Int J Clin Exp Pathol 2017;10(7):7551-7558
www.ijcep.com /ISSN:1936-2625/IJCEP0053505

Original Article

MiR-211 inhibits cell epithelial-mesenchymal transition by targeting MMP9 in gastric cancer

Xiao-Dong Wang[1], Fu-Xing Wen[2], Bai-Chun Liu[1], Ying Song[1]

[1]Department of Digestive Endoscopy, Second Affiliated Hospital of Jilin University, Jilin, China; [2]Department of General Medicine, The General Hospital of China National Petroleum Corporation in Jilin, Jilin, China

Received March 22, 2017; Accepted June 6, 2017; Epub July 1, 2017; Published July 15, 2017

Abstract: Recent studies have demonstrated that the dysregulation of miRNAs are frequently associated with cancer progression including gastric cancer (GC). MiR-211 was found to act as tumor suppressor in GC, however, the functional role of miR-211 involved in GC cell epithelial-mesenchymal transition (EMT) process still to be investigated. In the study, we demonstrated that miR-211 was lower expression in gastric cancer tissues compared with adjacent normal tissues. Lower miR-211 expression was positively associated with distant metastasis and lymph node metastasis in GC patients. Survival curve by Kaplan-Meier method and log rank test revealed that lower miR-211 expression indicated a poor outcome in GC patients. Function assays showed that miR-211 inhibited cell invasion and cell epithelial-mesenchymal transition (EMT) process in GC by upregulating E-cadherin expression and down-regulating twist1 and N-cadherin expression. Furthermore, we demonstrated that miR-211 suppressed cell EMT by targeting MMP9 expression in GC. These results showed that miR-211 acted as a tumor suppressor in GC and may be a potential target of GC treatment.

Keywords: Gastric cancer, miR-211, MMP9, epithelial-mesenchymal transition

Introduction

Gastric cancer (GC) is the fourth most common cancer worldwide and the second leading cause of cancer-related deaths in China [1, 2]. The major therapeutic option for GC is surgical resection. However, patients with advanced GC often show poor survival over time rate due to tumor recurence and metastasis [3, 4]. Therefore, it is essential to explore novel therapeutic strategies to improve GC treatment.

MicroRNAs (MiRNAs) are some endogenous small non-coding RNA molecules that could bind to the target messenger RNAs (mRNAs) in the 3'-untranslated regions (3'-UTRs) and regulated their expression [5]. MiRNAs play crucial roles in numerous biological processes including cell differentiation, cell proliferation, cell apoptosis and tumor progression including GC [6, 7]. For example, miR-124 interacts with the Notch1 signaling pathway and has therapeutic potential against gastric cancer [8]. MiR-495 and miR-551a inhibit the migration and invasion of human gastric cancer cells by directly interacting with PRL-3 [9]. MicroRNA-339, an epigenetic modulating target is involved in human gastric carcinogenesis through targeting NOVA1 [10]. MicroRNA-218 inhibits the proliferation, migration, and invasion and promotes apoptosis of gastric cancer cells by targeting LASP1 [11]. MiR-424-5p promotes proliferation of gastric cancer by targeting Smad3 through TGF-β signaling pathway [12]. Thus, these evidences showed that miRNAs play important role in GC.

MiR-211 had been found to act as a tumor suppressors or oncogenes in some tumors including GC progression, such as, miR-211 promotes non-small cell lung cancer proliferation by targeting SRCIN1 and regulating SRCIN1 expression [13]. MiR-211 suppresses cell proliferation by downregulating SATB2 expression in hepatocellular carcinoma [14]. MiR-211 modulates gemcitabine activity through downregulation of ribonucleotide reductase and inhibits the invasive behavior of pancreatic cancer cells [15].

Another study found that miR-211 inhibits cell proliferation and invasion of gastric cancer by down-regulating SOX4 expression [16]. However, the role of miR-211 in GC EMT process is still needed to be explored.

In the study, we demonstrated that miR-211 was lower expression in gastric cancer and associated with distant metastasis and lymph node metastasis in gastric cancer patients. Survival curve revealed that lower miR-211 expression indicated a poor outcome in GC patients. Furthermore, we demonstrated that miR-211 suppressed cell EMT process by regulating MMP9 expression. These results showed that miR-211 acted as a tumor suppressor in GC and may be a potential target of GC treatment.

