ORIGINAL ARTICLE

AEG-1 Is a Target of Perifosine and Is Over-Expressed in Gastric Dysplasia and Cancers

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Abstract

Background Perifosine, an alkylphospholipid, is an Akt inhibitor which inhibits the growth of diverse cancer cells. We have reported its inhibitory effects on the growth of gastric cancer cells recently, but its molecular mechanisms are still largely unknown.

Aims The purpose of this study was to investigate the effect and regulatory mechanism of perifosine in gastric cancer.

Methods Cell viability was determined by sulforhodamine B assay after transiently transfected with AEG-1 specific siRNAs. qRT-PCR and western blot assay were used to determine the mRNA expression and proteins levels of cell signaling molecules examined. Immunohistochemistry was used to detect the AEG-1 expression in 87 gastric carcinomas, 60 dysplasia, and 47 normal gastric mucosa.

Results Perifosine decreased AEG-1 gene expression along with inhibition of Akt/GSK3β/C-MYC signaling pathway. Knockdown of AEG-1 using siRNA led to significant down-regulation of cyclin D1 expression at both mRNA level and protein level, and inhibited the growth of gastric cancer cells. AEG-1 expression was elevated in gastric dysplasia and cancer tissues compared to normal gastric mucosa ($P < 0.01$). AEG-1 over-expression correlated with diffuse type of gastric cancer and advanced tumor stages.

Conclusions Perifosine inhibits the growth of gastric cancer cells possibly through inhibition of the Akt/GSK3β/C-MYC signaling pathway—mediated down-regulation of AEG-1 that subsequently down-regulated cyclin D1. AEG-1 may play an important role in the carcinogenesis and progression of gastric cancer and could be a therapeutic target of perifosine.

Introduction

Gastric cancer remains the fourth most common cancer in the world in 2008 [1]. It is the third leading cause of cancer death in men and the fifth leading cause in women [1]. In recent years, the morbidity and mortality from gastric cancer have decreased worldwide, however, the relative five-year survival rate remains low due to late diagnosis at advanced stages and lack of effective therapy [2, 3]. Understanding the molecular mechanisms is the key for early diagnosis and treatment of gastric cancer. Therefore, it is urgently needed for identifying the key molecules that play important roles in gastric cancer development and progression.

Perifosine is an oral Akt inhibitor [4]. It has been shown to inhibit the MAPK pathway, induce c-Jun NH$_2$-terminal kinase (JNK), and up-regulate death receptor 5 (DR5)

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[5–7]. As an alkylphospholipid, it perturbs the cell membrane and induces apoptosis, cell cycle arrest and autophagy [8]. Perifosine can also enhance the effects of histone deacetylase inhibitor, 3-Phosphoinositide-dependent protein kinase-1 (PDK1) inhibitor, epithelial growth factor receptor–tyrosine kinase inhibitors (EGFR-TKI), and some chemotherapeutic reagents [9–12]. However, the mechanisms of perifosine on cancer cells have not been fully elucidated.

Astrocyte elevated gene-1 (AEG-1) was originally identified as an inducible gene of human immunodeficiency virus (HIV)-1 in primary human fetal astrocytes in 2002 [13]. It has been reported to play an important role in many cellular processes, such as proliferation, apoptosis, differentiation, and invasion. AEG-1 has been shown to induce cell survival through activating PI3K/Akt signaling [14] and seems to be a downstream target of C-MYC. However, the relationship between perifosine and AEG-1 has not been studied.

In this study, we studied the effect of perifosine on gastric cancer cells and the relationship between perifosine and AEG-1. We also investigated the expression of AEG-1 in gastric cancers and its precancerous dysplasia.

Materials and Methods

Reagents

Perifosine was purchased from Selleck Chemicals LLC (Houston, TX, USA). It was dissolved in PBS at a concentration of 50 mmol/L, and stored at −20 °C. Stock solution was diluted to the appropriate concentrations with growth medium immediately before use. Lipofectamine 2000 transfection reagent was purchased from Life Technologies Co. Invitrogen (Carlsbad, CA, USA). AEG-1 and cyclin D1 antibodies were purchased from Zhongshan Goldenbridge Biotechnology Co., Ltd. (Beijing, China). p-Akt (Ser473), p-GSK3β (Ser9), Akt, and GSK3β antibodies were purchased from Cell Signaling Technology, Inc. (Beverly, MA). C-MYC antibody was purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). GAPDH antibody was purchased from Bioworld Technology Inc. (Louis Park, MN).

Human Tissue Samples

Specimens used in this study consisted of 87 cases of gastric adenocarcinoma, 30 cases of low-grade dysplasia, 30 cases of high-grade dysplasia, and 47 cases of normal gastric mucosa that were surgically resected at the Nanjing Medical University Affiliated Nanjing Hospital between July 2010 and Feb 2012. Median age of 87 cases of gastric

adenocarcinoma was 48.3 years (32–79 years), and 57 % of the patients were male. Tumors were histologically classified according to Lauren classification and tumor stages were classified according to the American Joint Committee on Cancer (2010 version). None of the patients received preoperative treatment such as chemotherapy or radiotherapy.

