RESEARCH ARTICLE

MicroRNA-498 inhibits the proliferation, migration and invasion of gastric cancer through targeting BMI-1 and suppressing AKT pathway

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Abstract

Recently, microRNA-498 (miR-498) plays important effect in human cancers. Nonetheless, the role of miR-498 is still unclear in gastric cancer (GC). Therefore, this study was designed to investigate the function of miR-498 in GC tissues and cell lines (SGC-7901, BGC-823, MGC-803). The expressions of miR-498 and BMI-1 were examined in GC tissues via the RT-qPCR assay. The function of miR-498 was investigated through MTT and transwell assays. The relationship between miR-498 and BMI-1 was testified by dual luciferase assay. The protein expression of EMT markers, AKT pathway markers and BMI-1 was measured through western blot. The expression of miR-498 was decreased in GC tissues which predicted poor prognosis of GC patients. Moreover, functional analyses show that the overexpression of miR-498 inhibited the progression of GC. Furthermore, BMI-1 was a direct target of miR-498 which was upregulated in GC. Especially, the upregulation of BMI-1 recovered the suppressive effect of miR-498 in GC. In addition, miR-498 inhibited the metastasis and proliferation of GC cells through blocking EMT and AKT pathway. MiR-498, by targeting BMI-1, presents a plethora of tumor suppressor activities in GC cells.

Keywords AKT pathway · BMI-1 · Gastric cancer · MiR-498

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Introduction

Gastric cancer (GC) is a malignant tumor of the digestive tract, accounting for 95% of the malignant tumors of the stomach [1]. It ranks the third most common human malignant tumors, which is the second leading cause of cancer-related human mortalities [2]. The onset age of GC is between 50 and 80 years old, and the ratio of male to female is 2:1 [3]. In addition, the incidence rate of GC in rural areas is 1.6 times that in urban areas [4]. Moreover, more than 70% of patients with early GC have no symptoms or only mild symptoms, and patients with advanced GC may experience abdominal discomfort or pain [5]. Most patients with early GC under endoscopy can obtain radical treatment and the 5-year survival rate more than 90% [6]. At present, the diagnostic rates of early GC lower than 10%, far less than Japan (70%) and Korea (50%) [7]. However, most of GC patients are diagnosed at advanced stage due to its atypical symptoms. And the 5-year survival rate of GC has been maintained at ~ 30% [8]. Moreover, early detection, early diagnosis and early treatment

of cancer are the main strategies to reduce mortality and improve survival [9]. Therefore, early diagnosis and treatment in high-risk groups of GC are efficient and feasible ways to change the severe situation of GC in China.

Increasing studies reported that microRNAs (miRNAs) involved in the pathogenesis of human cancers through binding genes with shared response elements [10]. Especially, many miRNAs had been found to participate in the progression of GC, such as miR-208a [11], miR-1297 [12] and miR-1236 [13]. Studies have indicated that miR-498 regulated different biological processes in human cancers and disease. For example, miR498 regulated herpes simplex virus 1 in Kaposi's sarcoma by targeting RTA [14], while miR-498 targeted FOXO3 and inhibited the proliferation in human ovarian cancer [15]. Inversely, miR-498 was proposed to promote cell proliferation and inhibit cell apoptosis in retinoblastoma by directly targeting CCPG1 [16]. Previous evidence has suggested that miR-498 was abnormally decreased in gastric cancer stem cells in the MKN-45 cancer cell line [17]. However, the specific mechanism of miR-498 in gastric cancer is still obscure. Thence, determining the exact molecular mechanisms of miR-498 in gastric cancer carcinogenesis might contribute to improving the diagnose and prognosis of patients with this tumor.

B-cell-specific moloney murine leukemia virus insertion site 1 (BMI-1) is an oncogene and upregulated in several human cancers, including glioma [18], nasopharyngeal carcinoma [19] and breast cancer [20]. Moreover, the interaction between BMI-1 and miRNAs had been demonstrated to influence the biological processes of human cancers. For instance, miR-139-5p inhibited bladder cancer proliferation and self-renewal by targeting the BMI-1 [21]. And Zhou et al. found that miR-183 was involved in cell proliferation, survival and poor prognosis of pancreatic ductal adenocarcinoma by regulating BMI-1 [22]. Furthermore, BMI-1 had been proposed to have great impact on the epithelial-mesenchymal transition (EMT) in endometrial cancer [23]. And miR-17 inhibition had been found to suppress EMT through a DEDD-dependent mechanism in GC [24]. Nevertheless, the carcinogenesis and regulatory mechanism of miR-498/BMI-1 in GC still need to be explored.

In addition, recent researches have revealed that the activation of AKT pathway was a therapeutic target in cancer [25]. Moreover, Cheng et al. demonstrated that miR-107 inhibited GC cell proliferation and metastasis by targeting PI3K/AKT pathway [26]. And AKT pathway has great effect on regulating cell proliferation, survival and growth [27]. Therefore, we want to investigate the effect of miR-498 on EMT and AKT pathway in GC. Moreover, the abnormal expression of miR-498 and the relationship between miR-498 and BMI-1 were also identified in GC. We hope these findings could provide fresh ideas for the treatment of GC.

