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MiR-1298 expression correlates with prognosis and inhibits cell proliferation and invasion of gastric cancer

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Abstract. – OBJECTIVE: To explore the association between miR-1298 expression and clinicopathological factors, prognosis of gastric cancer (GC) patients and biological functions underlying the GC progression.

PATIENTS AND METHODS: Expression of miR-1298 was examined by qRT-PCR in GC tissues and cells, the adjacent normal tissues and normal gastric cell line GES-1 cells were used as controls. Association of disease-free survival (DFS) and overall survival (OS) time with miR-1298 expression was analyzed by Kaplan-Meier analysis and log-rank test. Univariate and multivariate analysis were also performed to analyze relative prognostic risk factors of GC patients. Cell proliferation and invasion assays were used to examine cell proliferation and invasion capacities in vitro. The relative protein expression was analyzed by Western blot analysis.

RESULTS: MiR-1298 expression was lower in GC tissues and cells, compared to adjacent normal tissues and GES-1 cells, respectively. Lower miR-1298 expression levels were associated with lymph node metastasis and TNM stage. Kaplan-Meier analysis showed that lower miR-1298 expression predicted poor DFS and OS of GC patients. Furthermore, we demonstrated that lymph node metastasis, TNM stage, and lower miR-1298 expression were independent risk factors for DFS and OS in GC patients. *In vitro*, miR-1298 overexpression inhibited cell proliferation and invasion abilities. Additionally, our results revealed that miR-1298 overexpression suppressed PI3K/AKT signaling pathway in GC cells.

CONCLUSIONS: Our evidence indicated that miR-1298 may provide a specifically promising target for therapy of GC patients.

Key Words:
Gastric cancer, miR-1298, Cell proliferation, Cell invasion, Tumor prognosis.

Introduction

Gastric cancer (GC) has the highest morbidity and mortality worldwide and is listed as the second leading cause of cancer-related death[1]. Due to extensive invasion and lymphatic metastasis, patients who were diagnosed at advanced GC stage show poor prognosis[2,3]. Thus, it is necessary to identify more sensitive diagnostic biomarkers at the early stage of disease and novel targets of GC.

MicroRNAs (miRNA) are endogenous, short non-coding RNA molecules that regulate gene expression at post-transcriptional level by binding to the 3'-untranslated regions (3'-UTRs) of its targeted mRNAs[4,5]. Some miRNAs were identified as prognostic makers and therapeutic targets of gastric cancer. MiR-181a-5p promotes the progression of gastric cancer via RASSF6-mediated MAPK signaling activation[6]. miR-194 inhibits gastric cancer cell proliferation and tumorigenesis by targeting KDM5B[7]. MicroRNA-26b inhibits tumor metastasis by targeting the KPNA2/c-jun pathway in human gastric cancer[8]. Additionally, more and more microRNAs involved in gastric cancer were identified by researchers[9]. However, the clinical significance and biological functions for miR-1298 underlying the GC progression remain unknown.

In the study, we found that miR-1298 expression levels were lower in GC tissues and cells, respectively. Lower miR-1298 expression predicted a poor outcome of GC patients. *In vitro*, miR-1298 overexpression inhibited cell proliferation and invasion ability of GC cells. Thus, miR-1298 may serve as a prognostic maker and target for therapy of GC patients.

Patients and Methods

Patients

Human GC tissues and paired adjacent non-tumor tissues that were located at least 5 cm from tumors were obtained from 89 cases of patients

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who underwent radical surgery of GC at Affiliated Hospital of Qingdao University. Tissue samples were snap-frozen in liquid nitrogen and stored at -80°C for further analysis. All of the patients had no received radiotherapy or chemotherapy before surgery. The clinical data was listed in Table I. Written informed consent was obtained from all patients, and the study was approval from the Ethics Committee of Affiliated Hospital of Qingdao University.