Materials and methods

Patients and tissue sample

A total of 72 cases of patient tissues and adjacent normal tissues who received GC radical resection at the Second Hospital of Jilin University were used in this study. All of patients were confirmed as gastric adenocarcinoma by two pathologists. No patient received preoperative chemotherapy or radiotherapy. Tissues samples were immediately collected in liquid nitrogen and stored at -80°C for further analysis. Tumor stage was evaluated according to the criteria of the WHO Classification of Tumours of the Digestive System, 2010 edition. This study was proved by the Ethics Review Board of The Second Hospital of Jilin University and written inform was obtained from all patients.

Cell culture and cell transfection

Human GC cell lines AGS, BGC-823, MKN45, SGC7901 and a normal human gastric epithelial cell line GES-1 was purchased from the Chinese Academy of Sciences (Shanghai, China). Cell lines were cultured in RPMI-1640 medium (Thermo Fisher Scientific, Waltham, MA, USA) with 10% fetal bovine serum and antibiotics (1% penicillin/streptomycin; Thermo Fisher Scientific) in a humidified chamber with an atmosphere of 5% CO_2 at 37°C. MiR-211 mimic and miR-211 inhibitor and niR-211 negative control were collected from Ribobio (Guangzhou, China). Cell transfection was performed using the Lipofectamine 2000 Reagent (Invitrogen, USA) following to manufacturer's instruction.

Cell invasion assay

Cells invasive ability was measured by using Transwell invasion chambers coated with Matrigel (BD Biosciences, USA) according to the manufacturer's instruction. The transfected SGC-7901 and BGC-823 cells were collected and 1×10^5 cells/per well were resuspended in 100 μL serum-free medium and then added to the upper chambers. 500 μL medium supplemented with 10% FBS was added to the lower chamber. After incubation for 36 h, the membranes were fixed with methanol for 20 min and stained with 2% crystal violet for 20 min, and then be counted under a light microscope.

Quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA was isolated from tissues or cultured cells by using Trizol regent (Invitrogen) according to the manufacturer's instructions. The miScript II RT Kit (TAKAKA) was used to reverse RNA to cDNA. The qRT-PCR assay was performed using a miScript SYBR Green PCR Kit (TAKAKA) according to the manufacturer's instructions. The qRT-PCR analysis was conducted on ABI 7500 Real-Time PCR System (ABI, Foster City, CA, USA). The miRNA expression was calculated by normalization to the internal control U6 using the $2^{-\Delta\Delta Ct}$ method. Primers were as follows: β-actin forward, 5'-TGACGGGGTCACCCACACTGTGCCCATCTA-3', reverse, 5'-CTAGAAGCATTTGCGGTGGACGATGGAGGG-3'. miR-211 forward, 5'-TTCCCTTTGTCATCCTTCGCCT-3'.

Western blot analysis

Cells were harvested after transfected with miR-211 mimic, miR-211 inhibitor, miR-NC at 48 h and lysed in RIPA buffer (Sigma). Proteins were separated by a 10% or 12% polyacrylamide gel and transferred to a PVDF membrane. The membranes were probed with primary antibodies including anti-Twist1 (1:2000; Abcam, Cambridge, MA, USA), MMP9 (1:2000; Abcam, Cambridge, MA, USA), E-cadherin (Proteintech, Chicago, IL, USA), N-cadherin (Proteintech, Chicago, IL, USA), and GAPDH (Proteintech, Chicago, IL, USA) overnight 4°C fol-

