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Clinicopathological and prognostic significance of miR-4317 expression in gastric cancer patients

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Abstract. – OBJECTIVE: Previous studies have revealed that miR-4317 was abnormally expressed and functioned as a tumor suppressor in several tumors, including gastric cancer (GC). However, the clinical significance of miR-4317 in GC remains largely unclear. Our present study aimed at investigating the possibility of miR-4317 as a novel prognostic biomarker for GC patients.

PATIENTS AND METHODS: RT-PCR was performed to determine the levels of miR-4317 in GC tissues and matched normal gastric tissues. Association between miR-4317 levels and clinicopathological factors was also analyzed using Chi-square test. Kaplan-Meier survival analysis and univariate and multivariate assays were further conducted to investigate the correlation between miR-4317 expression and GC patients prognosis.

RESULTS: We showed that miR-4317 expression levels were significantly lower in GC cell lines compared to matched normal tissues ($p < 0.01$). Down-regulation of miR-4317 was observed to be associated with lymph node metastasis ($p = 0.019$), distant metastasis ($p = 0.011$) and TNM stage ($p = 0.007$). In addition, Kaplan-Meier analysis showed that patients with low miR-4317 expression had a shorter overall survival compared with the high miR-4317 expression group ($p = 0.0009$). Furthermore, multivariate analysis revealed that miR-4317 expression was an independent prognostic marker of overall survival of GC patients.

CONCLUSIONS: miR-4317 expression may be a novel and valuable prognostic factor in GC.

Key Words:
miR-4317, Gastric cancer, Prognosis.

Introduction

Gastric cancer (GC) is among the most common types of cancer in human and the second most important cause of tumor death after lung cancer worldwide, with 934,000 new cases occurring each year[1,2]. Early symptoms are variable in different GC patients and may include heartburn, upper abdominal pain and loss of appetite[3]. GC is frequently diagnosed at advanced stage accompanied by malignant proliferation and metastasis[4]. In spite of current advances in the chemotherapy and molecular targeting therapy for GC, the prognosis for patients diagnosed with advanced GC remains unfavorable and the 5-year survival rate of GC is lower than 25%[5,6]. The occurrence of local and systemic metastasis of GC cells was considered to be important reasons for poor prognosis of GC patients[7]. Because the molecular mechanisms underlying GC tumorigenesis and progression remain largely unclear, it is very difficult to develop the early diagnosis and accurate treatment methods for GC[8]. Up to date, more and more studies focused on the researches of identifying novel biomarkers that can be used to accurately predict the prognosis in GC patients. MicroRNAs (miRNAs), a novel class of small noncoding RNAs (19-22 nucleotides), control gene expressions by binding to the 3'-UTR of mRNAs[9]. miRNAs have been shown to regulate functional gene expression by various mechanism, and thus, they are considered to be novel regulators in several cellular processes such as cell growth, differentiation and apoptosis[10,11]. Recently, growing evidence

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indicated that miRNAs played an oncogenic or tumo-suppressive roles in the tumorigenesis and development of various tumors, including GC, and the dysregulated miRNAs can function as potential treatment targeting as well as potential biomarkers for GC patients[12-15]. However, because of the huge amounts of miRNAs, only a few of them were identified to be dysregulated and their functional remains largely unclear. miR-4317, located on 18p11.31, was a newly identified miRNA whose abnormally expression has been reported in several tumors, such as melanoma[16], laryngeal carcinoma[17], breast cancer[18] and non-small cell lung cancer[19], suggesting that the frequent dysregulation of miR-4371 may be involved in the regulation of tumor progression. Hu et al[20] firstly reported that miR-4317 expression was down-regulated in both GC tissues and cell lines, and its tumor-promotive roles were also confirmed *in vitro*. However, whether miR-4317 was frequently down-regulated in GC, and its clinical significance in GC patients, need to be further studied. In this study, we further offered evidence that miR-4317 was lowly expressed in our GC cases and was associated with poor long-term survival of GC patients, suggesting its potential as a novel biomarker for GC.

Patients and Methods

Patients and Clinical Specimens

A total of 191 GC tissues and adjacent non-tumor normal gastric tissues were obtained at Jining No.1 People's Hospital Affiliated to Jining Medical University between January 2009 and January 2013. The median patient age was 44 years (range 18-75 years). All collected tissue samples were snap-frozen in liquid nitrogen and preserved at -80°C until RT-PCR. All tissues were histologically diagnosed by two senior pathologists. All the patients did not receive prior anticancer treatment. Detailed information of each tissue donor is provided in Table I. The study was approved by the Ethic Committee of the Jining No.1 People's Hospital Affiliated to Jining Medical University and informed consent was obtained from each patient.

