MicroRNA-27a inhibitors alone or in combination with perifosine suppress the growth of gastric cancer cells

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Abstract. MicroRNA-27a (miR-27a) is an oncogene that contributes to drug resistance in various types of cancer. However, the involvement of miR-27a in gastric cancer has yet to be elucidated. Perifosine is an alkylphospholipid exhibiting antitumor activity as shown in both preclinical studies and clinical trials. The effects of perifosine on gastric cancer have yet to be determined. Therefore, this study was conducted to detect the role of miR-27a and perifosine in human gastric cancer. miR-27a was found to be expressed in human gastric cancer tissues and cell lines by quantitative reverse-transcription polymerase chain reaction (qRT-PCR). The correlation between miR-27a expression and clinicopathological characteristics of gastric cancer. We also explored the growth inhibitory effect of perifosine on human gastric cancer cells with or without co-targeting miR-27a by sulforhodamine B (SRB) assay. The results showed that miR-27a expression was significantly upregulated in gastric cancer tissues, compared with their non-tumor adjacent tissues. High expression levels of miR-27a were associated with poor tumor histological grade (P=0.037). MiR-27a inhibitors suppressed the growth of MGC-803 cells. Assay results showed that perifosine exerted its activity selectively on the AGS cell line and the growth inhibitory effect of perifosine was enhanced significantly in combination with miR-27a inhibitors in MGC-803 cells. In conclusion, our results demonstrated that miR-27a may be a therapeutic target and potential prognostic biological marker in gastric cancer. MiR-27a inhibitors alone or in combination with perifosine may be a novel therapeutic approach against gastric cancer.

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Introduction

Gastric cancer remains the second leading cause of cancer-related mortality worldwide (1,2). Even after a curative resection alone or after chemotherapy, more than 50% of patients are likely to experience local regional recurrence (3,4). Despite the development of more diagnostic techniques and new treatment modalities, the five-year survival rate of gastric cancer has not significantly changed. Therefore, it is critical to explore more specific and efficient therapies.

MicroRNAs (miRNAs) are important post-transcriptional regulators of gene expression. Additionally, they are a class of highly conserved, small RNA molecules that regulate key biological processes, including differentiation, development, proliferation and metabolism (5). Accumulating evidence suggests that the dysregulation of miRNAs expression contributed to malignant transformation. Furthermore, miRNAs have been confirmed to act as oncogenes or tumor suppressors in many types of cancer (6). miRNAs, such as miR-130a, miR-214, miR-27a and miR-451, have been reported to be associated with chemotherapy resistance (7-9). In particular, miR-27a, an oncogene, was found to be widely expressed in breast cancer, gastric adenocarcinoma, human uveal melanoma and colon cancer (10-13) and to contribute to drug resistance in various types of cancer (14-17). However, the role of miR-27a in gastric cancer remains to be determined.

Perifosine is an orally bioavailable alkylphospholipid exhibiting antitumor activity in both preclinical models and clinical trials (18,19). Its anticancer activity is known to target the cell membrane, inhibit Akt activity, and affect cell processes such as growth arrest, apoptosis and survival. In the preclinical studies, perifosine combined with UCN-01, the chemotherapeutic agent etoposide and other antitumor agents shows synergistic antitumor effects (20,21). In the clinical trials, perifosine shows substantial antitumor activity in sarcoma and

renal cell carcinoma (22). Combined with dexamethasone, it also has activity in relapsed/refractory multiple myeloma (23). However, the growth inhibitory effect of perifosine on gastric cancer has yet to be reported. Moreover, no related studies on the effect of combining miR-27a inhibitors with perifosine on gastric cancer are currently available.

Therefore, we hypothesized that miR-27a was an oncogene in gastric cancer and that miR-27a inhibitors or perifosine were able to suppress gastric cancer cell growth. In the present study, miR-27a expression was detected in gastric cancer tissues, their matched non-tumor adjacent tissues and gastric cancer cell lines. We also investigated the effect of miR-27a inhibitors or perifosine on human gastric cancer cell growth *in vitro*.

Materials and methods

Reagents. Perifosine was supplied by Selleck Chemicals LLC (Houston, TX, USA). This agent was dissolved in phosphate-buffered saline (PBS) and stored at -20˚C. Stock solution was diluted to the appropriate concentrations with growth medium immediately before use.

