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Original Article

Expression of microRNA-211 is a novel prognostic indicator of poor survival in human gastric cancer

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Abstract: Purpose: MicroRNA (miR)-211 was previously identified as a candidate biomarker for screening gastric cancer (GC) samples from noncancerous gastric samples based on miRNA microarray expression profiles. The current study aimed to explore the clinical significance of miR-211 expression in human GC. Methods: Expression levels of miR-211 in 160 pairs of human GC and matched normal mucosa tissues were detected by quantitative polymerase chain reaction. Then, the associations of miR-211 expression with various clinicopathological characteristics and patients' prognosis were evaluated. Results: The expression level of miR-211 in GC tissues was significantly higher than that in matched normal mucosa (GC vs. Normal: 4.05±1.04 vs. 2.10±0.92, P<0.001). In addition, the overexpression of miR-211 (miR-211-high) was more frequently found in GC tissues with positive lymph node metastasis (P = 0.01), advanced TNM stage (P = 0.002) and great depth of invasion (P = 0.03). Moreover, high miR-211 expression was significantly associated with poor disease-free and overall survivals. Furthermore, multivariate analysis revealed that high miR-211 expression was an independent predictive marker for a poor prognosis in patients with GC. Conclusions: These findings suggest that miR-211 overexpression may promote the aggressive progression and increase the risk of death and relapse of patients with GC. MiR-211 might be a novel promising indicator for survival of this disease.

Keywords: Gastric cancer, microRNA-211, clinicopathological feature, disease-free survival, overall survival

Introduction

Gastric cancer (GC) represents the fourth most common cancer and the second leading cause of cancer-related death worldwide (the third in males and the fifth in females) [1]. Over 70% of new cases and deaths of GC are occurred in developing countries, including China, and this cancer has become a major health problem [2]. Due to a lack of early specific symptoms, diagnosis of GC is often delayed, leading to the cancer cell invasion into the muscularis propria [3]. In recent years, great advances in early diagnosis, surgical techniques, perioperative management, and the use of combined systemic chemotherapy with radiotherapy and surgery have contributed to improving the outcomes of patients. However, it is difficult to cure effectively and the overall survival rate of patients with GC still remains low, partially due to the poorly understand of the pathogenesis and molecular mechanisms of this malignancy, although the considerable researches on carcinogenesis and progression of GC has been performed [4]. Therefore, there is an urgent need to identify novel diagnostic and prognostic biomarkers, and to characterize the underlying molecular mechanisms of GC, in order to aid prediction of clinical outcome and to implement individualized treatment for patients with this cancer.

MicroRNAs (miRNAs), a class of endogenous, small non-coding RNA molecules composed of 18-25 nucleotides, play various biological functions by inhibiting or inactivating target messenger RNAs (mRNAs) via base pairing between the seed region of miRNA and 3' un-translated regions of (3'-UTR) of target mRNAs [5]. MiRNAs have been indicated to be involved into specific cellular processes such as growth, differentiation, proliferation, migration, and survival [6].

Table 1. Associations of miR-211 expression with various clinicopathological characteristics of patients with gastric cancer (GC)

Clinical features	Case Number (%)	miR-211 expression		P
		High (n, %)	Low (n, %)	
Age (years)				
<58	50 (31.25)	25 (50.00)	25 (50.00)	NS
≥58	110 (68.75)	57 (51.82)	53 (48.18)	
Gender				
Male	108 (67.50)	52 (48.15)	56 (51.85)	NS
Female	52 (32.50)	30 (57.69)	22 (42.31)	
Tumor size (cm)				
<4	58 (36.25)	30 (51.72)	28 (48.28)	NS
≥4	102 (63.75)	52 (50.98)	50 (49.02)	
Lauren classification				
Diffuse type	99 (61.88)	50 (50.51)	49 (49.49)	NS
Intestinal type	61 (38.12)	32 (52.46)	29 (47.54)	
Differentiation				
Well or moderate	58 (36.25)	30 (51.72)	28 (48.28)	NS
Poor	102 (63.75)	52 (50.98)	50 (49.02)	
Lymph node metastasis				
Negative	100 (62.50)	37 (37.00)	63 (63.00)	0.01
Positive	60 (37.50)	45 (75.00)	15 (25.00)	
TNM stage				
I	16 (10.00)	0 (0.00)	16 (100.00)	0.002
II	40 (25.00)	6 (15.00)	34 (85.00)	
III	42 (26.25)	14 (33.33)	28 (66.67)	
IV	62 (38.75)	62 (100.00)	0 (0.00)	
Depth of invasion				
Mucosa or submucosa	26 (16.25)	2 (7.69)	24 (92.31)	0.03
Muscularis or subserosa	20 (12.50)	6 (30.00)	14 (70.00)	
Serosa	82 (51.25)	44 (53.66)	38 (46.34)	
Adjacent structure	32 (20.00)	30 (93.75)	2 (6.25)	