RNA Extraction and Real-Time PCR

All collected tissues were immersed in RNA later (Ambion, Xuhui, Shanghai, China) and stored at 20°C for RNA extraction. The miRNAs were extracted from frozen tissues (GC tissues and matched normal tissues) with a mirVana miRNA Isolation Kit (Applied Biosystems, Fos-

Table I. Association of miR-4317 expression with clinico-pathological features of GC.

Variables	Cases (N)	miR-4317 expression		*p*-value
		High	Low	
Age (years)				0.425
< 60	94	44	50	
≥ 60	97	51	46	
Gender				0.462
Male	115	55	60	
Female	78	41	36	
Differentiation degree				0.109
Well/Moderately	122	66	56	
Poorly	69	29	40	
Depth of invasion				0.415
T1/T2	119	57	52	
T3/T4	72	38	44	
Lymph node metastasis				0.019
N0/N1	136	75	61	
N2/N3	55	20	35	
Distant metastasis				0.011
Yes	139	77	62	
No	52	18	34	
TNM stage				0.007
I+II	127	72	55	
III+IV	64	23	41	

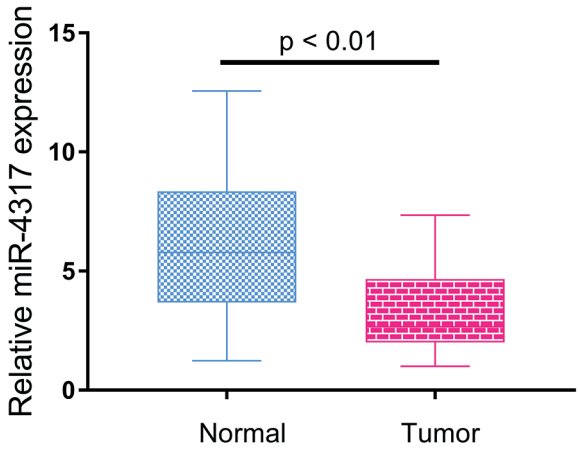

Figure 1. Expression of miR-4317 was decreased in GC tissues compared with matched normal gastric tissues through Real-time PCR ($p < 0.001$).

ter City, CA, USA). Reverse transcription of total miRNA was performed using miScript reverse Transcription Kit (Qiagen, Xuhui, Shanghai, China). Then, qRT-PCR was performed to determine the expression levels of miR-4317 using SYBR Premix Ex Taq (Thermo Fisher Scientific, Waltham, MA, USA) on Light Cycler 480 SYBR Green I Master (Roche, Basel, Switzerland). The primers were designed and synthesized by Genepharma Co., Ltd. (Xuhui, Shanghai, China), and its sequences were shown in Table II. Data were normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression. The average value of each triplicate was used to calculate the relative amount of miR-4317 using $2^{-\Delta\Delta Ct}$ methods.

Statistical Analysis

All data were carried out using SPSS 17.0 software package (version 17.0, SPSS Inc., Chicago, IL, USA). The significance of differences between two groups was estimated using the Student's *t*-test. The continuous data were analyzed using the Chi-square test. The overall survival was analyzed using the Kaplan-Meier method and compared by performing the log-rank test. The influence of variables on survival was evaluated by univariate and multivariate Cox proportional hazards model. A value of $p > 0.05$ was considered to be statistically significant.

Table II. Primers for RT-PCR in this study.

Name	Sequence (5'-3')
miR-4317-F	ATCCAGTGCGTGTCGTG
miR-4317-R	TGCGTCACATTGCCAGG
GAPDH-F	CAATGACCCCTTCATTGACC
GAPDH -R	GACAAGCTTCCCGTTCTCAG

Results

miR-4317 Expression was Downregulated in GC Tissues

Although it has been reported that miR-4317 was lowly expressed in GC patients, the clinical evidences were limited and further experiments were needed. In this study, we performed RT-PCR to detect the expression levels of miR-4317 in 191 GC tissues and matched normal tissues form our hospital. As shown in Figure 1A, we showed that miR-4317 expression was significantly downregulated in GC tissues compared to the normal tissue ($p < 0.01$). Thus, our results, together with previous results, suggested that miR-4317 was lowly expressed in GC and may serve a functional role in progression of GC.