Cell Line Culture

Five human GC lines (BGC-823, SGC-7901, MKN-45, AGS, and AZ-521) and normal gastric cell GES-1 were obtained from the Biochemistry and Cell Biology at the Chinese Academy of Sciences (Shanghai, China). Cells were cultured with Roswell Park Memorial Institute (RPMI) 1640 medium (HyClone, Logan, UT, USA) supplemented with 10% fetal bovine serum (FBS) (Gibco, Rockville, MD, USA). Cells were cultured at 37°C in a humidified atmosphere containing 5% CO_2.

Cell Transfection

The miR-1298 mimic and miR-1298 inhibitor or negative control were synthesized by Guangzhou RiboBio Company (Guangzhou, China). GC cells were transfected using Lipofectamine 2000 reagents (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions.

RNA Extraction and qRT-PCR

Total RNAs were extracted from fresh tissues or cells using TRIzol reagent (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions. RNA purity and concentration were determined using a ND-1000 NanoDrop spectrophotometer (NanoDrop, Wilmington, DE, USA) at the absorbance at 260 nm and 280 nm. A miScript-Reverse Transcription Kit and a standard SYBR Green PCR kit (Toyobo, Osaka, Japan) were used for RNA to reverse DNA. The mRNA expression levels were detected by SYBR1 Premix Dimmer Eraser kit (TaKaRa, Dalian, China) on Applied Biosystems 7500 Sequence Detection system. The primer sequence for miR-1298 was purchased from uGCT Inc. (Beijing, China). U6 were used as an internal control. MiR-1298 expression was calculated using $2^{-\Delta\Delta CT}$ methods.

Cell Counting Kit-8 Assay

SGC-7901 and MKN-45 cells were transfected with miR-NC, miR-1298 mimic or miR-1298 inhibitor and, then, were seeded in 96 well plates at a density of 2000 cells per well. Cell growth was detected using Cell Counting Kit-8 (CCK8) kit (Dojindo Laboratories, Kumamoto, Japan). Cells growth was detected at 24, 48, 72 h, and 96 h after cells transfection. 10 μL CCK8 solution were added into each well and cells were cultured for another 2 h at 37°C. The optical density was selected at 450 nm under a microplate reader (Sunrise™, Tecan, Männedorf, Switzerland).

Cell Invasion Assay

SGC-7901 and MKN-45 cells were transfected with miR-NC, miR-1298 mimic or miR-1298 inhibitor and, then, were seeded in 6 well plates at a density of 1×10^5 cells per well. Cells invasion were assessed in an 8.0 μm Pore Polycarbonate Membrane Insert (Corning, New York, NY, USA) following the manufacturer's instructions. The lower chambers were added with 500 μl of RPMI-1640 medium containing 10% FBS. The upper chambers were added with 300 μl of RPMI-1640 containing FBS and 1×10^5 cells per well. After cells were cultured for 48 h, cells were fixed with 75% methanol and, then, stained with 1% crystal violet. Cells were calculated in five random fields under a light microscope.

Western Blot Analysis

The protein was extracted from SGC-7901 and MKN-45 cells using radioimmunoprecipitation assay (RIPA) lysates (KeyGEN Company, Nan Jing, China) with protease inhibitor cocktail (Millipore, Billerica, MA, USA). Equal protein was separated on 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) gels and transferred to a polyvinylidene difluoride (PVDF) membrane. The membranes were blocked with 5% skimmed milk for 1 h at room temperature and, then, incubated with specific antibodies including PTEN (1:1000, Cell Signaling Technology, Danvers, MA, USA), p-AKT (1:1000, Cell Signaling Technology, Beverly, MA, USA) and AKT (1:1000, Cell Signaling Technology, Beverly, MA, USA) and GAPDH (1:1000, Cell Signaling Technology, Beverly, MA, USA) at 4°C overnight. The blot was analyzed using enhanced chemiluminescence (ECL) substrate (Pierce, Rockford, IL, USA).