Human tissue samples. In total, 67 pairs of human gastric tissue samples were obtained during surgery and used after informed consent was obtained from all 67 patients. The patients underwent surgical resection at the First Affiliated Hospital of Nanjing Medical University between 2009 and 2010 following diagnosis of gastric cancer based on histopathological evaluation. The matched non-tumor adjacent tissues were obtained from a segment of the resected specimens that was at a distance from the tumor (>5 cm). The samples were snap-frozen in liquid nitrogen and stored at -80˚C. No local or systemic treatment was conducted in these patients prior to surgery. The tumor histological grade was assessed according to World Health Organization criteria and was staged using the TNM staging of the International Union Against Cancer (UICC)/American Joint Committee on Cancer (AJCC) system (2002). The study was approved by the Research Ethics Committee of Nanjing Medical University, China. Sample data, including age, gender, weight, residence, hypertension, diabetes, smoke, tumor location, histological grade, depth of tumor invasion, lymph node metastasis and clinical stage were obtained from the clinical and pathologic records (Table I).

Cell lines and cell culture. The human gastric cancer cell lines (AGS and MGC-803) were obtained from the Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences (Shanghai, China). The cells were cultured in RPMI-1640 medium (Invitrogen Life Technologies Inc., Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (Gibco BRL, Grand Island, NY, USA) at 37˚C in a humidified atmosphere consisting of 5% CO_2.

miRNA transfection. Cells in the exponential phase of growth were plated in 60-mm plates at $1x10^6$ cells/plate and cultured for 16 h, and then transfected with miR-27a inhibitors or the negative control (NC) (100 nM) using Lipofectamine™ 2000 Reagent and OPTI-MEM I reduced serum medium (Invitrogen Life Technologies Inc.), following the manufacturer's protocol. The effects of miR-27a inhibitors or the NC were examined 48 h following transfection as described previously (9).

Growth inhibition assay. Cells were seeded in 96-well culture plates and treated on the second day with perifosine. At the end of a three-day treatment, the cell number was estimated by the sulforhodamine B (SRB) assay, as previously described (24).

RNA isolation and quantitative RT-PCR. Total RNA from specimens and cultured AGS and MGC-803 cells was extracted using TRIzol reagent (Invitrogen Life Technologies Inc.) according to the manufacturer's protocol. *Taq*Man microRNA assays (Applied Biosystems Inc., Carlsbad, CA, USA) were used to quantify miR-27a expression. Small nuclear RNA, U6B (Applied Biosystems Inc.), was treated as the normalization control. Real-time amplifications were measured in triplicate and performed with the ABI Prism® 7300 sequence detection system (Applied Biosystems Inc.). The fold-change of miR-27a was calculated using the $2^{-\Delta\Delta CT}$ method. Therefore, the value of the relative expression ratio >1.0 was considered as a high expression as compared to the non-tumor control where a ratio <1.0 was considered as a low expression of cancer.

Statistical analysis. Data were presented as the mean ± SD from at least three separate experiments. Statistical analysis was performed with the Student's t-test and Pearson's χ^2 test. The difference between the groups was determined by the one-way analysis of variance (ANOVA). Differences were considered statistically significant at $P<0.05$. The analyses were carried out with the SPSS 13.0 (SPSS Inc., Chicago, IL, USA) and were based on two-tailed probability.

Results

Expression of miR-27a in gastric cancer tissues and its correlation with clinicopathological characteristics of gastric cancer. Using a qRT-PCR method, miR-27a was detected in all 67 (100%) pairs of gastric cancer tissues and their matched non-tumor adjacent tissues. Of the 67 patients with gastric cancer, 42 (62.69%) cases revealed >50% increase in the miR-27a level as compared to their matched non-tumor adjacent tissues (Fig. 1).

The correlation between miR-27a expression and clinicopathological characteristics of gastric cancer was examined. The Pearson's χ^2 test revealed that the expression levels of miR-27a were associated with tumor histological grade ($P=0.037$) in gastric cancer patients (Table I). The patients with a high miR-27a expression tended to have a poor tumor histological grade.

Effect of downregulation of miR-27a on cell growth in vitro. The potential involvement of miR-27a in tumorigenesis was determined following the significant increase of miR-27a expression in gastric cancer samples. As an initial step, we examined the expression of miR-27a by qRT-PCR 48 h following transfection with miR-27a inhibitors or their respective NCs in MGC-803 and AGS cell lines. As shown in Fig. 2A and B, miR-27a inhibitors significantly inhibited

Table I. miR-27a expression and clinicopathological factors.