Relationship between miR-4317 Expression and GC Patients' Clinicopathologic Variables

In order to explore the clinical significance of miR-4317 in GC patients, we divided all of 191 patients into two groups: the low expression group (n =96) and the high expression group (n = 95) with the median fold change of miR-4317 used as a cutoff value. As shown in Table I, the results of Chi-square test showed that low miR-4317 expression was significantly associated with positively lymph node metastasis ($p = 0.019$), distant metastasis ($p = 0.011$) and advanced TNM stage ($p = 0.007$). However, there were no significant associations between miR-4317 expression and other clinical features including age, gender, differentiation degree and depth of invasion ($p > 0.05$). Thus, our findings revealed that miR-4317 levels may influence the clinical prognosis of GC patients.

Low Expression Level of miR-4317 was Significantly Associated with Poorer Prognosis in GC Patients

With a five-year follow-up, we collected the survival time from 191 GC patients and the overall survival rate of the patients was calculated with Kaplan-Meier method. As shown in Figure 2, we found that patients with decreased miR-4317 expression had shorter overall survival than those

Table III. Summary of univariate and multivariate Cox regression analyses of overall survival duration.

	Univariate analysis			Multivariate analysis		
Parameter	**HR**	**95% CI**	***p***	**HR**	**95% CI**	***p***
Age	1.623	0.723-2.231	0.325	-	-	-
Gender	1.428	0.843-2.146	0.216	-	-	-
Differentiation degree	2.145	0.682-2.644	0.114	-	-	-
Depth of invasion	1.956	0.894-2.742	0.143	-	-	-
Lymph node metastasis	3.321	1.436-4.864	0.009	2.758	1.233-4.428	0.016
Distant metastasis	3.653	1.557-5.327	0.003	3.157	1.258-5.138	0.007
TNM stage	3.945	1.734-5.663	0.001	3.326	1.242-4.886	0.011

with miR-4317 overexpression ($p = 0.0009$). Moreover, we performed a Cox regression analysis to assess the prognostic factors. As shown in Table III, the results of univariate Cox regression analysis indicated that both miR-4317 overexpression (HR =3.568, 95% CI: 1.549-5.231, $p = 0.004$), as well as lymph node metastasis, distant metastasis and TNM stage, was prime variables for the prognosis of GC patients. More importantly, multivariate analysis further confirmed that miR-4317 expression (HR =3.052, 95% CI: 1.266-4.648, $p = 0.008$) were significant independent predictors of poor survival of GC patients.

Discussion

Although GC is no longer the most common cancer worldwide, GC remains a serious burden in China and no effective treatment is available for this disease[21]. It has been known to us that accurate prediction of prognosis for GC patients is very important for the treatment of GC patients[22]. Up to date, several clinical factors, such as TNM stage, lymph node metastasis and clinical stage, have been well used in clinical practice to predict the prognosis of GC patients and help guide the individual treatment[23,24]. Unfortunately, these common clinical characteristics were not enough for the clinical requirements. Recent years, dysregulation of genes and ncRNA in tissues and blood highlighted their potential as biomarkers which can be used to establish a diagnosis, classify tumors and predict disease outcome[25,26]. Importantly, several well-studied miRNAs were considered to have huge potential as diagnostic and prognostic biomarkers for GC patients, such as microRNA-335-5p[27], miRNA-558[28] and miR-145-5p[29]. Interesting, the abnormally expressed miRNAs in blood were also frequently reported in more and more studies, suggesting that detection of miRNAs expression may be a novel and cheap method for screening of GC patients[30,31]. Previously, several studies have reported the expression pattern and biological function of miR-4317 in several tumors. In non-small cell lung cancer, it was reported that miR-4317 was lowly expressed and its high expression was associated with advanced clinical stages and exhibited better overall survival. Further functional investigations[19] confirmed its tumor-suppressive roles in lung cancer because its expression could suppress cells proliferation and metastasis by targeting fibroblast growth factor 9. On the other hand, in gastric cancer, miR-4317 expression was also found to be down-regulated, and its forced expression can suppress human gastric cancer cell proliferation by targeting ZNF322[20]. Previous results indicated that miR-4317 may act as a tumor-suppressive regulator in gastric cancer. However, the clinical sig-

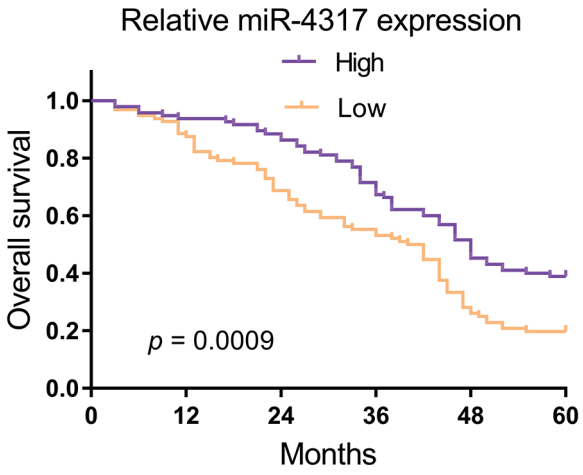

Figure 2. The association between GC patient survival and miR-4317 expression was estimated using the Kaplan-Meier method and the log-rank test.

