302 s in GC, and to determine their clinical implications. We first detected the expression levels of miR-302a, miR-302b, miR-302c and miR-302d by quantitative polymerase chain reaction (qPCR) using 160 pairs of human GC and matched normal mucosa tissues. Then, the associations of their expression with various clinicopathological characteristics and patients' prognosis were also statistically evaluated.

Materials and Methods

Ethics Statement

The present study was approved by the Ethics Committee of Huai'an First People's Hospital of Nanjing Medical University and Lianshui Third People's Hospital, China. Signed informed consent was also acquired. All specimens were handled and made anonymous based on the ethical and legal standards.

Patients and Tissue Samples

One hundred and sixty fresh GC and matched normal mucosa specimens were obtained from 160 patients with GC (108 male and 52 female; median age: 58 years, range: 28–86 years) in Department of Gastroenterology of Huai'an First People's Hospital from January 2005 to December 2010. All specimens were stored at −80 °C until use to detect relative expression levels of miR-302a, miR-302b, miR-302c and miR-302d. All participants in the current study did not receive any radiotherapy or chemotherapy before the surgery, and were classified based on the World Health Organization (WHO) Pathological Classification of Tumors. Of 160 cases, 58 (36.25%) were well or moderately differentiated tumors and 102 (63.75%) were poor or no differentiation. Regarding to the histological types, there were 10 cases of papillary adenocarcinoma, 92 cases of tubular adenocarcinoma, 50 cases of mucinous adenocarcinoma, and 8 cases of signet-ring cell carcinoma. There were 61 cases of intestinal histologic type and 99 cases of diffuse histologic type based on the Lauren classification. According to 2002 tumor-nodes-metastases (TNM) classification system, 16 cases were TNM stage I, 40 cases were stage II, 42 cases were stage III, and 62 cases were stage IV. The detail information on the clinicopathological characteristics of all 160 patients with GC was shown in Table 1.

All 160 patients with GC were given a follow-up exam ranging from 3 to 6 years. Patients who died from diseases other than GC or from unexpected events were excluded from the case collection in this study. For the analysis of survival and follow-up, the date of surgery was used to represent the beginning of the follow-up period. Overall survival was a

Table 1 Associations of miR-302a/b/c expressions with various clinicopathological characteristics of patients with gastric cancer

Clinical features	Case Number (%)	miR-302a expression		P	miR-302b expression		P	miR-302c expression		P
		High (n, %)	Low (n, %)		High (n, %)	Low (n, %)		High (n, %)	Low (n, %)	
Age (years)										
< 58	50 (31.25)	22 (44.00)	28 (56.00)	NS	22 (44.00)	28 (56.00)	NS	24 (48.00)	26 (52.00)	NS
≥ 58	110 (68.75)	56 (50.91)	54 (49.09)		55 (50.00)	55 (50.00)		55 (50.00)	55 (50.00)	
Gender										
Male	108 (67.50)	52 (48.15)	56 (51.85)	NS	52 (48.15)	56 (51.85)	NS	53 (49.07)	55 (50.93)	NS
Female	52 (32.50)	26 (50.00)	26 (50.00)		25 (48.08)	27 (51.92)		26 (50.00)	26 (50.00)	
Tumor size (cm)										
< 4	58 (36.25)	28 (48.28)	30 (51.72)	NS	28 (48.28)	30 (51.72)	NS	30 (51.72)	28 (58.28)	NS
≥ 4	102 (63.75)	50 (49.02)	52 (50.98)		49 (48.04)	53 (51.96)		49 (48.04)	53 (51.96)	
Lauren classification										
Diffuse type	99 (61.88)	48 (48.48)	51 (51.52)	NS	48 (48.48)	51 (51.52)	NS	50 (50.51)	49 (49.49)	NS
Intestinal type	61 (38.12)	30 (49.18)	31 (50.82)		29 (47.54)	32 (52.46)		29 (47.54)	32 (52.46)	
Differentiation										
Well or moderate	58 (36.25)	29 (50.00)	29 (50.00)	NS	28 (48.28)	30 (51.72)	NS	30 (51.72)	28 (48.28)	NS
Poor	102 (63.75)	49 (48.04)	53 (51.96)		49 (48.04)	53 (51.96)		49 (48.04)	53 (51.96)	
Lymph node metastasis										
Negative	100 (62.50)	60 (60.00)	40 (40.00)	0.008	60 (60.00)	40 (40.00)	0.008	60 (60.00)	40 (40.00)	0.008
Positive	60 (37.50)	18 (30.00)	42 (70.00)		17 (28.33)	43 (71.67)		19 (31.67)	41 (68.33)	
TNM stage										
I	16 (10.00)	16 (100.00)	0 (0.00)	<0.001	16 (100.00)	0 (0.00)	<0.001	16 (100.00)	0 (0.00)	<0.001
II	40 (25.00)	36 (90.00)	4 (10.00)		35 (87.50)	5 (12.50)		35 (87.50)	5 (12.50)	
III	42 (26.25)	26 (61.90)	16 (38.10)		26 (61.90)	16 (38.10)		28 (66.67)	14 (33.33)	
IV	62 (38.75)	0 (0.00)	62 (100.00)		0 (0.00)	62 (100.00)		0 (0.00)	62 (100.00)	
Depth of invasion										
Mucosa or submucosa	26 (16.25)	24 (92.31)	2 (7.69)	0.01	24 (92.31)	2 (7.69)	0.01	24 (92.31)	2 (7.69)	0.01
Muscularis or subserosa	20 (12.50)	13 (60.00)	8 (40.00)		12 (60.00)	8 (40.00)		12 (60.00)	8 (40.00)	
Serosa	82 (51.25)	37 (45.12)	45 (54.88)		37 (45.12)	45 (54.88)		37 (45.12)	45 (54.88)	
Adjacent structure	32 (20.00)	5 (15.63)	27 (84.37)		4 (12.50)	28 (87.50)		6 (18.75)	26 (81.25)	