Statistical Analysis

All of the data in the study was conducted with IBM SPSS Statistics (SPSS Inc., Chicago, IL, USA) software and results were given as mean ± SD from at least three independently ex-

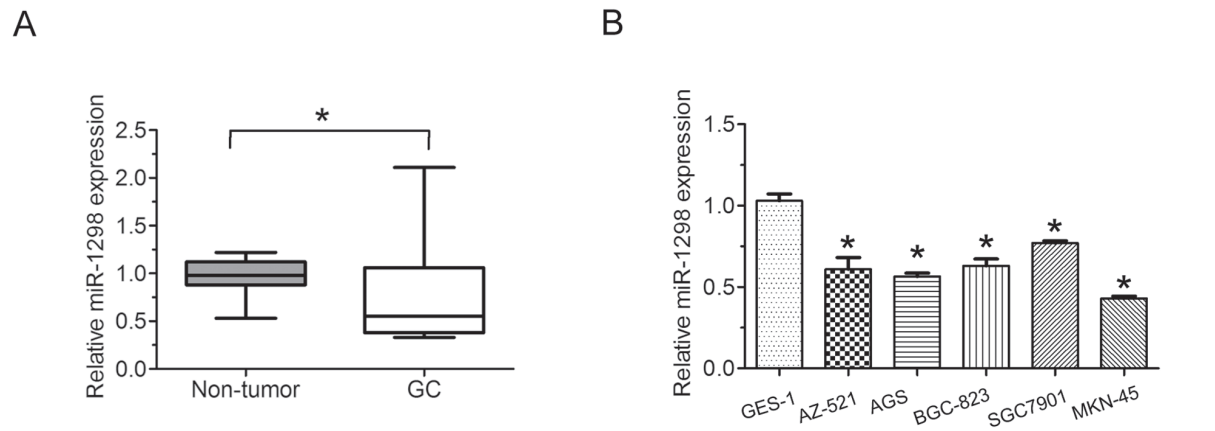

Figure 1. MiR-1298 expression is downregulated in GC tissues and cells. ***(A)*** Relative expression of miR-1298 was determined by qRT-PCR assay in 89 GC tissues and adjacent non tumor tissues. GAPDH was used as the internal control. ***(B)*** Relative expression of miR-1298 was determined by qRT-PCR assay in five human GC lines (BGC-823, SGC-7901, MKN-45, AGS, and AZ-521) and normal gastric cell line GES-1. GAPDH was used as the internal control. The experiments were repeated at least three times, *$p<0.05$.

periments. Difference between two groups was determined by using two-tailed paired Student's *t*-test or one-way analysis of variance (ANOVA) for comparisons of multiple groups. SNK test was used as a post-hoc test. *$p < 0.05$ were considered statistically significant.

Results

MiR-1298 Expression is Downregulated in GC Tissues and Cells

The expression level of miR-1298 in 89 pairs of GC tissues and matched adjacent non-tumor tissues were determined using qRT-PCR. As shown in Figure 1A, the miR-1298 expression was decreased in tumor tissues compared with adjacent non-tumor tissues ($p<0.05$). Furthermore, we observed that miR-1298 expression was significantly down-regulated in GC cells (BGC-823, SGC-7901, MKN-45, AGS, and AZ-521) than that in GES-1 cells (Figure 1B, $p<0.05$). According to miR-1298 median expression (0.52 fold) in GC tissues, we divided patients into two groups (higher miR-1298 expression and lower miR-1298 expression). Association of miR-1298 expression with clinicopathological data showed lymph node metastasis ($p=0.011$, Table I) and TNM stage ($p=0.016$, Table I) associated with miR-1298 expression in patients. Moreover, patients with higher miR-1298 expression had a good disease-free survival (DFS) (log rank test, $p<0.05$, Figure 2A) and overall survival (OS) time (log rank test, $p<0.05$, Figure 2B), compared with lower miR-1298 expression group. We performed the Univariate and Multivariate Cox model analysis to evaluate the prognostic factors of GC patients. The results showed that lymph node metastasis (HR, 1.982, 95%CI, 0.701-4.055, $p<0.05$), advanced tumor stage (HR, 1.899, 95%CI, 0.566-3.228, $p<0.05$), and lower miR-1298 expression (HR, 2.066, 95%CI, 0.855-4.011, $p<0.05$) were independent prognostic factors of DFS in GC patients (Table II). Consistently, the results also showed that lymph node metastasis (HR, 1.984, 95%CI, 0.644-3.896, $p<0.05$), advanced tumor stage (HR, 1.922, 95%CI, 0.833-3.188, $p<0.05$), and lower miR-1298 expression (HR, 2.129, 95%CI, 0.788–3.834, $p<0.05$) were independent prognostic factors of OS in GC patients (Table III). These results indicated that miR-1298 expression correlated with GC prognosis and serves as a biomarker of GC prognosis.