Note: 'NS' refers to the difference without statistical significance.

Especially, growing evidence shows that miRNAs play crucial roles as potential cancer biomarkers, and may also be considered to be oncogenes or tumor suppressors [7]. In recent years, several reports have highlighted the diagnostic and prognostic utilities of miRNA expression levels in diverse human cancers, including GC [8]. For example, Yan et al. [9] performed a systematic and integrative bioinformatics framework to identify GC-related miRNAs from the public miRNA and mRNA expression dataset generated by RNA-seq technology. They identified miR-211 as a candidate biomarker for screening GC samples from noncancerous gastric samples with the predictive accuracy of 0.936. However, the clinical significance of this miRNA in human GC has not been fully elucidated. Therefore, this study detected the expression levels of miR-211 in 160 pairs of human GC and matched normal mucosa tissues by quantitative polymerase chain reaction (qPCR). Then, the associations of miR-211 expression with various clinicopathological characteristics and patients' prognosis were evaluated.

Materials and methods

Ethics statement

This research 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 according to the ethical and legal standards.

Patients and tissue samples

A total of 160 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 level of miR-211 by quantitative RT-PCR. All participants in this study did not receive any radiotherapy or chemotherapy before the surgery, and were classified according to 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. Histologically, there were 10

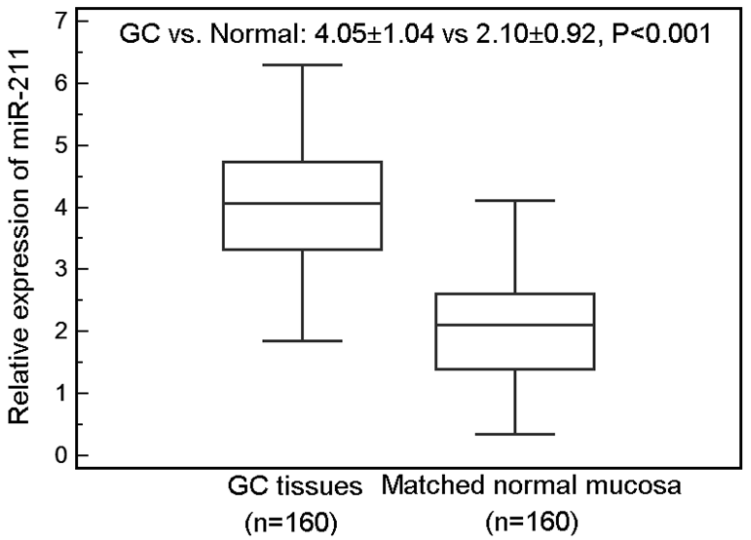

Figure 1. Relative expression of miR-211 in gastric cancer (GC) and matched normal mucosa.

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 according to 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 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). A total of 2 μg RNA was reverse transcribed using the SuperScript II RNase-Reverse Transcriptase System (Invitrogen). The cDNA was then subjected to real-time PCR with primers specific for miR-211 and U6 which was used as an internal control. PCR primers were designed as follows: miR-211 forward, 5'-TTC CCT TTG TCA TCC TTC GCC T-3'; miR-211 reverse, 5'-GTG CAG GGT CCG AGG TAT TC-3'; U6 forward, 5'-TGC GGG TGC TCG CTT CGG CAG C-3'; U6 reverse, 5'-CCA GTG CAG GGT CCG AGG T-3'. PCR cycles were as follows: 94°C for 4 min, followed by 40 cycles of 95°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). The amount of miR-211 relative to U6 was calculated as the average $2^{-\Delta Ct}$, where $\Delta Ct = Ct\text{-}Ct_{U6}$.