Factors	High expression group (n=42)		Low expression group (n=25)		P-value
	N	Percentage	N	Percentage	
Age (mean ± SD)	57.71±11.81		65.76±9.62		
Weight (mean ± SD)	62.85±10.87		65.33±15.21		
Gender					
Male	30	71.4	21	84.0	0.243
Female	12	28.6	4	16.0	
Hypertension					
Absent	31	73.8	18	72.0	0.872
Present	11	26.2	7	28.0	
Diabetes					
Absent	38	90.5	23	92.0	0.833
Present	4	9.5	2	8.0	
Smoke					
Absent	26	61.9	16	64.0	0.864
Present	16	38.1	9	36.0	
Location					
Cardia, fundus	23	54.8	9	36.0	0.137
Others	19	45.2	16	64.0	
Histological grade					
Well, moderately	25	59.5	21	84.0	0.037[a]
Poorly, others	17	40.5	4	16.0	
Depth of tumor invasion					
m, sm, mp	6	14.3	4	16.0	0.849
ss, se, si	33	85.7	21	84.0	
Lymph node metastasis					
Absent	9	21.4	5	32.0	0.905
Present	33	78.6	17	68.0	
Clinical stage					
I,II	10	23.8	10	40.0	0.161
III,V	32	76.2	15	60.0	
Residence					
Rural	23	54.8	9	36.0	0.137
Urban	19	45.2	16	64.0	

[a]Indicated statistical significance (P<0.05); m, mucosa; sm, submucosa; mp, muscularis propria; ss, subserosa; se, penetration of serosa; si, invasion of adjacent strucures.

the expression of miR-27a by 94.22 and 99.78% as compared with NCs in MGC-803 and AGS cells, respectively. According to the results of the SRB assay and growth curves, the MGC-803 cell line, which was transiently transfected with miR-27a inhibitors, was found to have a significant growth inhibition as compared with NC (P=0.005, Fig. 2C). On day 5, the cell number decreased to 0.57-fold in cells expressing the downregulation of miR-27a. However, this decrease in cell number did not suppress the growth of AGS cells (P=0.766, Fig. 2D). These findings suggest that the downregulation of miR-27a selectively inhibited cell growth in MGC-803 cells.

Expression of miR-27a in gastric cancer cell lines and the effect of perifosine. To explain the above finding, we detected the expression level of miR-27a in MGC-803 and AGS gastric cancer cell lines. Compared with AGS cells, the expression level of miR-27a was increased in MGC-803 cells (2.67±0.18-fold, Fig. 3A). These data suggest that the selective sensitivity of MGC-803 cells to miR-27a inhibitors is likely to be correlated with the high miR-27a expression levels.

In this study, the effect of perifosine as a single agent on the growth of the MGC-803 and AGS cells was investigated for the first time. Subsequent to treatment with varying

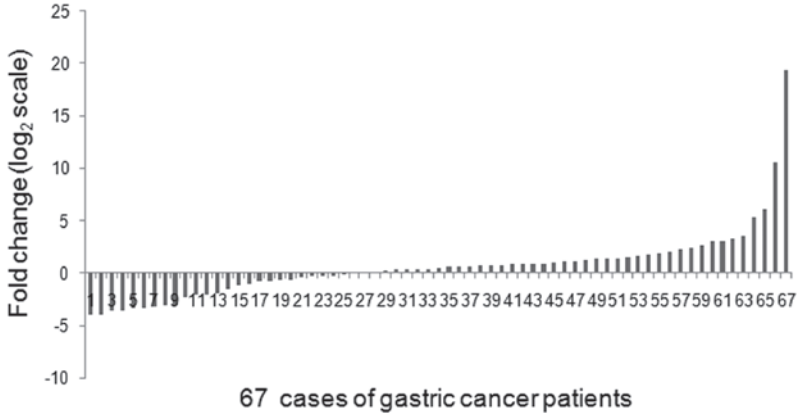

Figure 1. The expression of miR-27a in gastric cancer tissues. MiR-27a was detected in gastric cancer patients by quantitative real-time PCR. Data were presented as $\log_2$-fold change of gastric cancer relative to non-tumor adjacent tissues. The cases above the line ($\log_2(1)=0$) revealed >50% increase in the miR-27a level.