MiR-1298 Inhibits GC Cell Proliferation and Invasion In Vitro

To investigate the biological effects of miR-1298 in GC, we perform gain- and loss-of-function assays in MKN-45 and SGC-7901 cells, respectively. After cells transfection at 24 h, 48 h, 72 h, and 96 h, we evaluated cell growth rate by CCK8 assays. We observed that miR-1298 mimic significantly inhibited cell growth rate. However, miR-1298 inhibitor enhanced cell growth at 48 h, 72 h, and 96 h after cells transfection compared with control group in MKN-

Table I. Association between miR-1298 expression levels with clinicopathologic feathers.

Clinicopathologic feathers	Number of patients	Lower expression (n=48)	Higher expression (n=41)	*p*-value
Age (years)				0.358
≤60	67	38	29	
>60	22	10	12	
Sex				0.962
Female	48	26	22	
Male	41	22	19	
Tumor size				0.537
<3cm	60	31	29	
>3cm	29	17	12	
Local invasion				0.088
T1-T2	52	32	20	
T3-T4	37	16	21	
Histological grade				0.842
High	34	17	17	
Middle	32	18	14	
Poor	23	13	10	
Lymph node metastasis				
Negative	35	13	22	0.011[a]
Positive	54	35	19	
TNM stage				0.016[a]
I-II	53	23	30	
III-IV	36	25	11	

[a]p <0.05.

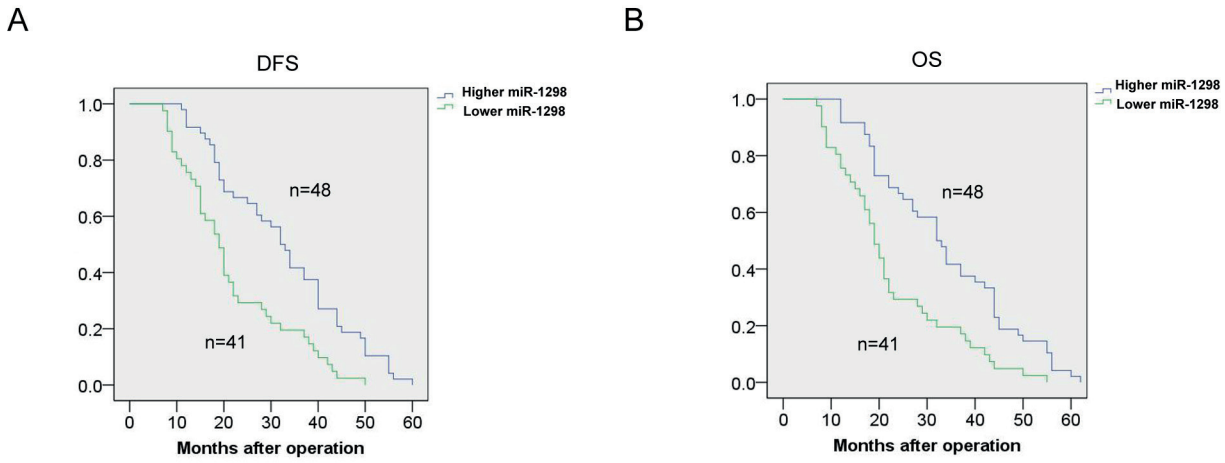

Figure 2. MiR-1298 expression associates with GC prognosis. ***(A)*** Patients with higher miR-1298 expression had a good disease free survival (DFS) (log rank test, p<0.05, and ***(B)*** overall survival (OS) time (log rank test, p<0.05), compared with lower miR-1298 expression group.