Statistical analysis

All statistical analyses were performed using the software of SPSS version 11.0 for Windows (SPSS Inc, IL, USA). Continuous variables were expressed as Mean ± S.D. The differences of miR-211 expression between GC tissues and matched normal mucosa were analyzed by the paired-t test. The associations of miR-211 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 $P<0.05$.

Results

Expression of miR-211 was upregulated in human GC tissues

MiR-211 expression levels in 160 fresh GC tissues and matched normal mucosa were measured by qPCR. As shown in **Figure 1**, the relative expression values of miR-211 in GC tissues

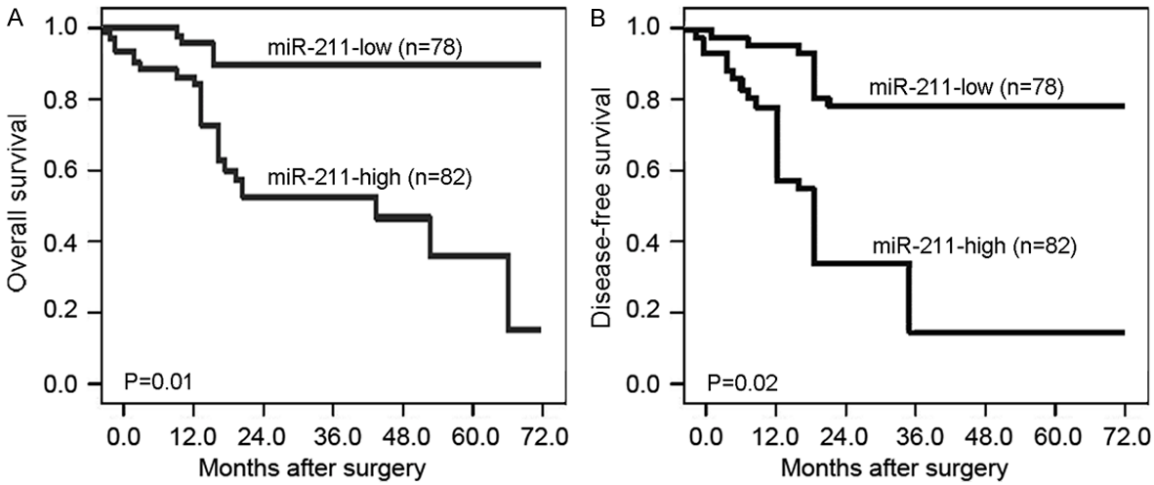

Figure 2. Kaplan-Meier survival curves of overall survival and disease-free survival for miR-211 expression in human gastric cancer (GC).

Table 2. Prognostic value of miR-211 expression for overall survival and disease-free survival of patients with gastric cancer (GC) in univariate 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-211 expression	4.398 (0.912-9.651)	0.01	3.976 (0.866-8.813)	0.02

were higher than those in matched normal mucosa (GC vs. Normal: 4.05±1.04 vs. 2.10± 0.92, P<0.001). According to our scoring system, the median value (4.06) of miR-211 expression in GC tissues was used as a cut-off point for dividing all 160 GC patients into miR-211-low and miR-23b-high groups. GC patients with the relative expression levels of miR-211 exceeding the median value were deemed to be miR-211-high group; all other tissues were considered to be miR-211-low group. Of 160 GC patients, 82 (51.25%) displayed high expression of miR-211 and 78 (48.75%) showed low expression of miR-211.

Overexpression of miR-211 associations with aggressive progression of human GC

To assess the associations between miR-211 expression and various clinicopathological characteristics, q-PCR was performed in 160 GC samples. As listed in **Table 1**, the overexpression of miR-211 (miR-211-high) was more frequently found in GC tissues with positive lymph node metastasis (P = 0.01), advanced TNM stage (P = 0.002) and great depth of invasion (P = 0.03). However, there was no significant relation between miR-211 and other clinicopathological characteristics such as age, gender, tumor size, lauren classification, and tumor differentiation (all P>0.05, **Table 1**).

Overexpression of miR-211 associations with poor prognosis in human GC

Kaplan-Meier analysis showed that GC patients with high miR-211 expression had both shorter overall survival and disease-free survival than those with low miR-211 expression (P = 0.01 and 0.02, respectively; log-rank test; **Figure 2**).