Materials and methods

Clinical tissues

The sixty-two human GC tissues and adjacent noncancerous tissues were acquired from The Affiliated Hospital of Qingdao University from September 2012 to July 2014. And all patients with GC have not received any treatment before the operation. Theses tissues were frozen in liquid nitrogen and then stored in the $-80\ °C$ refrigerator. All the patients provided the signed informed consent. This experiment was approved by the Institutional Ethics Committee of The Affiliated Hospital of Qingdao University and was performed according to the guidelines of the Declaration of Helsinki.

Cell lines culture

The SGC-7901, BGC-823, MGC-803 cell lines and human gastric mucosa epithelial line GES-1 were used for this experiment. These cell lines were acquired from ATCC (Manassas, VA). Then these cell lines were seeded in RPMI-1640 medium with 10% fetal bovine serum (FBS) which were cultured at 37 °C with 5% CO_2 in a humidified incubator.

Cell transfection

The miR-498 mimics or inhibitor and negative control (NC) were obtained from Fulengen (Guangzhou, China). Then they were transferred into SGC-7901 and MGC-803 cells, respectively, with Lipofectamine 2000 (Invitrogen, Carlsbad, USA) at a concentration of 10 nM based on the manufactures' protocol.

RT-qPCR analysis

TRIzol reagent (Invitrogen, Carlsbad, USA) was applied to extract total RNA in GC. The quality and concentration of the isolated RNA were analyzed using a Nano drop 2000 (Thermo Fisher Scientific, Waltham, MA, USA). And the synthesis of cDNA was performed using Taqman® miRNA Reverse Transcription kit (Thermo Fisher Scientific, Inc.). We conducted RT-qPCR through using the SYBR® Green qPCR Assay kit (Thermo Fisher Scientific, Inc.) on ABI 7500 Fast system (Applied Biosystems, CA, USA). U6 or GAPDH was used as control for miR-498 or BMI1. And their expressions were calculated using the $2^{-\triangle\triangle ct}$ method.

The primers used were as followed: miR-498 forward, 5′-GAAAAACGCCCCCUGGCUUGAAA-3′, reverse, and 5′-CUUUUUGCGGGGGGACCGAACUUU-3′; U6 forward, 5′-CTCGCTTCGGCAGCACATATACT-3′, and reverse,

5′-ACGCTTCACGAATTTGCGTGTC-3′. BMI-1 forward 5′-CTGGTTGCCCATTGACAGC-3′, and reverse, 5′-CAG AAAATGAATGCGAGCCA-3′. GAPDH forward, 5′-GAC TCATGACCACAGTCCATGC-3′, and reverse, 5′-AGA GGCAGGGATGATGTTCTG-3′.

MTT assay

First, 2×10^3 SGC-7901 and MGC-803 cells with miR-498 mimics or inhibitor were seeded into a 96-well plate. Then at, 10 μl MTT (5 mg/l) was added each well and cultured for 24, 48, 72, and 96 h. Then, these cells were destroyed with 100 μl DMSO after incubation at 37 °C for 4 h. In the end, OD values were measured at 490 nm.

Transwell assay

Transwell chambers (8 μm pore size membranes) were employed to perform cell migration and invasion assays. The lower chamber was added with 10% FBS and incubated at 37 °C with 5% CO_2. Then the upper surface with matrigel (BD Biosciences, USA) was used for cell invasion. And cell migration assay was conducted without matrigel. After the transfection of SGC-7901 and AGS cells for 24 h, the cells were cultured in the upper chamber with serum-free medium. 48 h later, the migrated or invasive cells were fixed with methanol and stained with crystal violet. Finally, we counted the number of cells that migrated to the bottom of the filter using a microscope.

Dual luciferase assay

The wild or mutant type of 3′-UTR of BMI1 was inserted into pcDNA3.1 plasmid vector (Promega, Madison, USA) to perform luciferase reporter experiments. Then, wild or mutant type of 3′-UTR of BMI1 and miR-498 mimic were transfected into SGC-7901 and MGC-803 cells. Subsequently, the luciferase activity was measured through dual luciferase assay system (Promega, USA).

Western blot analysis

The protein samples were obtained using RIPA lysis buffer from SGC-7901 and MGC-803 cells. Then the proteins were separated through a 10% SDS-PAGE and incubated with 5% skim milk in PVDF membranes at room temperature. Next we incubated the membranes overnight at 4 °C with EMT markers (E-cadherin, N-cadherin, vimentin), AKT pathway markers (AKT, p-AKT), BMI1 and GAPDH antibodies. After washing, they were incubated with corresponding secondary antibodies for 2 h at room temperature. Then, protein expression levels were measured by ECL (Pierce Biotechnology, USA).