45 and SGC-7901 cells (Figure 3A-3B, p<0.05). Next, transwell invasion assays revealed that miR-1298 overexpression significantly inhibited cell invasion ability, whereas miR-1298 inhibitor enhanced cell invasion ability in MKN-45 and SGC-7901 cells (Figure 3C-3F). These results suggested that miR-1298 affected cell growth and invasion of GC *in vitro*.

MiR-1298 Regulated GC Progression Through PI3K/AKT In Vitro

PI3K/AKT signaling is essential for the biological functions of GC[10]. According to miR-1298 expression in GC cells, we performed gain of function in MKN-45 cells, while loss-of-function in SGC-7901 cells. As shown in Figure 4A, we demonstrated that miR-1298 overexpression si-

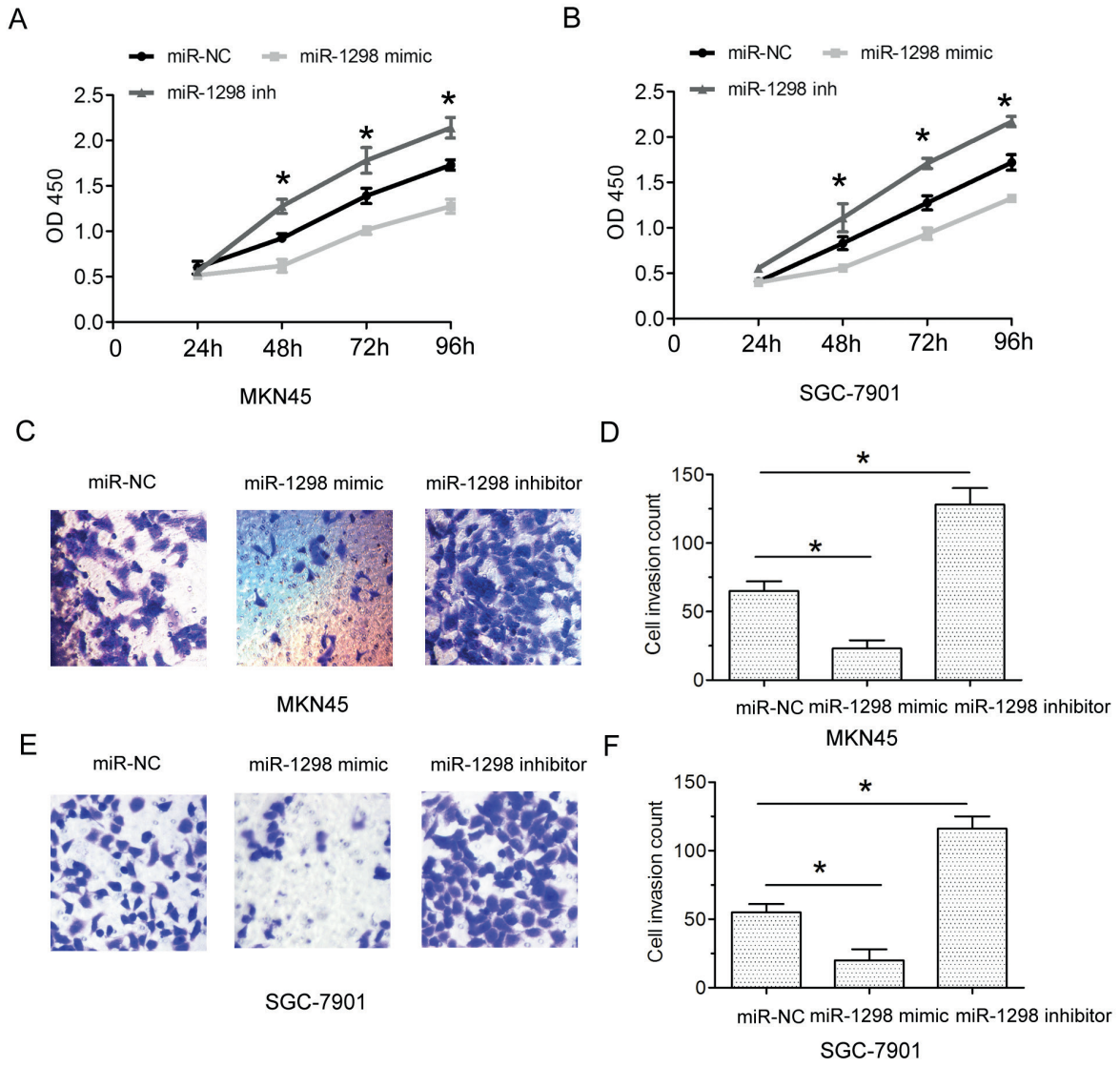

Figure 3. MiR-1298 inhibits cell proliferation and invasion capacities *in vitro*. ***(A-B)*** CCK8 cell assay was used to detect the cell proliferation ability after miR-1298 mimic or miR-1298 inhibitor were transfected into MKN-45 or SGC-7901 cells. ***(C-E)*** Transwell cell invasion assay was used to detect the cell proliferation ability after miR-1298 mimic or miR-1298 inhibitor were transfected into MKN-45 or SGC-7901 cells. The experiments were repeated at least three times, *$p<0.05$.