To determine whether miR-211 expression was an independent risk factor for prognosis, the Cox proportional hazard regression model was used. As shown in **Table 2**, Univariate analysis found that positive lymph node metastasis (both P = 0.01), advanced TNM stage (both P<0.001), great depth of invasion (P = 0.02 and 0.03, respectively) and high miR-211 expression (P = 0.01 and 0.02, respectively) 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] and miR-211 expression [for overall survival, HR: 4.022, 95% CI: 0.828-9.176, P = 0.01; for disease-free survival, HR: 3.182, 95% CI: 0.733-8.892, P = 0.02] were independent prognostic markers for predicting poor prognosis in patients with GC.

Discussion

Growing evidence shows that a favorable clinical outcome of patients with GC need early and accurate diagnosis, proper and effective therapeutic strategies, and efficient estimation of prognosis during the clinical practice, thus, it is an urgent necessary to identify and clarify the precise molecular mechanism involved in the development and progression of GC. In the current study, our data revealed that miR-211 was overexpression in GC tissues compared with matched normal mucosa. The elevated expression level of miR-211 was observed correlated significantly with aggressive progression of patients with GC. More importantly, the aberrant expression of miR-211 was one of the independent prognostic factors for patient's patients with GC. These results suggest that the expression of miR-211 may play a crucial role in cancer progression, migration and prognosis in patients with GC.

MiR-211, encoded within the sixth intron of TRPM1 gene at 15q13-q14, has been indicated as a cancer related gene which can impact cell migration, invasion and proliferation of multiple human malignancies, and it acts as either an onco-miRNA or a tumor suppressive miRNA in a cancer-dependent manner [10]. For example, Ye et al. [11] found that miR-211 expression was upregulated in human non-small cell lung cancer (NSCLC) cell lines and tissues, and its overexpression enhanced NSCLC cell proliferation, colony formation, and invasion via regulating its target gene SRC kinase signaling inhibitor 1; Cai et al. [12] reported that enforced expression of miR-211 could promote tumor cell growth of colorectal cancer, at least in part, by downregulating the expression level of the chromodomain-helicase-DNA-binding protein 5 tumor suppressor; Cai et al. [13] determined based on clinical data that in colon cancer patients with low expression of miR-221, the survival time was longer, while patients with high expression of miR-221 had shorter survival, suggesting that this miRNA could serve as a molecular marker for the prognosis patients with colon cancer. In contrast, Asuthkar et al. [14] reported that miR-211 was suppressed in grade IV glioblastoma multiforme specimens, and found an acute inhibitory effect of miR-211 on glioma cell invasion and migration via suppression of MMP-9; Mazar et al. [15] found that miR-211 expression was downregulated in non-pigmented melanoma and was regulated by the microphthalma associated transcription factor gene, which might be important molecular events for melanoma development and/or progression; Consistently, Levy et al. [16] implicated miR-211 as a suppressor of melanoma invasion whose expression was silenced (or selected against) via suppression of the entire melastatin locus during human melanoma progression; Jiang et al. [17] revealed that miR-211 was decreased in hepatocellular carcinoma tissues compared with adjacent normal tissues, and also found that overexpression of miR-211 repressed proliferation and invasion in HepG2 and SMMC7721 cells by downregulating its direct target special AT-rich sequence-binding protein-2; Xia et al. [18] reported that miR-211 was found to arrest cells in the G0/G1-phase, inhibit proliferation and induce apoptosis through controling the expression of Cyclin D1 and CDK6, which could restore proliferative ability. According to these previous findings, the roles of miR-211 in carcinogenesis is still contentious and may exhibit cell-specific regulatory manner.

To date, the clinical significance, biological roles, and the potentially involved molecular mechanisms of miR-211 in human GC remain unexplored. Here, our study described the clinical significance of miR-211 in GC for the first time. We found that miR-211 expression was upregulated in GC tissues compared with matched normal tissues and significantly correlated with several clinicopathological characteristics including lymph node metastasis, TNM stage and depth of invasion. Moreover, miR-211 could serve as an independent prognostic indicator of patients' overall survival and disease-free survival by Cox's proportional hazards model. Our data are in accordance with previous reports which confirm the role of miR-211 as an important oncogene in human cancers.

In conclusion, our results suggest that miR-211 overexpression may promote the aggressive progression and increase the risk of death and relapse of patients with GC. MiR-211 might be a novel promising indicator for survival of this disease.

Disclosure of conflict of interest

None.

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