Statistical analysis

The data was shown as mean ± SD. SPSS 19.0 or Graphpad Prism 6 was employed to analyze these data. The correlation of miR-498 with clinic pathological characteristics of GC was calculated through the Chi-squared test. The difference between the groups was calculated through Tukey's one-way ANOVA. The survival curves were drawn by Kaplan–Meier analysis, and log-rank test was used to compare the survival differences. Differences with a value of $P < 0.05$ or < 0.01 were regarded as statistically significant.

Results

The expression of miR-498 was decreased in GC tissues

The expression of miR-498 was examined in GC tissues through RT-qPCR assay. The results showed that the expression of miR-498 was obviously reduced in GC tissues in comparison to the normal tissues (Fig. 1a). Moreover, we also found that miR-498 was associated with differentiation ($P = 0.008$), lymph node metastasis ($P = 0.045$), and TNM stage ($P = 0.013$; Table 1). Besides that, low miR-498

Fig. 1 The expression of miR-498 was decreased in GC tissues. **a** The expressions of miR-498 in GC tissues were measured by RT-qPCR. **b** High miR-498 expression was correlated with longer overall survival of GC patients by Kaplan–Meier analysis. The experiments were repeated three times.*$P < 0.05$, **$P < 0.01$

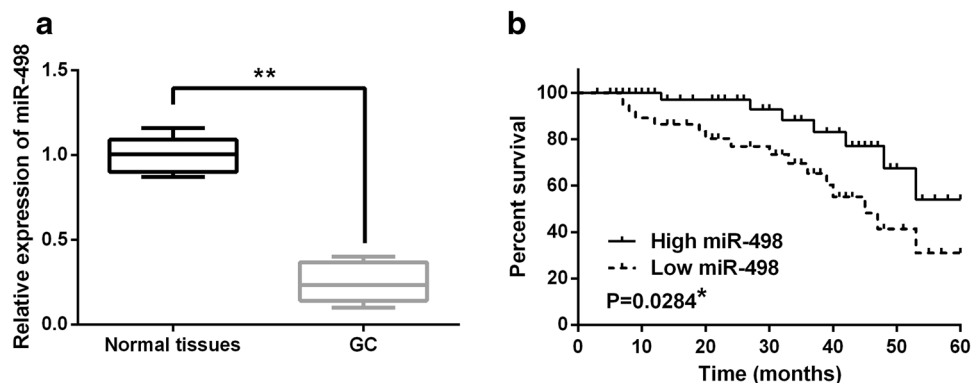**Table 1** Relationship between miR-498 expression and their clinic-pathological characteristics of GC patients

Characteristics	Cases	MiR-498		P value
		High	Low	
Age (years)				0.064
≥60	26	8	18	
<60	36	15	21	
Gender				0.193
Male	38	13	25	
Female	24	10	14	
Tumor size (mm)				0.104
≤5.0	29	12	17	
>5.0	33	11	22	
Differentiation				0.008*
Well/moderate	22	8	14	
Poor	40	15	25	
Lymph node metastasis				0.045*
Yes	42	15	27	
No	20	8	12	
TNM stage				0.013*
I–II	18	9	9	
III–IV	44	14	30	

Statistical analyses were performed by the χ2 test

*$P < 0.05$ was considered significant

expression was found to predict the poor prognosis of GC patients ($P = 0.0284$; Fig. 1b). Based on these findings, miR-498 was suspected to participate in the pathogenesis of GC.

Overexpression of miR-498 inhibited the progression of GC

Next, the expression of miR-498 was observed in SGC-7901, BGC-823, MGC-803 and GES-1 cell lines. And the downregulation of miR-498 was also detected in SGC-7901, BGC-823 and MGC-803 cell lines in comparison to GES-1 cells (Fig. 2a). Then, miR-498 mimics or inhibitor was transfected into SGC-7901 and MGC-803 cells to explore its role in GC. MiR-498 was upregulated by its mimics and downregulated by its inhibitor as shown in Fig. 2b. Furthermore, the overexpression of miR-498 was identified to suppress the proliferation of SGC-7901 and MGC-803 cells (Fig. 2c, d). Inversely, the downregulation of miR-498 promoted cell proliferation in SGC-7901 and MGC-803 cells (Fig. 2c, d). Similarly, the upregulation of miR-498 suppressed cell migration (95 vs. 24) while knockout of miR-498 promoted the migration of SGC-7901 and MGC-803 cells (85 vs. 184) (Fig. 2e). And the same effect of miR-498 was also identified for cell invasion in SGC-7901 cells (105 vs. 33; 96 vs. 192) (Fig. 2f). These results indicated that miR-498 played a suppressive role in GC.