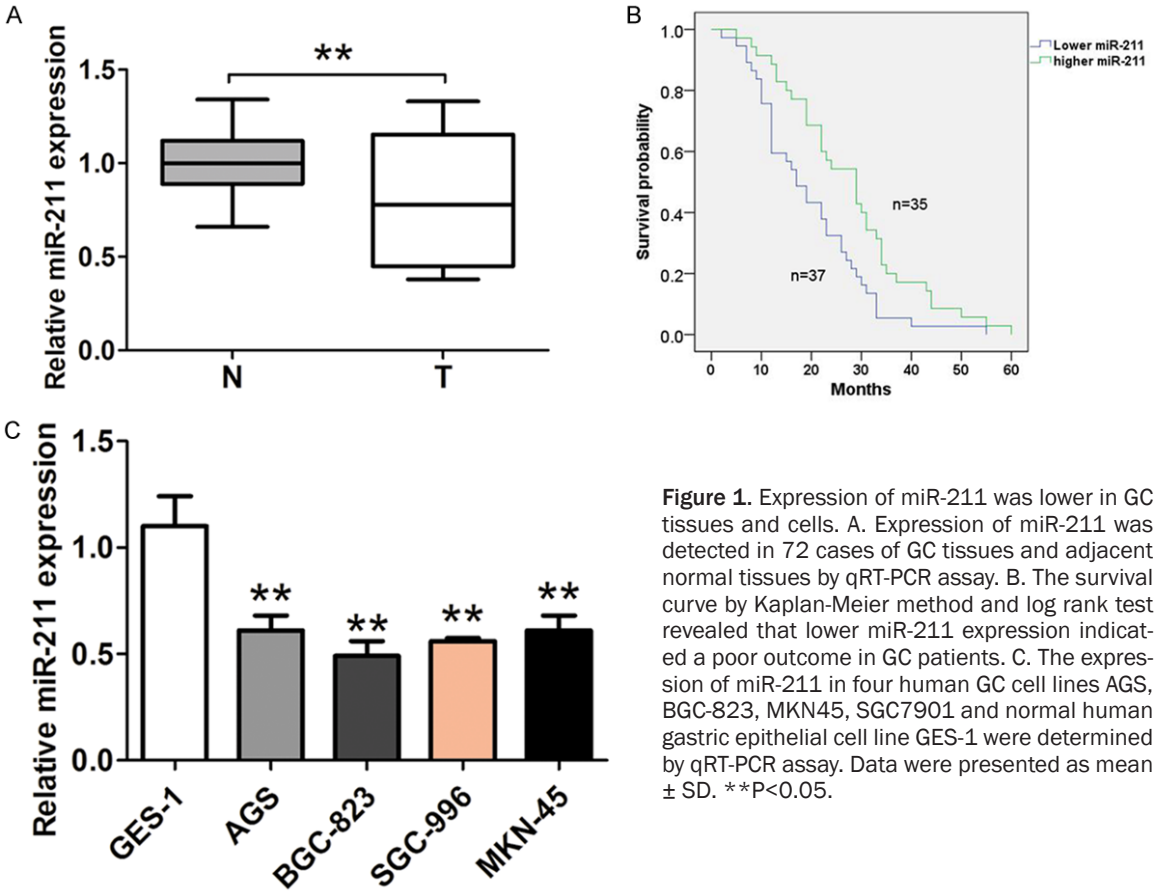

Figure 1. Expression of miR-211 was lower in GC tissues and cells. A. Expression of miR-211 was detected in 72 cases of GC tissues and adjacent normal tissues by qRT-PCR assay. B. The survival curve by Kaplan-Meier method and log rank test revealed that lower miR-211 expression indicated a poor outcome in GC patients. C. The expression of miR-211 in four human GC cell lines AGS, BGC-823, MKN45, SGC7901 and normal human gastric epithelial cell line GES-1 were determined by qRT-PCR assay. Data were presented as mean ± SD. **P<0.05.

lowed by incubation with horseradish peroxidase-conjugated secondary antibodies. Enhanced chemiluminescence (ECL) solution was added onto the membranes and protein expression was measured using Image J software. GAPDH was used as a internal control.

Dual luciferase reporter assay

The fragment of wild-type MMP9 3'-UTR (3'-UTR-WT) containing predicted miR-211 target sites was amplified by PCR, and mutant MMP9 3'-UTR (3'-UTR-MUT) was generated by mutating the conserved binding sites for miR-211 using overlap-extension PCR method. The 3000 SGC-7901 cells/per well were seeded in 96-well plates before cell were transfected. Cell were transfected with miR-211 mimic cotransfected with wild or mutated 3'-UTR MMP9 reporter vector, Luciferase activity was measured after transfected at 48 h using the Dual-Glo luciferase assay system (Promega, Fitchburg, WI, USA) according to the manufacture's protocol.

Statistical analysis

All statistical analyses were performed by using the SPSS 20.0 version (SPSS Inc., Chicago, IL, USA). Data were tested using two-tailed Student's t-test and one-way ANOVA. P<0.05 was considered to be statistically significant.