Cell Lines and Cell Culture

The human gastric epithelial cell lines (GES-1) and gastric cancer cell lines (SGC7901, MGC803, MKN45, AGS) were obtained from the Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, China. The cells were cultured in RPMI-1640 medium supplemented with 10 % fetal bovine serum (Gibco BRL, Grand Island, NY) at 37 °C in a humidified atmosphere consisting of 5 % CO_2.

Growth Curve of Tumor Cells

Cells after transfection with siRNA for 24 h were seeded in 96-well plates at 1500/well density and plates were fixed with 10 % (w/v) trichloroacetic acid at 4 °C each day for 5 days. Cell number was determined by the sulforhodamine B (SRB) assay as previously described [15, 16].

Western Blot Analysis

Radio-immunoprecipitation assay (RIPA) lysis buffer was purchased from Cell Signaling Technology, Inc. (Beverly, MA) and was stored at 4 °C. Protease inhibitors were added just before use. Cells were harvested at the end of treatment and subjected for western blotting assay as described previously [16].

Gene Silencing by siRNA

Control (non-target) small interfering RNA (siRNA) was purchased from Invitrogen. AEG-1 siRNAs that target 5′- AAC AGA AGA AGA AGA ACC GGA -3′ were described previously and synthesized by Invitrogen [14]. Cells in the exponential phase of growth were plated in 6-well plates at 5×10^5 cells/well and transfected with AEG-1 siRNA or control siRNA with a final concentration of 100 nmol/L for 48 h using Lipofectamine™ 2000 Reagent (Invitrogen Life Technologies Inc., Carlsbad, CA), according to the manufacturer's protocol. Then the total RNAs and whole-cell lysates were prepared and subjected for qRT-PCR assay and Western blotting assay, respectively. To determine the cell number, cells after transfection for 24 h were reseeded into 96-well plates and subjected for a 5-day SRB assay.

RNA Isolation and Quantitative RT-PCR

Total RNA from cells was extracted using Trizol reagent (Gibco Carlsbad, CA, USA) according to the manufacturer's protocol. The reverse transcription was conducted according to standard procedure using M-MLV reverse transcriptase from Promega (Madison, WI, USA). Quantitative PCR (qRT-PCR) reactions were conducted using Power SYBR Green PCR Master Mix from Applied Biosystems, Inc. (Foster, CA, USA), according to manufacturer's instructions. Forward (F) and reverse (R) primers were used as follows: AEG-1, F: 5′- ACGACCTGGCCTT GCTGAAGAATCT -3′ and R: 5′- CGGTTGTAAGTTGC TCGGTGGTAA -3′; cyclin D1, F: 5′- ACAAACAGATCA TCCGCAAACAC -3′ and R: 5′- TGTTGGGGCTCCT CAGGTTC -3′; GAPDH, F: 5′- ATGGGGAAGGTGAAGGT CG -3′, and R: 5′- GGGGTCATTGATGGCAACAATA -3′, and synthesized by Invitrogen [14, 17, 18]. All real-time amplifications were performed with the ABI Prism 7300 sequence detection system in triplicate (Applied Biosystems, Inc.). The fold-change of the target genes were calculated using the $2^{-\Delta\Delta CT}$ method.

Immunihistochemistry

All tissues had been fixed in 10 % neutral-buffered formalin and embedded in paraffin using standard surgical pathology protocols. Immunohistochemical staining for AEG-1 (polyclonal rabbit anti-AEG-1, 1:100 dilution, Abcam Corportation, USA) was done using an EnVsion procedure after autoclave-based antigen retrieval. Sections were incubated for 5 min in 3 % hydrogen peroxide to abolish endogenous peroxidase activity. Immunostaining was performed on a BenchMark XT (Ventana Medical System, Inc., Tucson, AZ) according to the manufacturer's protocol. Staining was performed with diaminobenzidine and countstaining with hematoxylin. Negative control staining was carried out by substituting PBS for the primary antibody. A tissue section of breast cancer was used as a positive control. All immunohistochemical staining results were evaluated semiquantitatively. The staining intensity for AEG-1 was graded as 0 (negative), 1 (pale yellow), 2 (yellow), and 3 (dark yellow). The frequency (number of tumor cells stained) was scored as 0 (negative), 1 (<10 %), 2 (10–50 %), and 3 (>50 %). The staining index was calculated as staining intensity score multiplied by frequency of positive tumor cells. AEG-1 expression was divided into high expression (≥4) and low expression (<4) according to the staining index score.

Statistical Analysis

All data was represented as the mean ± SD from three separate experiments. Statistical analysis was performed using the two-sided unpaired Student's t tests. The correlation between the positive expression of AEG-1 and clinicopathological parameters was analyzed using the χ^2 test or the Fisher exact test. Differences were considered statistically significant at $P < 0.05$. The IC_{50} value of perifosine was calculated by Bliss software.