BMI1 was a direct target of miR-498 in GC

Then, the target genes of miR-498 were investigated in GC. BMI1 was found to have binding sites with miR-498 according to bioinformatics analysis online TargetScan (http://www.targetscan.org/) (Fig. 3a). To confirm that, luciferase reporter assay was performed. The luciferase activity of Wt-BMI1 was found to be reduced by miR-498 mimics in SGC-7901 and MGC-803 cells as shown in Fig. 3b. And the luciferase activity of Mut-BMI1 was not affected by miR-498 mimics in SGC-7901 and MGC-803 cells (Fig. 3b). In addition, BMI1 expression had negative association with miR-498 in GC tissues ($P < 0.0001$, $R^2 = 0.5811$; Fig. 3c). Moreover, BMI1 expression was observed in SGC-7901 and MGC-803 cells with miR-498 mimics or inhibitor. We found that miR-498 mimics reduced BMI1 expression (Fig. 3d), on the contrary miR-498 inhibitor promoted BMI1 expression in SGC-7901 and MGC-803 cells (Fig. 3e). In brief, BMI1 was a direct target of miR-498 which had negatively association with miR-498 in GC.

BMI1 was upregulated in GC tissues

The alternation of BMI1 expression was detected in GC tissues. We found that BMI1 was upregulated in GC tissues compared to normal tissues (Fig. 4a). Similarly, the upregulation of BMI1 was observed in human GC cell lines (SGC-7901, BGC-823, MGC-803) compared with GES-1 cell (Fig. 4b). Besides that, BMI1 was found have correlation with GC patient' prognosis. The results of Kaplan–Meier analysis indicated that high BMI1 expression was related to shorter overall survival of GC patients ($P = 0.0311$; Fig. 4c). Thus, BMI1 was considered to involve in the progression of GC.