'NS' refers to the difference without statistical significance

endpoint which was calculated as the amount of time between the date of surgery and the date of death, regardless of the cause. Disease-free survival was defined as the time from randomization until recurrence of tumor or death from any cause. Surviving patients were censored on March 31, 2013.

QPCR

Total RNA was isolated with Trizol (Invitrogen, Carlsbad, CA) based on the instruction of manufacturers. During this process, RNAse-free DNase was used to remove DNA contamination. The concentration and quantity of total RNA were evaluated by using a DNA/Protein Analyzer (GeneQuant pro RNA/DNA). A total of 2 μg RNA was reverse transcribed using the SuperScript II RNase-Reverse Transcriptase System (Invitrogen, Carlsbad, CA). The cDNA was then subjected to real-time PCR with primers specific to the target miRNAs. U6 was used as an internal control to normalized the expression levels of miR-302 s. PCR cycles were as follows: 95 °C for 3 min, followed by 38 cycles of 94 °C for 1 min, 60 °C for 1 min and 72 °C for 1 min. The SYBR Premix Ex TaqTM kit (TaKaRa, Otsu, Shiga, Japan) was used to measure the amplified DNA, and real-time PCR was performed using an iQ5 real-time PCR detection system (Bio-Rad). All primers were obtained from Sangon (Shanghai, China). The amount of miR-302 s relative to U6 was calculated as the average $2^{-\Delta Ct}$, where $\Delta Ct = Ct\text{-}Ct_{u6}$.

Statistical Analysis

The software of SPSS version 11.0 for Windows (SPSS Inc., IL, USA) were used for statistical analyses in the current study. Continuous variables were expressed as Mean ± Standard Deviation (S.D). The differences of miR-302 s expression levels between GC tissues and matched normal mucosa were evaluated by the paired-*t* test. The associations of miR-302 s expression with various clinicopathological characteristics of patients with GC were analyzed by Fisher's exact test for any 2 × 2 tables and Pearson χ^2 test for non-2 × 2 tables. The survival analysis was estimated by the Kaplan-Meier method and was compared by using the log-rank test. Multivariate analysis was performed using the Cox proportional hazard model. A difference was considered significant when $P < 0.05$.