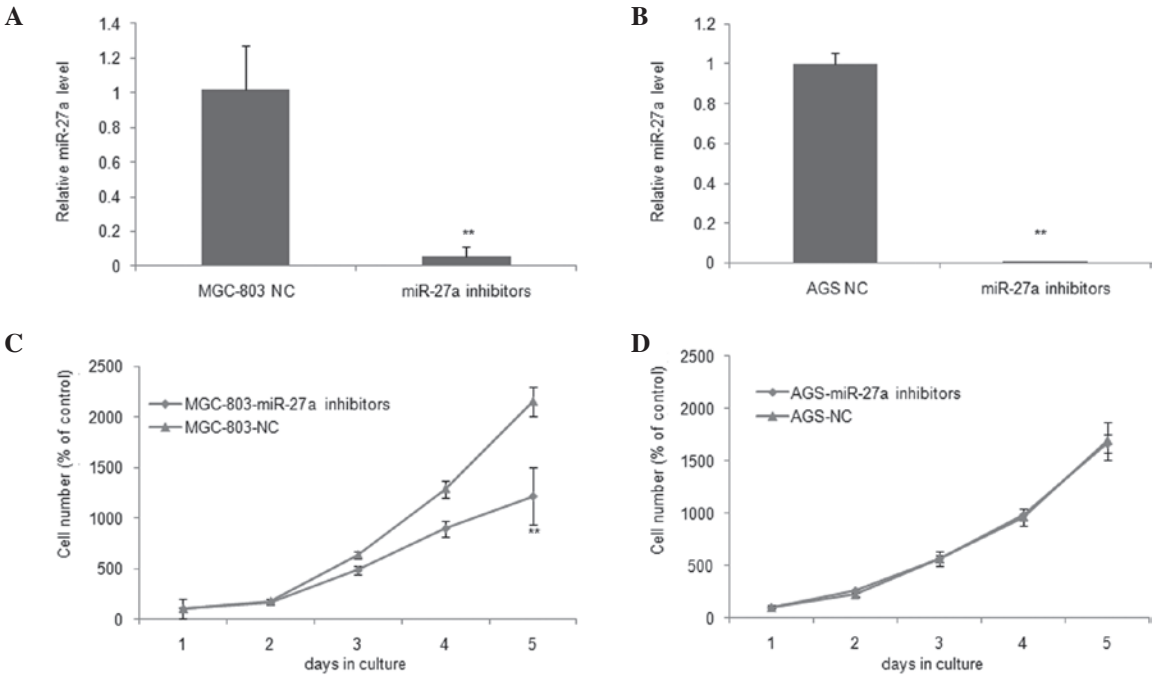

Figure 2. Downregulation of miR-27a inhibits cell proliferation *in vitro*. (A and B) Relative level of miR-27a in the MGC-803 and AGS cell lines following transfection of miR-27a inhibitors. Total cell RNA extracted from each group was examined by quantitative real-time PCR. (C and D) MGC-803 and AGS cell lines were transfected with miR-27a inhibitors or negative controls (NCs). Cells were subjected to SRB assay to estimate cell viability. Each point is the mean ± SD. Results were reproducible in three independent experiments. $^{**}P<0.01$.

concentrations of perifosine for 72 h, the results of SRB assay showed that perifosine decreased MGC-803 and AGS cell growth by 52.44±2.50 and 74.72±0.29% in 10 μM/l group compared to control cells, respectively (Fig. 3B). Additionally, the 50% inhibitory concentration (IC_{50}) values of perifosine in MGC-803 and AGS cells were 9.84 and 2.76 μM/l, respectively, for the same intervals. These results suggest that perifosine inhibited the growth of gastric cancer cells in a dose-dependent manner, but AGS cells were more sensitive to perifosine compared to MGC-803 cells. We hypothesized that the insensitivity of MGC-803 cells to perifosine may also be correlated with the high miR-27a expression levels and that the downregulation of miR-27a may enhance the effects of perifosine on MGC-803 cells.

Growth inhibitory effect of perifosine following transfection with miR-27a inhibitors. To confirm that perifosine insensitivity is correlated with miR-27a expression in MGC-803 cells, cells were transfected with either the miR-27a inhibitors or the NC and treated with varying concentrations of perifosine. Cell viability was then measured using the SRB assay. As shown in Fig. 4, at the maximum concentration of 10 μM/l perifosine, the cell viability of the MGC-803 cells with miR-27a transfection was reduced by 60.53±1.99%, whereas the NC-transfected cell viability was reduced by 50.04±1.17%. The IC_{50} of perifosine was 4.93 and 9.21 μM/l in MGC-803 cells with miR-27a inhibitors and the NC-transfected cells, respectively (Fig. 4). The IC_{50} of perifosine was significantly decreased after miR-27a inhibitor transfection ($P<0.001$). These data suggest