Results

Expression of miR-211 was lower in GC tissues

To investigate the expression of miR-211 in GC, we detected 72 paired tumor tissues and adjacent normal tissues in patients by performing qRT-PCR. As shown in **Figure 1**, the miR-211 expression levels were lower in GC tissues compared to adjacent normal tissues (50/72). We then determined whether miR-211 was associated with clinicopathological factors. The results showed that miR-211 was closely association with distant metastasis and lymph node metastasis in gastric cancer patients (**Table 1**), but no significance between miR-211 and others factors including age, gender, tumor size,

Table 1. Correction between expression of miR-211 and clinical factors in 72 cases GC patients

Factors	Patients number	miR-211 expression		*p*-value
		Lower (n=37)	Higher (n=35)	
Gender				0.159
Female	37	22	15	
Male	35	15	20	
Age				0.246
≤55	46	26	20	
>55	26	11	15	
Tumor size				0.984
<5 cm	39	20	19	
>5 cm	33	17	16	
Histological grade				0.479
Well and morderately	36	20	16	
Poorly	36	17	19	
Depth of tumor				0.859
T1-T2	26	13	13	
T3-T4	46	24	22	
Distant metastasis				0.007[a]
Positive	28	20	8	
Negative	44	17	27	
Lymphatic metastasis				0.002[a]
Positive	32	23	9	
Negative	40	14	26	
TNM stage				0.584
I-II	27	15	12	
III-IV	45	22	23	

[a]P<0.05.

and so on (**Table 1**). Moreover, the survival curve by Kaplan-Meier method and log rank test revealed that lower miR-211 expression indicated a poor outcome in GC patients (log rank =7.738, P=0.005, **Figure 1B**). These results showed that miR-211 was lower in GC and lower miR-211 expression was associated with poor survival in GC patients, which indicated that miR-211 may act as tumor suppressor in GC.

MiR-211 suppressed cell invasion and epithelial-mesenchymal transition (EMT) in GC

To uncover the function of miR-211 in the progression of GC cells, we analyzed the expression of miR-211 in four human GC cell lines AGS, BGC-823, MKN45, SGC7901 and normal human gastric epithelial cell line GES-1. The results showed that miR-211 was down-regulated in GC cell compared with the expression in GES-1 cells (**Figure 1C**). Furthermore, we found that up-regulation of miR-211 suppressed the cells invasion ability compared with miR-NC group in SGC-7901 and BGC-823 cells (**Figure 2A-D**). Moreover, we detected the association between miR-211 expression and EMT in GC cells. The results showed that up-regulation of miR-211 in SGC-7901 and BGC-823 cells led to relatively higher mRNA and protein levels of E-cadherin, but down-regulated transcription factors Twist1 and related markers N-cadherin in SGC-7901 and BGC-823 cells (**Figure 3A-D**). Thus, these results showed that miR-211 suppressed cell invasion and epithelial-mesenchymal transition (EMT) in GC cells.

MiR-211 inhibits cell EMT by target MMP9 in GC

Furthermore, we used the bioinformatics algorithms TargetScan and miR and a to search for potential targets of miR-211. The results showed that the 3'-UTR region of MMP9 mRNA contained a highly conserved binding site with a miR-211 seed sequence (**Figure 4A**). To demonstrated that MMP9 was a direct target of miR-211 in GC, we performed the luciferase activity reporter assays in SGC-7901 cells by constructing wild-type MMP9 3'-UTR (3'-UTR-WT) containing predicted miR-211 target sites that was amplified by PCR, and mutant MMP9 3'-UTR (3'-UTR-MUT) that was generated by mutating the conserved binding sites for miR-211 using overlap-extension PCR method (**Figure 4A**). The results showed that miR-211 mimic significantly reduced the luciferase activity of the MMP9-3'-UTR-WT reporter, but did not affect the MMP9-3'-UTR-Mut reporter (**Figure 4B**). Furthermore, the qRT-PCR analysis demonstrated that the MMP9 mRNA expression levels were down-regulated after transfected with miR-211 mimic into BGC-823 and SGC-7901 cells, but up-regulated after transfected with miR-211 inhibitor into BGC-823 and SGC-7901 cells (**Figure 4C**, **4D**). Furthermore, the MMP9 protein expression was also confirmed down-regulated after transfected with miR-211 mimic