MiR-498 inhibited the development of GC through targeting BMI-1

Then, miR-498 mimics and BMI-1 vector were co-transfected into SGC-7901 and MGC-803 cells to investigate the relationship between miR-498 and BMI-1. The results of qRT-PCR assay showed that the decreased BMI-1 expression induced by miR-498 mimics was recovered by BMI-1 vector in SGC-7901 and MGC-803 cells (Fig. 5a). Furthermore, the suppressive effect of miR-498 on cell proliferation was hindered by BMI-1 vector in SGC-7901 and MGC-803 cells (Fig. 5b). As for cell migration and invasion, the same results were also identified in SGC-7901 and MGC-803 cells (Fig. 5c, d). In brief, miR-498 was considered to impede the proliferation, migration and invasion of GC through targeting BMI-1.
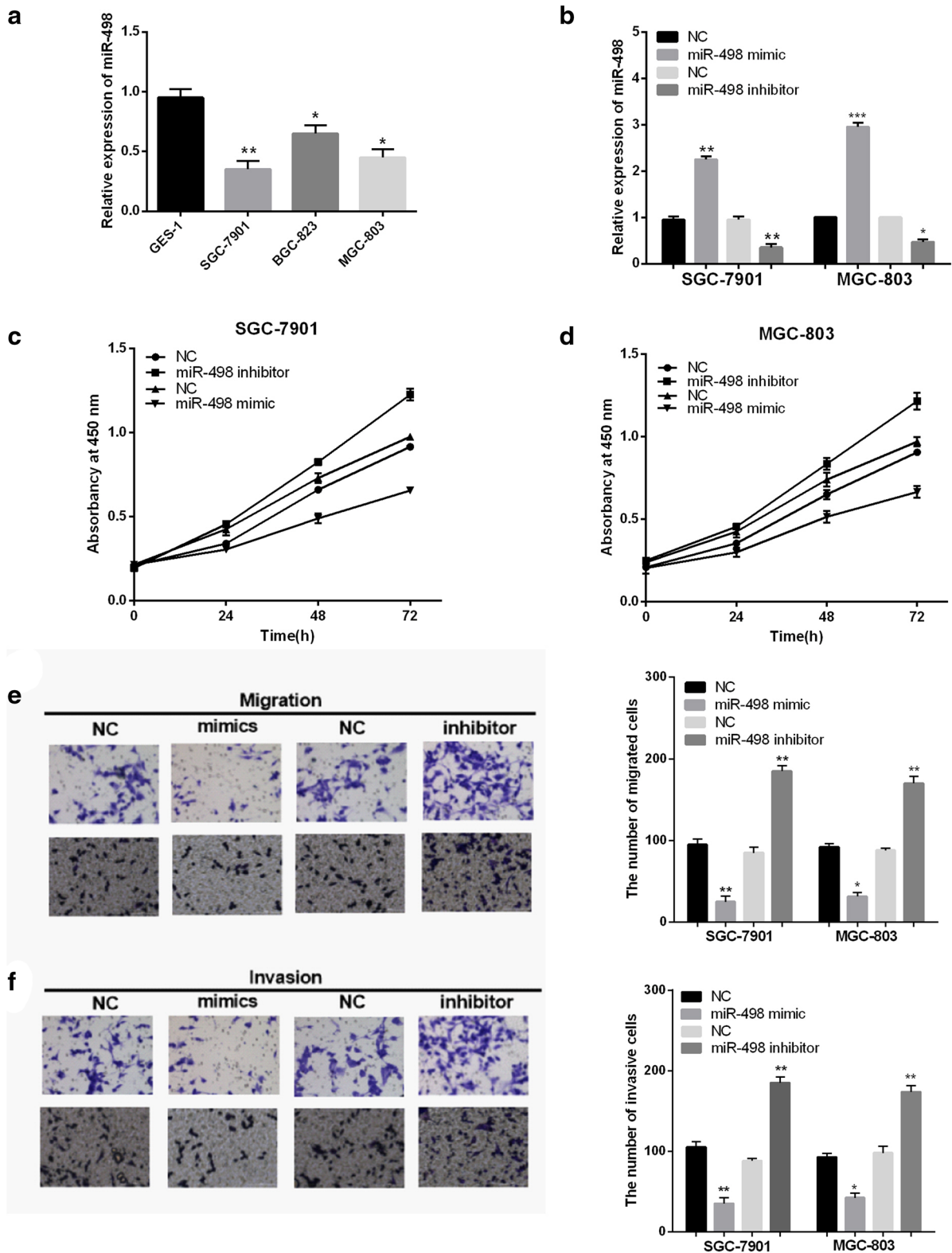
Fig. 2 Overexpression of miR-498 inhibited the progression of GC. **a** The miR-498 expression in SGC-7901, BGC-823, MGC-803 and GES-1 cell lines was measured by RT-qPCR. **b** The expression of miR-498 was examined in SGC-7901 and MGC-803 cells with miR-498 mimics or inhibitor. **c**, **d** The cell proliferation was measured in cells containing miR-498 mimics or inhibitor by MTT assay. **e**, **f** Cell migration and invasion analysis was conducted in SGC-7901 and MGC-803 cells containing miR-498 mimics or inhibitor. The experiments were repeated three times. *$P < 0.05$, **$P < 0.01$
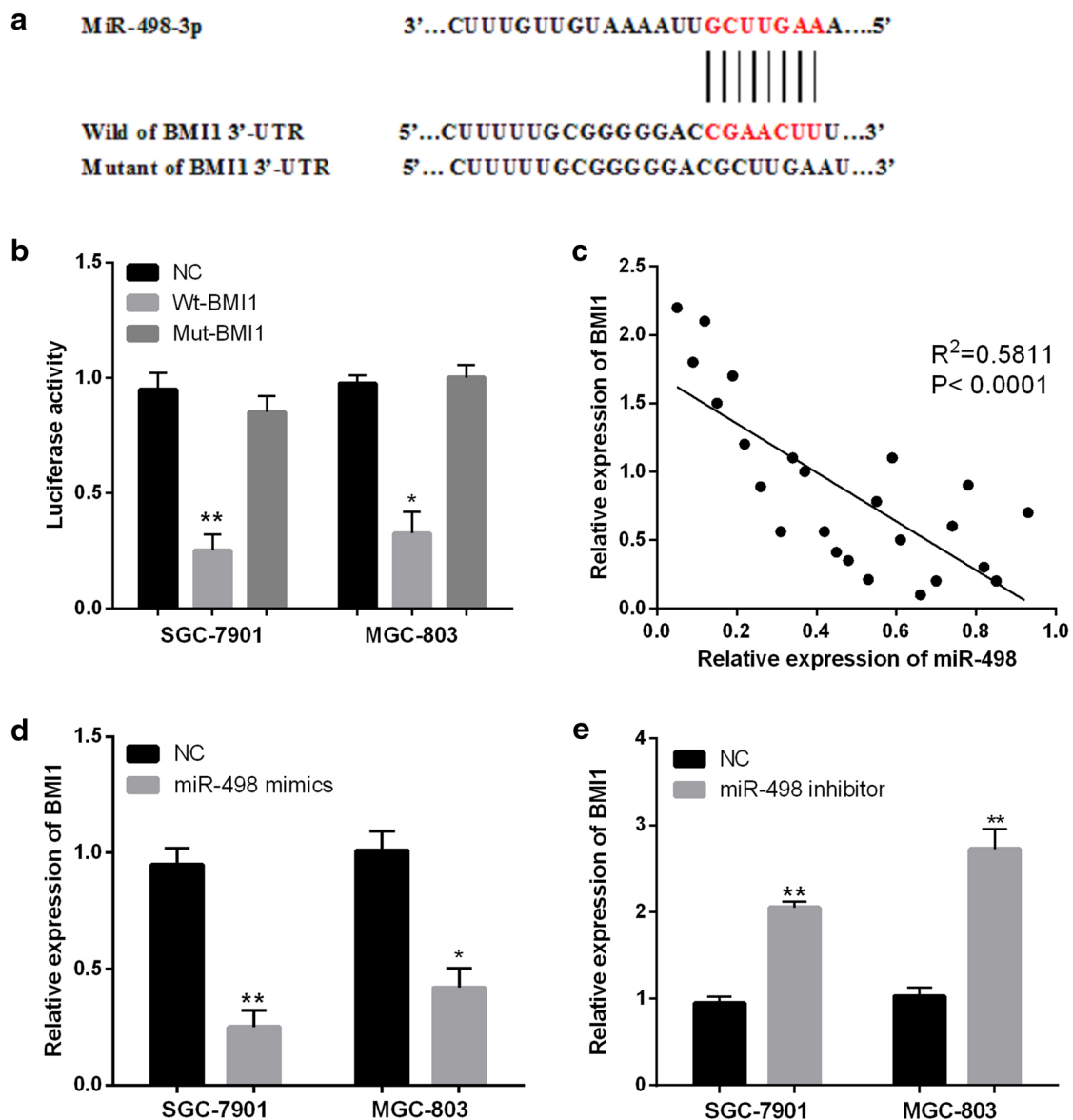
Fig. 3 BMI1 was a direct target of miR-498 in GC. **a** A putative miR-498 binding site in the BMI1 3′UTR is indicated with red characters. This site was identified using TargetScan. **b** Human SGC-7901 and MGC-803 cells were pre-transduced with miR-NC or miR-498 mimic, inhibitor, followed by transfection of firefly luciferase vectors BMI1 or BMI1/mutant. The luciferase activity was normalized to Renilla luciferase activity. Results represent the mean ± SD of triplicate wells and are representative of three independent experiments. **c** MiR-498 had negative correlation with BMI1. **d**, **e** The expression of BMI1 was observed in SGC-7901 and MGC-803 cells containing miR-498 mimics or inhibitor measured by RT-qPCR. The experiments were repeated three times. **P < 0.01