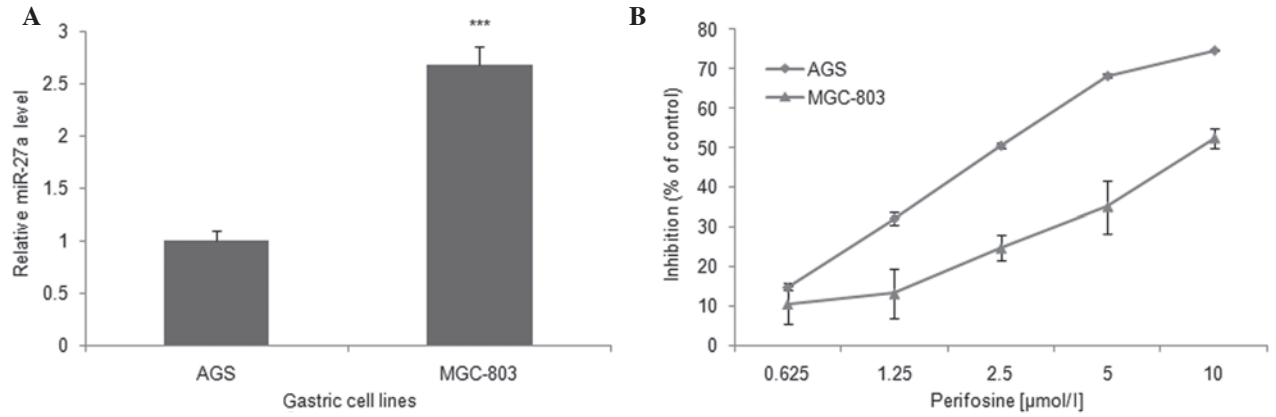

Figure 3. Effect of perifosine on human gastric cancer cell growth and the expression of miR-27a. (A) The relative expression of miR-27a was determined by normalization to U6B control using quantitative real-time PCR in the MGC-803 gastric cancer cell line as compared to AGS. Each point is the mean ± SD. Results were reproducible in three independent experiments. ***P<0.001. (B) Survival of AGS and MGC-803 cells was determined by sulforhodamine B (SRB) assay after 72 h stimulation with or without varying concentrations of perifosine. Each point is the mean ± SD. Results were reproducible in three independent experiments.

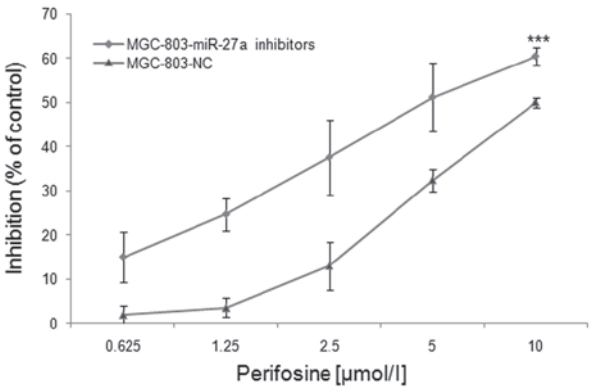

Figure 4. The growth inhibitory effect of perifosine following transfection with miR-27a inhibitors. MGC-803 cells with miR-27a inhibitors or negative control (NC)-transfected cells were treated with varying concentrations of perifosine ranging from 0.625 to 10 μM/l. After 72 h, the cells were subjected to SRB assay to estimate cell viability. Each point is the mean ± SD. Results were reproducible in three independent experiments. ***P<0.001.

the downregulation of miR-27a enhanced the inhibitory effects of perifosine on gastric cancer MGC-803 cells.

Discussion

It is well known that surgical resection is the most effective treatment for gastric cancer and is effective in prolonging the survival of patients with early gastric cancer, whereas the prognosis for individuals with advanced gastric cancer remains poor (25). Although numerous chemotherapy drugs have been studied, there is no internationally recognized standard of care for chemotherapy strategy (26). Therefore, new therapeutic approaches should be developed.

MiRNAs are important post-transcriptional regulators of gene expression. Moreover, they are a class of highly conserved, small RNA molecules that regulate key biological processes, while the dysregulation of miRNA expression contributes to malignant transformation (5,6). Recent evidence shows that the dysregulation of miRNAs may be important in cancer initiation, progression and prognosis (27,28). Therefore, the study of their clinical potential in gastric cancer is imperative.