Table II. Univariate and multivariate analysis of DFS in 89 GC patients.

	Univariate Cox analysis		Multivariate Cox analysis	
Factor	**HR (95% CI)**	***p***	**HR (95% CI)**	***p***
Age (years)	0.677 (0.373-1.229)	0.884		
Sex	0.877 (0.438-1.466)	0.672		
Tumor size	1.055 (0.688-1.732)	0.504		
Local invasion	0.922 (0.663-1.388)	0.598		
Histological grade	1.032 (0.786-1.665)	0.472		
Lymph node metastasis	2.386 (0.772-4.268)	0.001[a]	1.982(0.701-4.055)	0.001[a]
TNM stage	2.089 (0.633-3.899)	0.002[a]	1.899(0.566-3.228)	0.006[a]
Lower miR-1298	2.566 (0.996-4.344)	0.001[a]	2.066(0.855-4.011)	0.001[a]

[a]$p<0.05$.

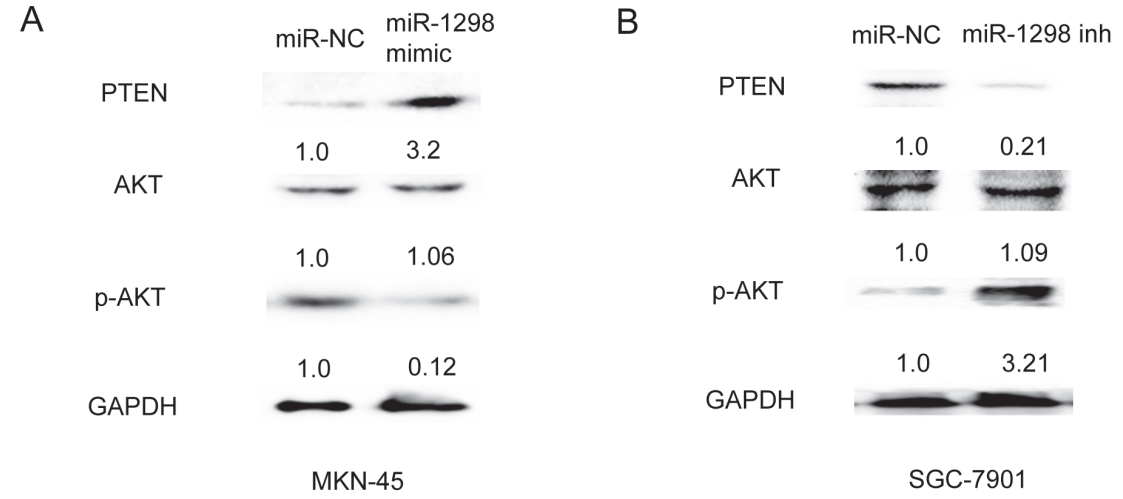

Figure 4. MiR-1298 inhibits PI3K/AKT signaling in GC. ***(A)*** The relative protein expression of PTEN, AKT and p-AKT was analyzed by Western blot analysis after miR-1298 mimic were transfected into MKN-45 cells. (***B)*** The relative protein expression of PTEN, AKT, and p-AKT was analyzed by Western blot analysis after miR-1298 inhibitor were transfected into SGC-7901 cells. The experiments were repeated at least three times.