MiR-498 suppressed EMT and AKT pathway in GC

Furthermore, we investigated the effect of miR-498 on EMT and AKT pathway in GC. And the overexpression of miR-498 promoted E-cadherin expression and inhibited N-cadherin and Vimentin expressions in SGC-7901 and MGC-803 cells as shown in Fig. 6. On the contrary, the downregulation of miR-498 was found having opposite effect on these markers (Fig. 6). Additionally, miR-498 overexpression was identified to inhibit p-AKT expression (Fig. 6) while the downregulation of miR-498 promoted p-AKT expression in SGC-7901 and MGC-803 cells (Fig. 6). However, the expression of AKT was not influenced by miR-498 mimics or inhibitor in SGC-7901 and MGC-803 cells. Hence, miR-498 was inferred to inhibit the metastasis and proliferation of GC cells through blocking EMT and AKT pathway.
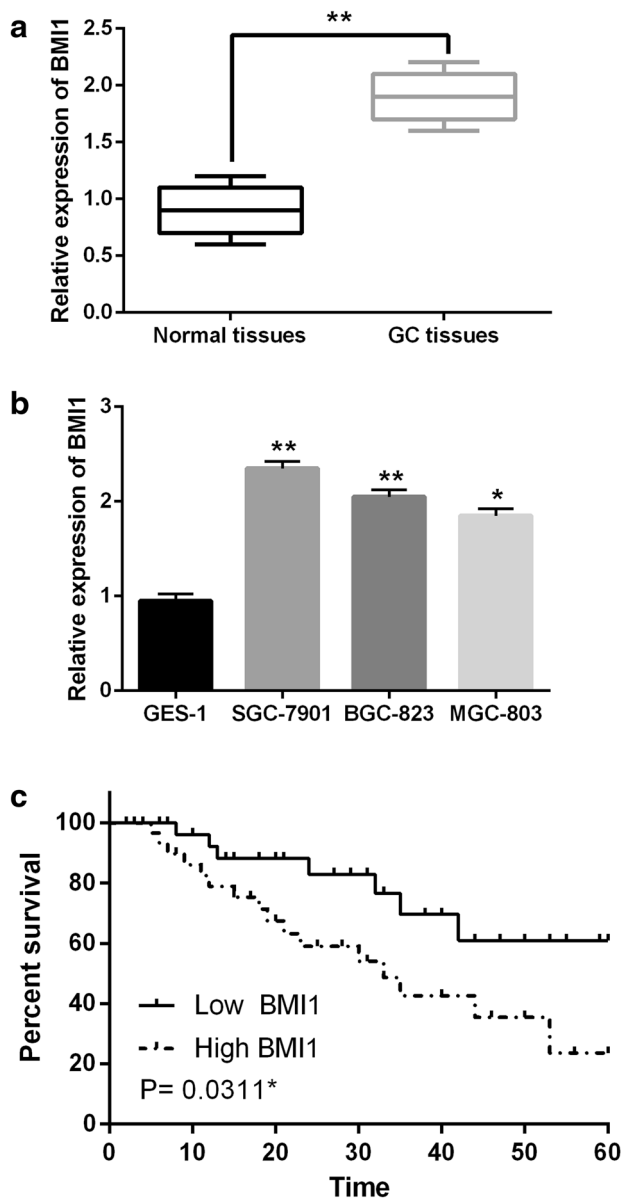
Fig. 4 BMI1 was upregulated in GC tissues. **a** The expression of BMI1 in GC tissues was measured by RT-qPCR. **b** The BMI1 expression in SGC-7901, BGC-823, MGC-803 and GES-1 cell lines was measured by RT-qPCR. **c** High BMI1 expression was related to shorter overall survival of GC patients. The experiments were repeated three times.*$P < 0.05$, **$P < 0.01$. *NC* negative control