MiR-27a, which is considered to be a carcinogenesis, has been found to be widely expressed in many tumors (10-13). Liu *et al* (12) have shown that miR-27a was upregulated in human gastric adenocarcinoma and MGC-803 gastric cancer cells. In this study, we found a significant high expression of miR-27a in gastric cancer tissues compared with their matched non-tumor adjacent tissues, which was consistent with previous reports (12). Taken together, we have further suggested that miR-27a may be involved in carcinogenesis as an oncogene in human gastric cancer. Furthermore, miR-27a expression was associated with tumor histological grade (P=0.037) in gastric cancer patients. The patients with a high miR-27a expression tended to have a poor tumor histological grade, which was an independent prognostic factor in patients with cancer. Additionally, miR-27a may be involved in gastric cancer progression and serve as a potential prognostic marker for gastric cancer patients.

The correlation of cell growth and the expression of miR-27a was detected. In present experiments, we transfected MGC-803 and AGS cells with the miR-27a inhibitors or the NCs and yielded a high transfection efficiency. Moreover, results of the SRB assay revealed that the downregulation of miR-27a significantly inhibited cell growth in the MGC-803 cell line, but did not suppress the growth of AGS cells. The reason for the downregulation of miR-27a inhibiting cell growth of MGC-803 versus AGS cells was also investigated, and the results suggest varying miR-27a expression levels. Our findings also confirmed that the expression of miR-27a was higher in the MGC-803 cell line as compared to the AGS cell line. Thus, miR-27a acts as an oncogene in gastric cancer and downregulation of its expression significantly inhibited gastric cancer cell growth. However, the lack of a significant effect on AGS cells may be due to the low miR-27a expression levels.

Perifosine is a synthetic, orally bioavailable alkylphospholipid analogue that acts primarily at the cell membrane targeting signal transduction pathways. Perifosine has been shown to exert anticancer activity, which is partly associated with its ability to inhibit Akt activity and the mTOR signaling

pathway, and to affect multiple cell processes, including proliferation, survival and apoptosis (19,29,30). Perifosine has recently received wide attention due to its potential health beneficial effects (31). Therefore, to improve our understanding of the antitumor activity of perifosine, we measured its effects on human gastric cancer cells. Results of the present study have shown for the first time that perifosine inhibited human gastric cancer cell growth *in vitro* in a concentration-dependent manner. However, perifosine exerted its activity selectively on AGS cells, which is a miR-27a low expression gastric cancer cell line. These data therefore suggest that the insensitivity of perifosine is correlated with the high expression levels of miR-27a in gastric cancer cell lines.

In their study, Zhu *et al* (9) reported that miR-27a and miR-451 were involved in activating the multidrug resistant gene 1 (MDR1) P-glycoprotein expression, which was associated with cancer cell resistance to a series of chemotherapeutics. Zhang *et al* (15) also reported that the downregulation of miR-27a has the potential to reverse MDR of esophageal squamous cell carcinoma. Additionally, the mechanism is likely to decrease the expression of P-glycoprotein, Bcl-2, and the transcription of MDR1, but upregulate the expression of Bax. Notably, the growth inhibitory effect of perifosine was enhanced when combined with miR-27a inhibitors. The result of SRB assay indicated that the downregulation of miR-27a promoted perifosine sensitivity of the MGC-803 cell line, in which miR-27a expression was higher than that in AGS cell line. This finding may be partly due to the fact that miR-27a was associated with chemotherapy resistance and that downregulation of miR-27a modulated MDR in cancer cells. Taken together, miR-27a is able to potentially participate in the establishment of a drug-resistant network of perifosine in gastric cancer. Co-targeting the miR-27a might provide a new therapy to increase chemotherapy sensitivity, with the possible mechanism being that the downregulation of miR-27a was capable of significantly decreasing the expression of P-glycoprotein, which functioned as an ATP-dependent drug-efflux pump (15).

In conclusion, miR-27a is an oncogene in gastric cancer whose downregulation has the potential to inhibit the growth of MGC-803 cells *in vitro*. Moreover, a high miR-27a expression has been associated with poor tumor histological grade in gastric cancer patients. Furthermore, our study has demonstrated for the first time that perifosine exerts growth inhibitory activities in gastric cancer cells *in vitro*, and that the downregulation of miR-27a may significantly increase the growth inhibitory effect of perifosine. Therefore, miR-27a may be a therapeutic target and potential prognostic biological marker in gastric cancer. MiR-27a inhibitors alone or in combination with perifosine may be a novel therapeutic approach against gastric cancer. However, more investigations should be conduced to clarify the mechanism underlying the correlation between the miR-27a and tumor chemoprevention of perifosine.

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