nificance of miR-4317 has not been investigated. In this study, we performed RT-PCR to detect the expression of miR-4317 in our GC cases, finding that miR-4317 expression was significantly lower in GC tissues than that in matched normal tissues, which was consistent with previous study by Hu et al[20]. Then, the results of Chi-square test showed that low miR-4317 expression was significantly associated with lymph node metastasis, distant metastasis and advanced TNM stage, suggesting that miR-4317 may act as a negative regulator in clinical progression of GC. Furthermore, we collected five-year survival data and performed Kaplan-Meier method to explore the influence of miR-4317 on long-term of GC patients, finding that lower levels of miR-4317 expression had poorer survival than those with higher levels of miR-4317 expression. Of note, multivariate analysis further revealed that miR-4317 expression was a significant independent predictor of poor survival in GC patients. Overall, our findings highlighted the potential of miR-4317 as a novel biomarker for GC patients. Some limitations of this study should be noted. First, because the sample sizes are small, further studies on more patients are required to confirm our results. Second, our study focused on the prognostic value of miR-4317 in GC patients, and so the functional assays were not performed. Further gain-function assay and los-function assays were needed to further study the roles of miR-4317 in progression of GC. Finally, the potential mechanism by which miR-4317 influences the clinical prognosis of GC patients was not been studied. In the future, we will explore the potential mechanism.

Conclusions

We indicated that miR-4317 was significantly downregulated in GC and associated with aggressive progression and poor prognosis of GC patients, suggesting that it may be a potential biomarker and a therapeutic target of GC.

Conflict of Interest

The Authors declare that they have no conflict of interest.

References

1) Siegel RL, Miller KD, Jemal A. Cancer statistics, 2016. CA Cancer J Clin 2016; 66: 7-30.
2) Hashim D, Boffetta P, La Vecchia C, Rota M, Bertuccio P, Malvezzi M, Negri E. The global decrease in cancer mortality: trends and disparities. Ann Oncol 2016; 27: 926-933.
3) Marques-Lespier JM, Gonzalez-Pons M, Cruz-Correa M. Current perspectives on gastric cancer. Gastroenterol Clin North Am 2016; 45: 413-428.
4) Kanagavel D, Fedyanin M, Tryakin A, Tjulandin S. Second-line treatment of metastatic gastric cancer: current options and future directions. World J Gastroenterol 2015; 21: 11621-11635.
5) Lee SY, Oh SC. Changing strategies for target therapy in gastric cancer. World J Gastroenterol 2016; 22: 1179-1189.
6) Shen L, Shan YS, Hu HM, Price TJ, Sirohi B, Yeh KH, Yang YH, Sano T, Yang HK, Zhang X, Park SR, Fujii M, Kang YK, Chen LT. Management of gastric cancer in Asia: resource-stratified guidelines. Lancet Oncol 2013; 14: e535-547.
7) Poh AR, O'Donoghue RJ, Ernst M, Putoczki TL. Mouse models for gastric cancer: Matching models to biological questions. J Gastroenterol Hepatol 2016; 31: 1257-1272.
8) Miao RL, Wu AW. Towards personalized perioperative treatment for advanced gastric cancer. World J Gastroenterol 2014; 20: 11586-11594.
9) Tian T, Wang J, Zhou X. A review: microRNA detection methods. Org Biomol Chem 2015; 13: 2226-2238.
10) Vishnoi A, Rani S. miRNA biogenesis and regulation of diseases: an overview. Methods Mol Biol 2017; 1509: 1-10.
11) Erson-Bensan AE, Begik O. m6A modification and implications for microRNAs. MicroRNA 2017; 6: 97-101.
12) Wang LL, Zhang XH, Zhang X, Chu JK. MiR-30a increases cisplatin sensitivity of gastric cancer cells through suppressing epithelial-to-mesenchymal transition (EMT). Eur Rev Med Pharmacol Sci 2016; 20: 1733-1739.
13) Yu FY, Zhou CY, Liu YB, Wang B, Mao L, Li Y. miR-483 is down-regulated in gastric cancer and suppresses cell proliferation, invasion and protein O-GlcNAcylation by targeting OGT. Neoplasma 2018; 65: 406-414.
14) Qu Y, Zhang H, Sun W, Han Y, Li S, Qu Y, Ying G, Ba Y. MicroRNA-155 promotes gastric cancer growth and invasion by negatively regulating transforming growth factor-β receptor 2. Cancer Sci 2018; 109: 618-628.
15) Yan YF, Gong FM, Wang BS, Zheng W. MiR-425-5p promotes tumor progression via modulation of CYLD in gastric cancer. Eur Rev Med Pharmacol Sci 2017; 21: 2130-2136.
16) Sand M, Skrygan M, Sand D, Georgas D, Gambichler T, Hahn SA, Altmeyer P, Bechara FG. Comparative microarray analysis of microRNA expression profiles in primary cutaneous malignant melanoma, cutaneous malignant melanoma metastases, and benign melanocytic nevi. Cell Tissue Res 2013; 351: 85-98.