Discussion

The discovered miRNAs modulate the expression of various genes involved in GC progression and carcinogenesis. For instance, miR-592 promoted GC proliferation, migration, and invasion through the PI3K/AKT and MAPK/ERK signaling pathways by targeting Spry2 [28]. On the contrary, miR-361-5p inhibited the mobility of GC cells through suppressing EMT via the Wnt/β-catenin pathway [29]. Previous

study has shown that miR-498 was downregulated in GC cell lines and tissues, acting as a tumor suppressor, which was similar to our research [30]. In this study, we demonstrated that the overexpression of miR-498 inhibited the migration, invasion, EMT and proliferation of GC cells. In addition, miR-498 was also found to predict unfavorable overall survival of GC patients, suggesting that miR-498 was a valuable marker for the tumorigenesis and prognosis of GC.

Previous study has proved that EMT plays a fundamental role in the initial stage of metastatic progression of cancer cells [31]. Moreover, it has been found that 90% failures of cancer treatment are due to metastasis [32]. Interestingly, several studies proposed that EMT had tightly association with the expression of microRNAs in GC. Which same as our results, miR-630 inhibited EMT by regulating Wnt/β-catenin pathway in GC cells [33]. Besides that, miR-498 was downregulated in colorectal cancer which had some direct or indirect effect in the development of colorectal adenocarcinoma [34]. Moreover, Cong et al. reported that low miR-498 expression levels were associated with poor prognosis in ovarian cancer [35]. And miR-498 was demonstrated to regulate FOXO3 expression and inhibit the proliferation of human ovarian cancer cells [15]. In current research, western blot analysis showed that overexpression of miR-498 promoted E-cadherin expression and inhibited N-cadherin and Vimentin expressions in SGC-7901 and MGC-803 cells.

It has been reported that miRNAs have roles in a number of cancers via complementary base pairing with the 3′UTR of their target genes [36]. There are many genes was conformed as direct targets of miR-498, such as HMGA2 [37]. BMI-1 was reported to positively correlate with tumor size, degree of tumor differentiation, invasion and lymph node metastasis [38], and silencing BMI-1 enhances the senescence and decreases the metastasis of human gastric cancer cells [39]. By bioinformatics analysis, we identified many potential targets of miR-498 and selected BMI-1 for further analysis. BMI-1 was firstly confirmed as a direct target of miR-498 in this study, we found that miR-498 mimics reduced BMI1 expression and miR-498 inhibitor promoted BMI1 expression in SGC-7901 cells. Moreover, several miRNAs had been proposed to directly target BMI-1 which was consistent with our results, such as miR128-1 [40], miR-203 [41] and miR-376c [42]. Furthermore, Li et al. demonstrated that miR-200c inhibited EMT by targeting the BMI-1 gene through the AKT pathway in endometrial carcinoma cells in vitro [43].