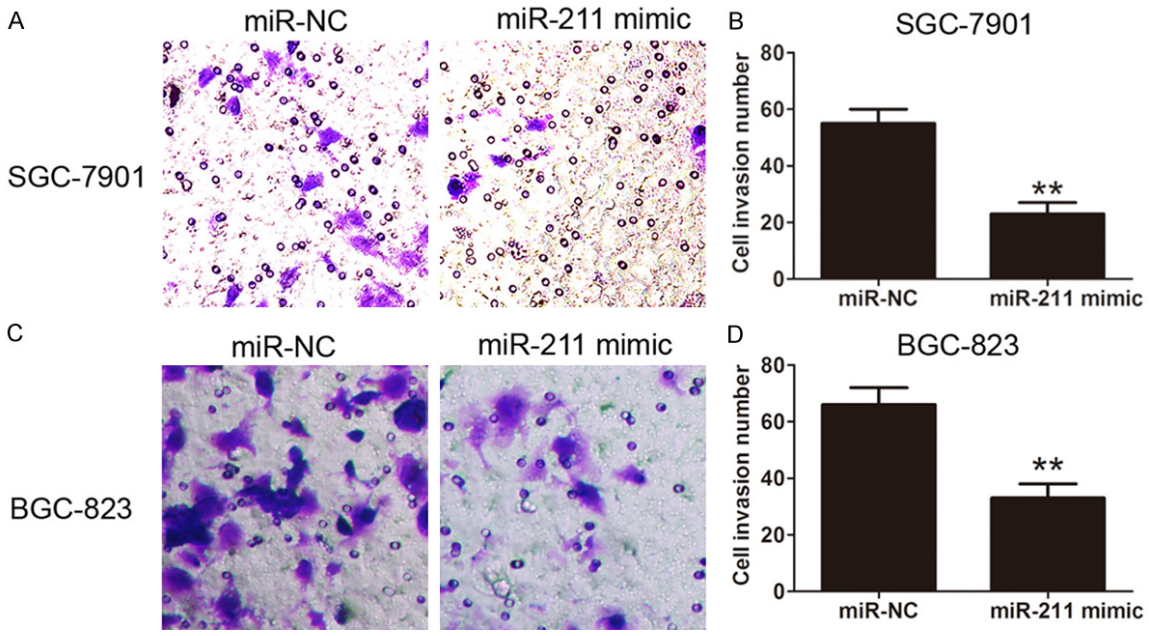

Figure 2. MiR-211 suppressed GC cell invasion. A, B. Cell invasion was evaluated by transfecting miR-211 mimic or miR-211 NC into SGC-7901 cell by transwell cell invasive assay. C, D. Cell invasion was evaluated by transfecting miR-211 mimic or miR-211 NC into BGC-823 cell by transwell cell invasive assay. Data were presented as mean ± SD. **P<0.05.

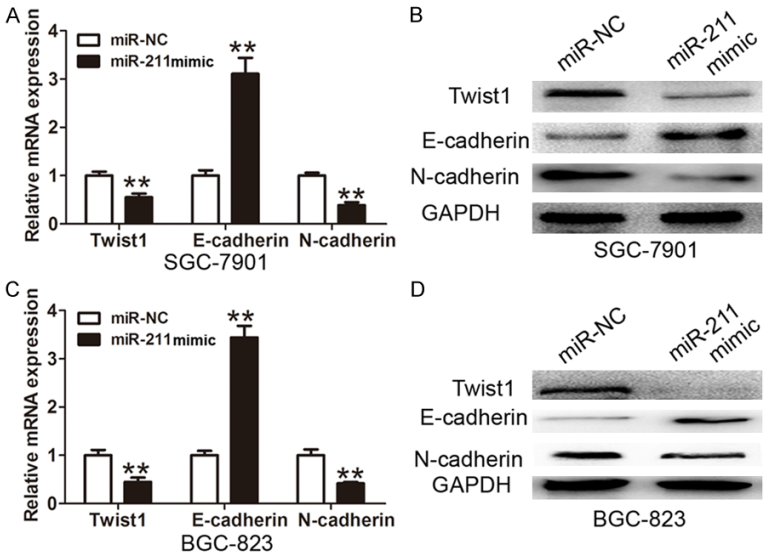

Figure 3. MiR-211 suppressed cell epithelial-mesenchymal transition (EMT) in GC. A, B. The mRNA and protein expression of Twist 1, E-cadherin and N-cadherin were detected by qRT-PCR and western-blot assays in SGC-7901 cells. C, D. The mRNA and protein expression of Twist 1, E-cadherin and N-cadherin were detected by qRT-PCR and western-blot assays in BGC-823 cells. Data were presented as mean ±SD. **P<0.05.

into BGC-823 and SGC-7901 cells, but up-regulated after transfected with miR-211 inhibitor into BGC-823 and SGC-7901 cells (**Figure 4E**, **4F**). Thus, these results showed that miR-211 inhibits cell EMT by target MMP9 in GC.