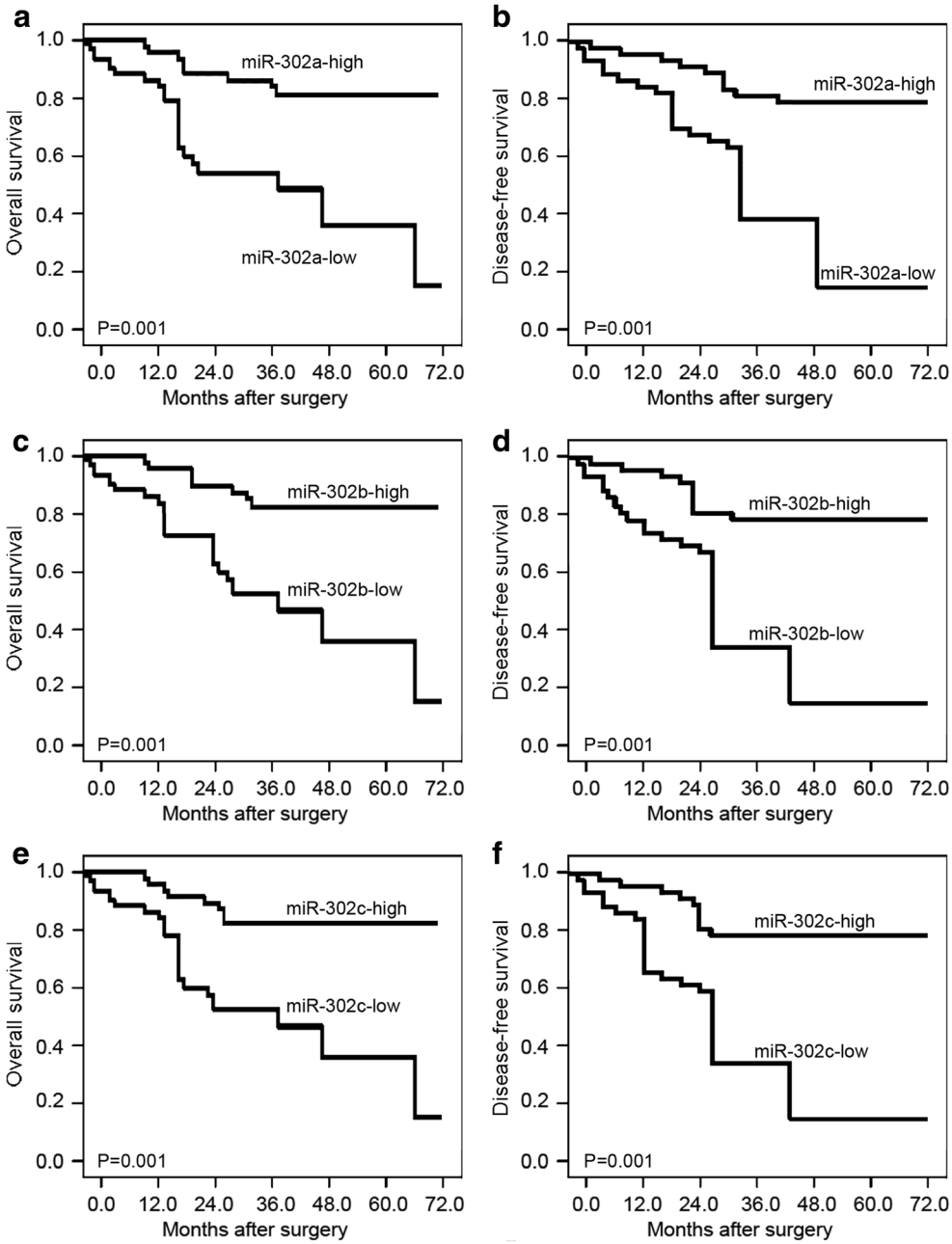

Fig. 1 Kaplan-Meier survival curves of overall survival and disease-free survival for miR-302a (**a** and **b**, respectively), miR-302b (**c** and **d**, respectively) and miR-302c (**e** and **f**, respectively) expression in human gastric cancer

Results

Expression of miR-302a/b/c/d in Human GC Tissues

The expression levels of miR-302a, miR-302b, miR-302c and miR-302d in 160 fresh GC tissues and matched normal mucosa were firstly detected in this study by qPCR. As a result, the relative expression levels of miR-302a (GC vs. Normal: 1.69 ± 0.80 vs 3.73 ± 1.04, $P < 0.001$), miR-302b (GC vs. Normal: 1.61 ± 1.17 vs 3.67 ± 1.22, $P < 0.001$) and miR-302c (GC vs. Normal: 2.42 ± 1.19 vs 4.22 ± 1.30, $P < 0.001$) in GC tissues were all significantly lower than those in matched normal mucosa, but miR-302d (GC vs. Normal: 1.58 ± 1.18 vs 2.72 ± 0.79, $P > 0.05$) expression had no significant differences between cancer and normal groups. Thus, we would like to assess the clinical significance of miR-302a, miR-302b and miR-302c expression in human GC in the following sections.