The AKT is usually dysregulated in human cancer which belongs to intracellular signaling pathways [44]. AKT signaling pathway had been found to regulate the cell growth, proliferation and apoptosis in Wilms' tumors [45]. Long et al. indicated that miR-374b promoted proliferation and inhibited apoptosis of human gastrointestinal stromal tumors through the activation of the AKT pathway [46]. Xiao et al.
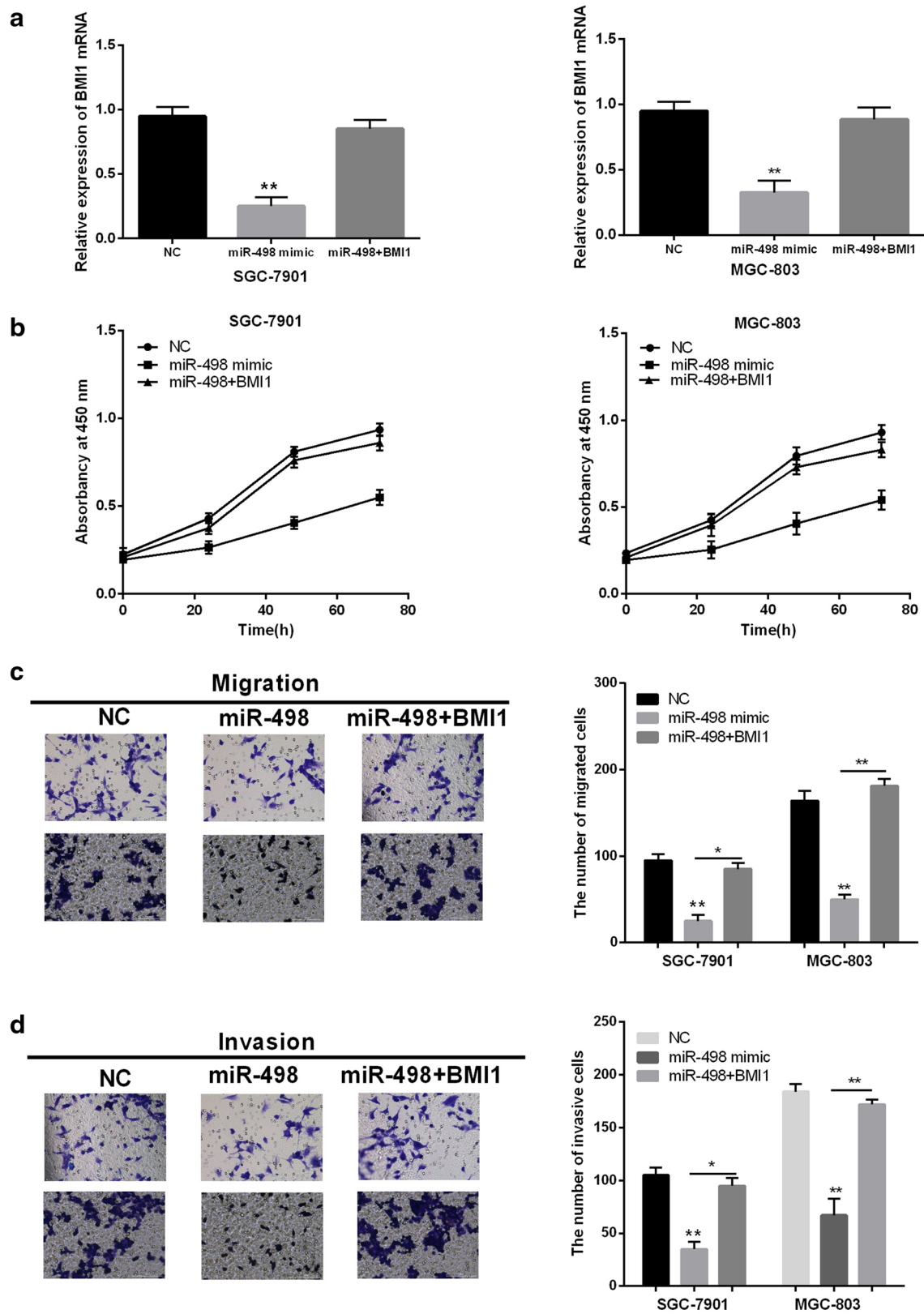
Fig. 5 MiR-498 inhibited the development of GC through targeting BMI1. **a** The expression of BMI1 was measured by RT-qPCR in SGC-7901 and MGC-803 cells with BMI1 vector and miR-498. **b** The cell proliferation was measured by MTT assay in SGC-7901 and MGC-803 cells with BMI1 vector and miR-498. **c, d** The cell migration and invasion in SGC-7901 and MGC-803 cells with BMI1 vector and miR-498. The experiments were repeated three times. **$P<0.01$

Fig. 6 MiR-498 suppressed EMT and AKT pathway in GC. Western blot analysis was used to measure the expression of E-cadherin, N-cadherin, vimentin, AKT and p-AKT in SGC-7901 and MGC-803 cells contained miR-498 mimics or inhibitor
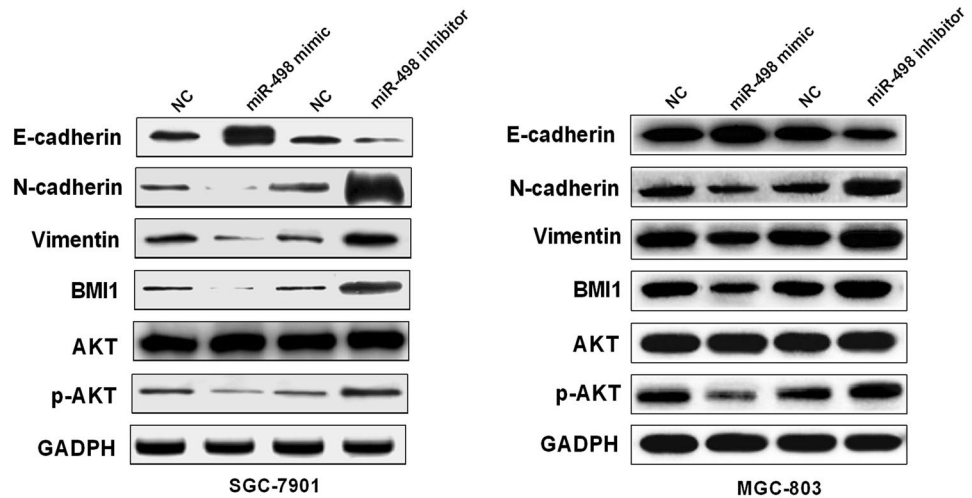
proposed that miR-28-5p inhibited the migration and invasion of GC cells by suppressing AKT phosphorylation [47]. In this study, western blot analysis showed that miR-498 was also conformed to negatively activate AKT pathway in GC. Here, we also identified that miR-498 inhibit the metastasis and proliferation of GC cells through blocking AKT phosphorylation.

Conclusion

In the present study, the downregulation of miR-498 was detected in GC which was related to shorter overall survival of GC patients. Moreover, miR-498 was found to inhibit the progression of GC through targeting BMI1 and suppressing EMT and AKT pathway. These findings would open the new path for the diagnosis and therapies of GC.

Compliance with ethical standards

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