Discussion

In GC, genetic and epigenetic alterations are involved in the pathogenesis of cancerous lesions. Abundant miRNAs regulate a wide range of biological processes in GC progression [17]. In the study, we demonstrated that miR-211 was lower expression in gastric cancer and negatively associated with distant metastasis and lymph node metastasis in gastric cancer patients. Survival curve revealed that lower miR-211 expression indicated a poor outcome in GC patients. These results showed that miR-211 function as a potentially prognostic maker for GC.

Function assays showed that miR-211 inhibited cell invasion and cell epithelial-mesenchymal transition (EMT) process. EMT is a crucial process for GC invasion and metastasis. MiR-211 has been reported to be associated with EMT process in some tumors. Such as, the expression of miR-

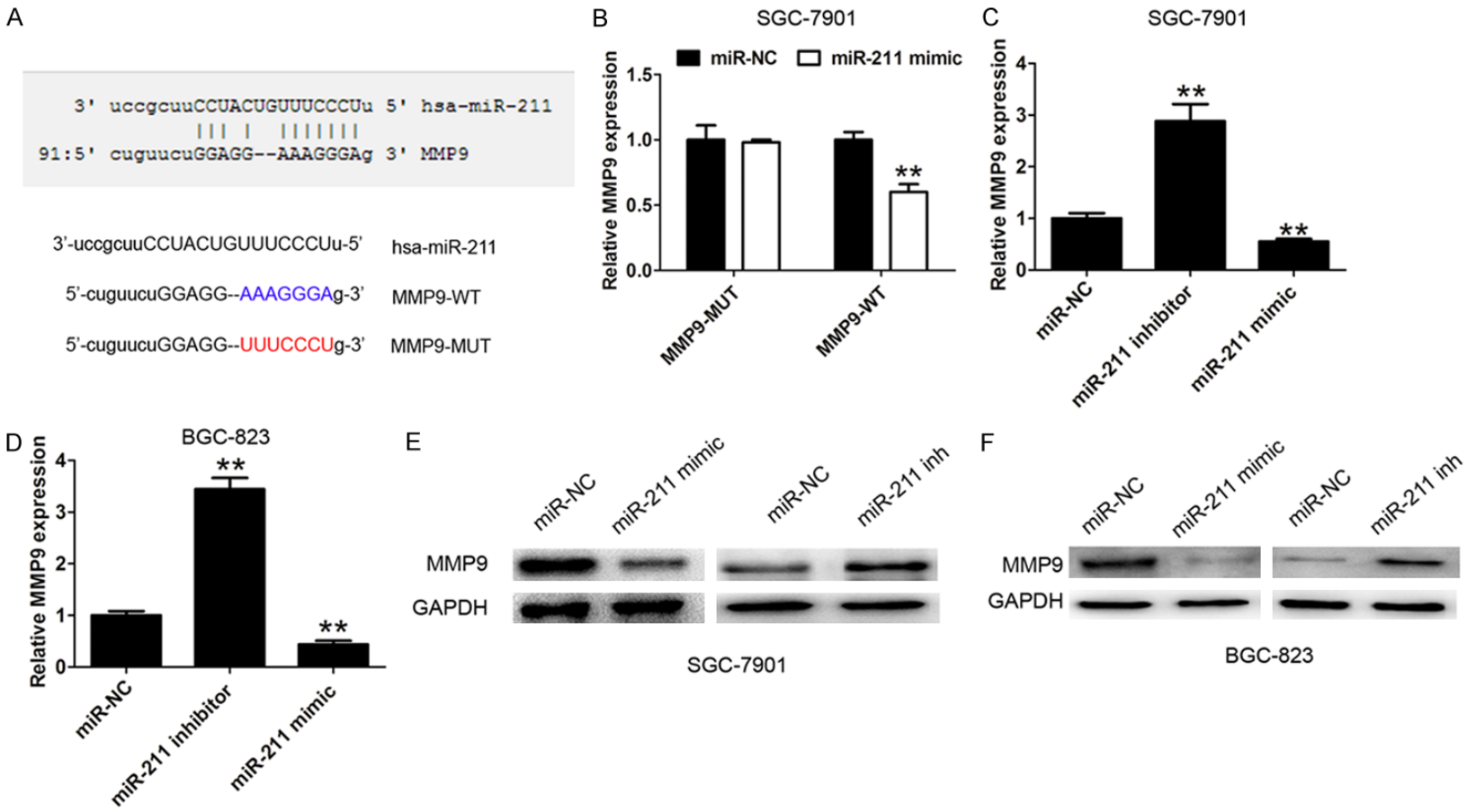

Figure 4. MiR-211 inhibits cell EMT by target MMP9 in GC. A. 3'-UTR region of MMP9 mRNA contained a highly conserved binding site with a miR-211 seed sequence. B. MiR-211 mimic significantly reduced the luciferase activity of the MMP9-3'-UTR-WT reporter, but did not affect the MMP9-3'-UTR-Mut reporter. C, D. The mRNA expression of MMP9 were detected by qRT-PCR assays by transfecting miR-211 inhibitor, miR-211 mimic or miR-211 NC into SGC-7901 and BGC-823 cells. E, F. The protein expression of MMP9 were detected by western-blot assays by transfecting miR-211 inhibitor, miR-211 mimic or miR-211 NC into SGC-7901 and BGC-823 cells. Data were presented as mean ± SD. **P<0.05.

211 in melanoma cell lines was down-regulated and modulates epithelial mesenchymal transition (EMT) of melanoma cells via downregulating RAB22A [18]. MiR-211 inhibits invasion and epithelial-to-mesenchymal transition (EMT) of cervical cancer cells via targeting MUC4 [19]. Consistent with these findings, in the study, we demonstrated that knockdown of miR-211 led to relatively higher expresion level of E-cadherin, but down-regulated transcription factors twist1 and related markers N-cadherin expresson levels in GC cells.