Table III. Univariate and multivariate analysis of OS in 89 GC patients.

	Univariate Cox analysis		Multivariate Cox analysis	
Factor	**HR (95% CI)**	***p***	**HR (95% CI)**	***p***
Age (years)	0.588 (0.306-1.011)	0.921		
Sex	0.678 (0.356-1.066)	0.787		
Tumor size	1.109 (0.578-1.994)	0.491		
Local invasion	0.822 (0.433-1.599)	0.664		
Histological grade	0.955 (0.710-1.584)	0.566		
Lymph node metastasis	2.188 (0.899-4.455)	0.001a	1.984 (0.644-3.896)	0.001[a]
TNM stage	2.233 (0.901-3.899)	0.001a	1.922 (0.833-3.188)	0.001[a]
Lower miR-1298	2.337 (0.882-4.154)	0.001a	2.129 (0.788-3.834)	0.001[a]

[a]$p < 0.05$.

gnificantly decreased the protein expression of phosphorylated AKT (p-AKT) but increasing the protein expression of PTEN in MKN-45 cells. However, miR-1298 inhibitor increased the protein expression of p-AKT and decreased the PTEN expression level in SGC-7901 cells (Figure 4B). Thus, these results indicated that miR-1298 inhibited the activation of PI3K/AKT signaling pathway in GC cells.

Discussion

MicroRNAs have been implicated in the regulation of human disease development and progression. Some microRNAs including miR-1[11], miR-122[12], miR-101[13], miR-125b-2, miR-451a, and so on were reported to be involved in GC progression. MiR-1298 was little known in GC, some researchers showed the important roles of miR-1298 involved in some diseases including tumors. MicroRNA-1298 is regulated by DNA methylation and affects vascular smooth muscle cell function by targeting connexin 43[14]. MiR-1298 inhibits mutant KRAS-driven tumor growth by repressing FAK and LAMB3[15]. MiR-1298 affected cell proliferation and apoptosis of rat tumor cells by targeting SETD7[16]. In the study, we found that MiR-1298 expression levels were lower in GC tissues and cells, compared to adjacent normal tissues and GES-1 cells, respectively. Lower miR-1298 expression levels were associated with lymph node metastasis and TNM stage. Kaplan-Meier analysis showed that lower miR-1298 expression predicted a poor outcome of GC patients. *In vitro*, miR-1298 overexpression inhibited cell proliferation and cell invasion ability.

PTEN acts as tumor suppressor and negatively regulates the activity of PI3K/AKT pathway. Loss of tumor-suppressor PTEN and subsequent activation of PI3K/AKT pathway promotes GC progression. In previous study[17], miR-137 effects on gastric carcinogenesis are mediated by targeting Cox-2-activated PI3K/AKT signaling pathway. MiR-19a promotes epithelial-mesenchymal transition through PI3K/AKT pathway in gastric cancer[18]. Upregulation of miR-34a by diallyl disulfide suppresses invasion and induces apoptosis in SGC-7901 cells through inhibition of the PI3K/Akt signaling pathway[19]. We demonstrated that miR-1298 overexpression significantly decreased the expression level of phosphorylated AKT in MKN-45 cells and increased the PTEN expression. However, miR-1298 inhibitor increased the expression of phosphorylated AKT and decreased the PTEN expression in SGC-7901 cells. Thus, these results indicated that miR-1298 inhibited the activation of PI3K/AKT signaling pathway in GC cells.

Conclusions

We found that miR-1298 expression was reduced in GC tissues and cells. Lower miR-1298 expression predicted poor prognosis and served as a biomarker of GC prognosis. Furthermore, we demonstrated that miR-1298 inhibited cell proliferation and invasion by regulating PI3K/AKT signaling in GC. These results indicated that miR-1298 may be a prognostic maker and potential target of GC treatment.

Acknowledgements

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Conflict of Interest

The Authors declare that they have no conflict of interest.

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