Downregulation of miR-302a, miR-302b and miR-302c Associate with Aggressive Progression of Human GC

The associations of miR-302a, miR-302b and miR-302c expression levels with various clinicopathological characteristics were evaluated. The median values of miR-302a (1.53), miR-302b (1.25) and miR-302c (2.44) expression in GC tissues were used as cutoff points for dividing all

Table 2 Prognostic value of miR-302a/b/c expressions for overall survival and disease-free survival of patients with gastric cancer in multivariate analysis by Cox Regression

Features	Overall survival		Disease-free survival	
	Hazard ratio(95%CI)	P	Hazard ratio(95%CI)	P
Age	1.005 (0.101–2.008)	NS	0.798 (0.122–1.689)	NS
Gender	1.379 (0.303–2.743)	NS	1.199 (0.336–2.306)	NS
Tumor size	0.462 (0.202–1.039)	NS	0.729 (0.308–1.692)	NS
Lauren classification	2.232 (0.566–4.676)	NS	1.928 (0.416–4.086)	NS
Differentiation	1.019 (0.272–2.069)	NS	1.012 (0.262–2.016)	NS
Lymph node metastasis	4.658 (0.913–9.621)	0.01	4.126 (0.893–9.282)	0.01
TNM stage	10.039 (1.925–20.791)	<0.001	9.629 (1.902–19.391)	<0.001
Depth of invasion	3.939 (0.825–8.791)	0.02	3.027 (0.628–7.128)	0.03
miR-302a expression	4.282 (0.869–9.623)	0.01	3.686 (0.728–8.822)	0.02
miR-302b expression	4.081 (0.832–9.261)	0.01	3.582 (0.722–7.986)	0.02
miR-302c expression	4.128 (0.862–9.628)	0.01	3.860 (0.802–8.656)	0.02

160 GC patients into miR-302a-low/high, miR-302b-low/high and miR-302c-low/high groups, respectively. GC patients with the relative expression of miR-302a/b/c exceeding the corresponding median values were deemed to be miR-302a/b/c-high groups; all other tissues were considered to be miR-302a/b/c-low groups. Of 160 GC patients, 78 (48.75%) displayed high expression of miR-302a and 82 (51.25%) showed low expression of miR-302a; 77 (48.13%) displayed high expression of miR-302b and 83 (51.87%) showed low expression of miR-302b; 79 (49.38%) displayed high expression of miR-302c and 81 (50.62%) showed low expression of miR-302c. Data in Table 1 showed that GC patients with low miR-302a, miR-302b and miR-302c expression more frequently had positive lymph node metastasis (all $P < 0.05$), advanced TNM stage (all $P < 0.05$) and great depth of invasion (all $P < 0.05$). However, there was no significant relations between miR-302a/b/c expression and other clinicopathological characteristics such as age, gender, tumor size, lauren classification, and tumor differentiation (all $P > 0.05$, Table 1).

Downregulation of miR-302a, miR-302b and miR-302c Associate with Poor Prognosis in Human GC

To further evaluate the prognostic implications of miR-302a, miR-302b and miR-302c expression in human GC, Kaplan-Meier analysis were performed and found that GC patients with low miR-302a expression/ low miR-302b expression/ low miR-302c expression had both shorter overall survival and disease-free survival than those with high miR-302a expression (both $P = 0.001$, log-rank test, Fig. 1) / high miR-302b expression (both $P = 0.001$, log-rank test, Fig. 1) / high miR-302c expression (both $P = 0.001$, log-rank test, Fig. 1).

Then, the Cox proportional hazard regression model was used to determine whether miR-302a, miR-302b and miR-302c expression were independent prognostic factors. As a result, Univariate analysis showed that the presence of lymph node metastasis (both $P = 0.01$), higher TNM stage (both $P < 0.001$), greater depth of invasion ($P = 0.02$ and 0.03, respectively), low miR-302a expression (both $P = 0.001$), low miR-302b expression (both $P = 0.001$) and low miR-302c expression (both $P = 0.001$) were significantly associated with poor overall survival and disease-free survival.