Matrix metalloproteinases (MMPs) are secreted during the growth, invasion, metastases, and angiogenesis of tumors [20]. MMP9 had been reported to promote cell EMT process, miR-520c and miR-373 upregulate MMP9 expression by targeting mTOR and SIRT1, and activate the Ras/Raf/MEK/Erk signaling pathway and NF-κB factor in human fibrosarcoma cells [21]. MiR-34a inhibits migration and invasion of tongue squamous cell carcinoma via targeting MMP9 and MMP14 [22]. Furthermore, luciferase activity reporter assays showed that MMP9 was a direct target of miR-211 and miR-211 suppressed cell EMT process by regulating MMP9 expression.

In conclusion, our results showed that miR-211 was lower in GC, and lower miR-211 predicted a poor prognosis of GC patients, Furthermore, cell invasion and EMT process was suppressed by up-regulation of miR-211. Meanwhile, we demonstrated that miR-211 inhibited cell invasion by regulated in GC cells. Thus, these results showed that miR-211 acted as a tumor suppressor in GC and may be a potential target of GC treatment.

Acknowledgements

This work was supported by Young Scholars Fund of Bethune Medical Scientific Research Program (No. 2013206042), and Special Health Projects of Jilin Province.

Disclosure of conflict of interest

None.

Address correspondence to: Bai-Chun Liu and Ying Song, Department of Digestive Endoscopy, The Second Hospital of Jilin University, 218 Ziqiang Street, Changchun 130041, Jilin, China. Tel: 86-431-88796555; Fax: 86-431-88796555; E-mail: liubaicqq1@163.com (BCL); songying12@21cn.com (YS)

References

[1] Bickenbach K and Strong VE. Comparisons of gastric cancer treatments: east vs. west. J Gastric Cancer 2012; 12: 55-62.

[2] Gigek CO, Chen ES, Calcagno DQ, Wisnieski F, Burbano RR and Smith MA. Epigenetic mechanisms in gastric cancer. Epigenomics 2012; 4: 279-294.

[3] Szakacs G, Paterson JK, Ludwig JA, Booth-Genthe C and Gottesman MM. Targeting multidrug resistance in cancer. Nat Rev Drug Discov 2006; 5: 219-234.

[4] Leja M, You W, Camargo MC and Saito H. Implementation of gastric cancer screening-the global experience. Best Pract Res Clin Gastroenterol 2014; 28: 1093-1106.

[5] Bartel DP. MicroRNAs: genomics, biogenesis, mechanism, and function. Cell 2004; 116: 281-297.