17) Janiszewska J, Szaumkessel M, Kostrzewska-Poczekaj M, Bednarek K, Paczkowska J, Jackowska J, Grenman R, Szyfter K, Wierzbicka M, Giefing M, Jarmuz-Szymczak M. Global miRNA expression profiling identifies miR-1290 as novel potential oncomiR in laryngeal carcinoma. PLoS One 2015; 10: e0144924.

18) Danza K, De Summa S, Pilato B, Carella M, Palumbo O, Popescu O, Paradiso A, Pinto R, Tommasi S. Combined microRNA and ER expression: a new classifier for familial and sporadic breast cancer patients. J Transl Med 2014; 12: 319.

19) He X, Chen SY, Yang Z, Zhang J, Wang W, Liu MY, Niu Y, Wei XM, Li HM, Hu WN, Sun GG. miR-4317 suppresses non-small cell lung cancer (NSCLC) by targeting fibroblast growth factor 9 (FGF9) and cyclin D2 (CCND2). J Exp Clin Cancer Res 2018; 37: 230.

20) Hu X, Zhang M, Miao J, Wang X, Huang C. miRNA-4317 suppresses human gastric cancer cell proliferation by targeting ZNF322. Cell Biol Int 2018; 42: 923-930.

21) Braam HJ, Schellens JH, Boot H, van Sandick JW, Knibbe CA, Boerma D, van Ramshorst B. Selection of chemotherapy for hyperthermic intraperitoneal use in gastric cancer. Crit Rev Oncol Hematol 2015; 95: 282-296.

22) Ashraf N, Hoffe S, Kim R. Adjuvant treatment for gastric cancer: chemotherapy versus radiation. Oncologist 2013; 18: 1013-1021.

23) Bekaii-Saab T, El-Rayes B. Identifying and targeting cancer stem cells in the treatment of gastric cancer. Cancer 2017; 123: 1303-1312.

24) Venerito M, Link A, Rokkas T, Malfertheiner P. Gastric cancer - clinical and epidemiological aspects. Helicobacter 2016; 21 Suppl 1: 39-44.

25) Hamashima C. Current issues and future perspectives of gastric cancer screening. World J Gastroenterol 2014; 20: 13767-13774.

26) Tahara T, Arisawa T. DNA methylation as a molecular biomarker in gastric cancer. Epigenomics 2015; 7: 475-486.

27) Sandoval-Bóroquez A, Polakovicova I, Carrasco-Véliz N, Lobos-González L, Riquelme I, Carrasco-Avino G, Bizama C, Norero E, Owen GI, Roa JC, Corvalán AH. MicroRNA-335-5p is a potential suppressor of metastasis and invasion in gastric cancer. Clin Epigenetics 2017; 9: 114.

28) Zheng L, Jiao W, Song H, Qu H, Li D, Mei H, Chen Y, Yang F, Li H, Huang K, Tong Q. miRNA-558 promotes gastric cancer progression through attenuating Smad4-mediated repression of heparanase expression. Cell Death Dis 2016; 7: e2382.

29) Zhang Y, Wen X, Hu XL, Cheng LZ, Yu JY, Wei ZB. Downregulation of miR-145-5p correlates with poor prognosis in gastric cancer. Eur Rev Med Pharmacol Sci 2016; 20: 3026-3030.

30) Hou CG, Luo XY, Li G. Diagnostic and prognostic value of serum MicroRNA-206 in patients with gastric cancer. Cell Physiol Biochem 2016; 39: 1512-1520.

31) Imaoka H, Toiyama Y, Okigami M, Yasuda H, Saigusa S, Ohi M, Tanaka K, Inoue Y, Mohri Y, Kusunoki M. Circulating microRNA-203 predicts metastases, early recurrence, and poor prognosis in human gastric cancer. Gastric Cancer 2016; 19: 744-753.