Furthermore, multivariate analysis showed that status of lymph node metastasis [for overall survival, hazard ratio (HR): 4.055, 95% CI: 0.839–8.228, $P = 0.01$; for disease-free survival, HR: 3.167, 95% CI: 0.702–6.832, $P = 0.02$], TNM stage [for overall survival, HR: 9.462, 95% CI: 1.908–19.918, $P < 0.001$; for disease-free survival, HR: 8.363, 95% CI: 1.802–18.089, $P < 0.001$], depth of invasion [for overall survival, HR: 3.062, 95% CI: 0.768–6.918, $P = 0.03$; for disease-free survival, HR: 2.618, 95% CI: 0.682–6.138, $P = 0.03$], miR-302a expression [for overall survival, HR: 4.282, 95% CI: 0.869–9.623, $P = 0.01$; for disease-free survival, HR: 3.686, 95% CI: 0.728–8.822, $P = 0.02$], miR-302b expression [for overall survival, HR: 4.081, 95% CI: 0.832–9.261, $P = 0.01$; for disease-free survival, HR: 3.582, 95% CI: 0.722–7.986, $P = 0.02$] and miR-302c expression [for overall survival, HR: 4.128, 95% CI: 0.862–9.628, $P = 0.01$; for disease-free survival, HR: 3.860, 95% CI: 0.802–8.656, $P = 0.02$] were independent prognostic markers for predicting unfavorable clinical outcome of GC patients (Table 2).

Discussion

Identification of novel and efficient molecular markers may improve the diagnostic and therapeutic levels of human GC.

Accumulating studies have identified various miRNAs which are differentially expressed in GC tissues and patients' sera, and some of which may be further evaluated as early diagnostic markers, prognostic factors and therapeutic targets of human GC. In the current study, we performed qPCR analysis and found that the expression levels of miR-302a, miR-302b and miR-302c in GC tissues were all significantly lower than those in matched normal mucosa (all $P < 0.001$), but miR-302d expression had no significant differences between cancer and normal groups. In addition, GC patients with low miR-302a expression, low miR-302b expression and low miR-302c expression more frequently had positive lymph node metastasis (all $P < 0.05$), advanced TNM stage (all $P < 0.05$) and great depth of invasion (all $P < 0.05$). More importantly, low miR-302a, miR-302b and miR-302c expression in GC tissues were significantly associated with shorter disease-free and overall survivals of GC patients (all $P < 0.05$). Further multivariate analysis identified miR-302a, miR-302b and miR-302c as independent prognostic markers for GC patients. These findings highligh the diagnostic and prognostic implications of miR-302a/b/c in human GC.

The miR-302 family has been reported to play an important role in stemness maintenance, cellular self-renewal, differentiation and reprogramming, and to be involved into various biological and pathological processes, mainly through regulating the corresponding target genes during cell cycle, cellular signalling transduction and epigenetic regulation [10]. MiR-302a has been indicated to be downregulated in malignant cells of breast cancer [11], ovarian cancer [12], colorectal cancer [13], testicular embryonal carcinoma [19], and play an important role in regulation of invasion and metastasis of cancer cells. Consistent with these human malignancies, our data found the decreased expression of miR-302a in GC tissues compared to normal mucosa and the closed correlation of miR-302a downregulation with aggressive tumor progression, and short overall and disease-free survivals of patients with GC. Recent tumor-related miRNA studies have proved the potential function of miR-302b as a tumor suppressor in different cancer types, such as glioma [20], esophageal squamous cell carcinoma [21], breast cancer [22], hepatocellular carcinoma [14, 16] and epithelial ovarian cancer [15]. In line with our data, as an embryonic stem cell-specific miRNA, miR-302b expression was found to be downregulated in high-grade GC and its potential tumor-suppressor role was also determined in tumorigenesis of gastric tissues. Regarding to miR-302c, growing evidence show that miR-302c may suppress tumor growth, invasion and migration of glioma [23] and hepatocellular carcinoma [24]. Chen et al. [17] indicated that the loss of the receptor for activated C-kinase could promote metastasis of GC by inducing a miR-302c/IL-8 signaling loop. Our study further confirmed the clinical significance of miR-302c in human GC.

In conclusion, GC patients with the decreased expression of miR-302a, miR-302b and miR-302c may had aggressive cancer progression and unfavorable prognosis. Further rigorous validation based on a large cohort of clinical cases should be performed. Since miRNAs exert the biological functions via regulating the corresponding target genes, the identification of candidate targets for miR-302a/b/c and the investigation of underlying mechanisms of these miRNAs acting on tumor progression and prognosis in human GC should also be carried out in our future research.

Author Contributions YX and SA: participated in study design and coordination, analysis and interpretation of data, and supervised study. MG performed most of the experiments and statistical analysis and drafted the manuscript. LQ and DW: carry out the experiment and sample collection. All authors read and approved the final manuscript.

Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

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