[6] Zhang B, Pan X, Cobb GP and Anderson TA. microRNAs as oncogenes and tumor suppressors. Dev Biol 2007; 302: 1-12.

[7] Ruan K, Fang X and Ouyang G. MicroRNAs: novel regulators in the hallmarks of human cancer. Cancer Lett 2009; 285: 116-126.

[8] Jiang L, Lin T, Xu C, Hu S, Pan Y and Jin R. miR-124 interacts with the Notch1 signalling pathway and has therapeutic potential against gastric cancer. J Cell Mol Med 2016; 20: 313-322.

[9] Li Z, Cao Y, Jie Z, Liu Y, Li Y, Li J, Zhu G, Liu Z, Tu Y, Peng G, Lee DW and Park SS. miR-495 and miR-551a inhibit the migration and invasion of human gastric cancer cells by directly interacting with PRL-3. Cancer Lett 2012; 323: 41-47.

[10] Shen B, Zhang Y, Yu S, Yuan Y, Zhong Y, Lu J and Feng J. MicroRNA-339, an epigenetic modulating target is involved in human gastric carcinogenesis through targeting NOVA1. FEBS Lett 2015; 589: 3205-3211.

[11] Wang LL, Wang L, Wang XY, Shang D, Yin SJ, Sun LL and Ji HB. MicroRNA-218 inhibits the proliferation, migration, and invasion and promotes apoptosis of gastric cancer cells by targeting LASP1. Tumour Biol 2016; 37: 15241-15252.

[12] Wei S, Li Q, Li Z, Wang L, Zhang L and Xu Z. miR-424-5p promotes proliferation of gastric cancer by targeting Smad3 through TGF-beta signaling pathway. Oncotarget 2016; 7: 75185-75196.

[13] Ye L, Wang H and Liu B. miR-211 promotes non-small cell lung cancer proliferation by targeting SRCIN1. Tumour Biol 2016; 37: 1151-1157.

[14] Jiang G, Cui Y, Yu X, Wu Z, Ding G and Cao L. miR-211 suppresses hepatocellular carcinoma by downregulating SATB2. Oncotarget 2015; 6: 9457-9466.
[15] Maftouh M, Avan A, Funel N, Frampton AE, Fiuji H, Pelliccioni S, Castellano L, Galla V, Peters GJ and Giovannetti E. miR-211 modulates gemcitabine activity through downregulation of ribonucleotide reductase and inhibits the invasive behavior of pancreatic cancer cells. Nucleosides Nucleotides Nucleic Acids 2014; 33: 384-393.
[16] Wang CY, Hua L, Sun J, Yao KH, Chen JT, Zhang JJ and Hu JH. MiR-211 inhibits cell proliferation and invasion of gastric cancer by down-regulating SOX4. Int J Clin Exp Pathol 2015; 8: 14013-14020.
[17] Lu J, Getz G, Miska EA, Alvarez-Saavedra E, Lamb J, Peck D, Sweet-Cordero A, Ebert BL, Mak RH, Ferrando AA, Downing JR, Jacks T, Horvitz HR, Golub TR. MicroRNA expression profiles classify human cancers. Nature 2005; 435: 834-888.
[18] Yu H and Yang W. MiR-211 is epigenetically regulated by DNMT1 mediated methylation and inhibits EMT of melanoma cells by targeting RAB22A. Biochem Biophys Res Commun 2016; 476: 400-405.
[19] Xu D, Liu S, Zhang L and Song L. MiR-211 inhibits invasion and epithelial-to-mesenchymal transition (EMT) of cervical cancer cells via targeting MUC4. Biochem Biophys Res Commun 2017; 485: 556-562.
[20] Franco C, Patricia HR, Timo S, Claudia B, Marcela H. Matrix metalloproteinases as regulators of periodontal inflammation. Int J Mol Sci 2017; 18.
[21] Liu P and Wilson MJ. miR-520c and miR-373 upregulate MMP9 expression by targeting mTOR and SIRT1, and activate the Ras/Raf/MEK/Erk signaling pathway and NF-kappaB factor in human fibrosarcoma cells. J Cell Physiol 2012; 227: 867-876.
[22] Jia LF, Wei SB, Mitchelson K, Gao Y, Zheng YF, Meng Z, Gan YH, Yu GY. miR-34a inhibits migration and invasion of tongue squamous cell carcinoma via targeting MMP9 and MMP14. PLoS One 2014; 9: e108435.