Results

Perifosine Inhibited AEG-1/Cyclin D1 Signaling Pathway Along with the Down-Regulation of Akt and C-MYC Signaling

We recently reported that perifosine inhibited the growth of gastric cancer cells, but its mechanisms were still elusive [19, 20]. Since perifosine is a well-known Akt inhibitor, we explored its effects on the Akt/GSK3β/C-MYC signaling pathway. As expected, perifosine decreased p-Akt (Ser 473), p-GSK3β (Ser 9), and C-MYC levels in SGC7901 and MGC803 cells (Fig. 1). Interestingly, the expression of AEG-1 and cyclin D1 was also inhibited by perifosine along with other Akt pathway molecules. These results suggest that AEG-1 is also a target of perifosine in gastric cancer and is probably regulated through inhibition of the Akt/GSK3β/C-MYC signaling pathway.

To confirm this hypothesis, we examined AEG-1 mRNA expression by qRT-PCR assay after perifosine treatment for 24, 48, and 72 h. As shown in Fig. 2, perifosine treatment
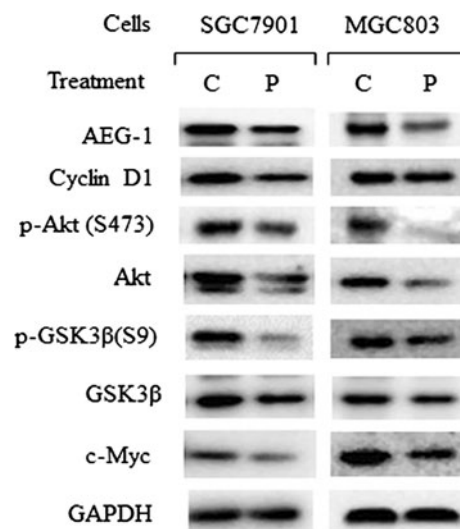

Fig. 1 Perifosine inhibited Akt/GSK3β/C-MYC signaling pathway and AEG-1protein. SGC7901 and MGC803 cells were treated with 0.75 or 10 μmol/L perifosine for 48 h, respectively, and whole-cell protein lysates were purified and subjected for western blot analysis. Western blotting assay was done using specific antibodies against the proteins indicated. GAPDH was used as loading control. *C* control, *P* perifosine treatment

significantly decreased AEG-1 expression in SGC7901 cells in both a time-dependent manner (a) and a dose-dependent manner (b). At the same time, we observed a decrease of cyclin D1 transcript by perifosine in a similar pattern as AEG-1 (Fig. 2c, d). Western blotting assay confirmed the down-regulation of AEG-1 and cyclin D1 expression at the protein level at different time points and doses of perifosine treatments (Fig. 2e, f). These results suggest that perifosine down-regulates AEG-1 and cyclin D1 expression in gastric cancer cells through transcriptional regulation.

Cyclin D1, but Not C-MYC, Is Regulated by AEG-1 in Gastric Cancer Cells

To explore the relationship among AEG-1, cyclin D1 and C-MYC, we knocked down AEG-1 expression by using AEG-1 specific targeting siRNA. As shown in Fig. 3, AEG-1 siRNA significantly down-regulated AEG-1 expression in SGC7901 and MGC803 cells (a, b). Interestingly, we observed significant down-regulation of cyclin D1 expression at both transcription level (c, d) and protein level (g) upon knockdown of AEG-1. However, the mRNA (e, f) and protein (g) levels of C-MYC were not decreased after AEG-1 knockdown, rather the mRNA of C-MYC increased in MGC803 cells (f) after

AEG-1 knockdown ($P < 0.05$). These results indicate that cyclin D1 may be a downstream target of AEG-1. However, C-MYC is not a downstream target of AEG-1, rather an upstream regulator of AEG-1 in gastric cancer cells examined.

Down-Regulation of AEG-1 Expression Inhibits the Growth of Gastric Cancer Cells

To explore AEG-1's function in gastric cancer, we transiently transfected control siRNAs or AEG-1 specific siRNAs into SGC7901 and MGC803 cells, and the cell number was determined by a 5-day SRB assay. Our results showed that knockdown of AEG-1 expression inhibited the growth of tumor cells (Fig. 4).

AEG-1 Expression Was Elevated in Gastric Dysplasia and Cancers

We examined AEG-1 expression levels in the gastric cancer cell lines, SGC7901, MGC803, AGS, and MKN45, as well as in human gastric epithelial cell line, GES-1. AEG-1 mRNA expression was higher in all the gastric cancer cells examined than in GES-1 cells ($P < 0.05$) (Fig. 5a), and AEG-1 protein expression was over-expressed in 3 of 4 gastric cancer cell

Fig. 2 Perifosine decreased the expression of AEG-1 and cyclin D1 in gastric cancer cells. SGC7901 cells were treated with 0.75 μmol/L perifosine for different times as indicated (a, c) or with different concentrations of perifosine as indicated for 48 h (b, d). Total RNA was extracted and subjected to qRT-PCR assay to detect the mRNA expression of AEG-1 and cylcin D1. Data are expressed as mean ± SD of three replicate determinations; * $P < 0.05$. e, f SGC7901 cells were treated with 0.75 μmol/L perifosine for different times as indicated or treated with different concentrations of perifosine as indicated for 24 h. Whole-cell protein lysates were purified and subjected to western blot analysis. C control, P perifosine treatment
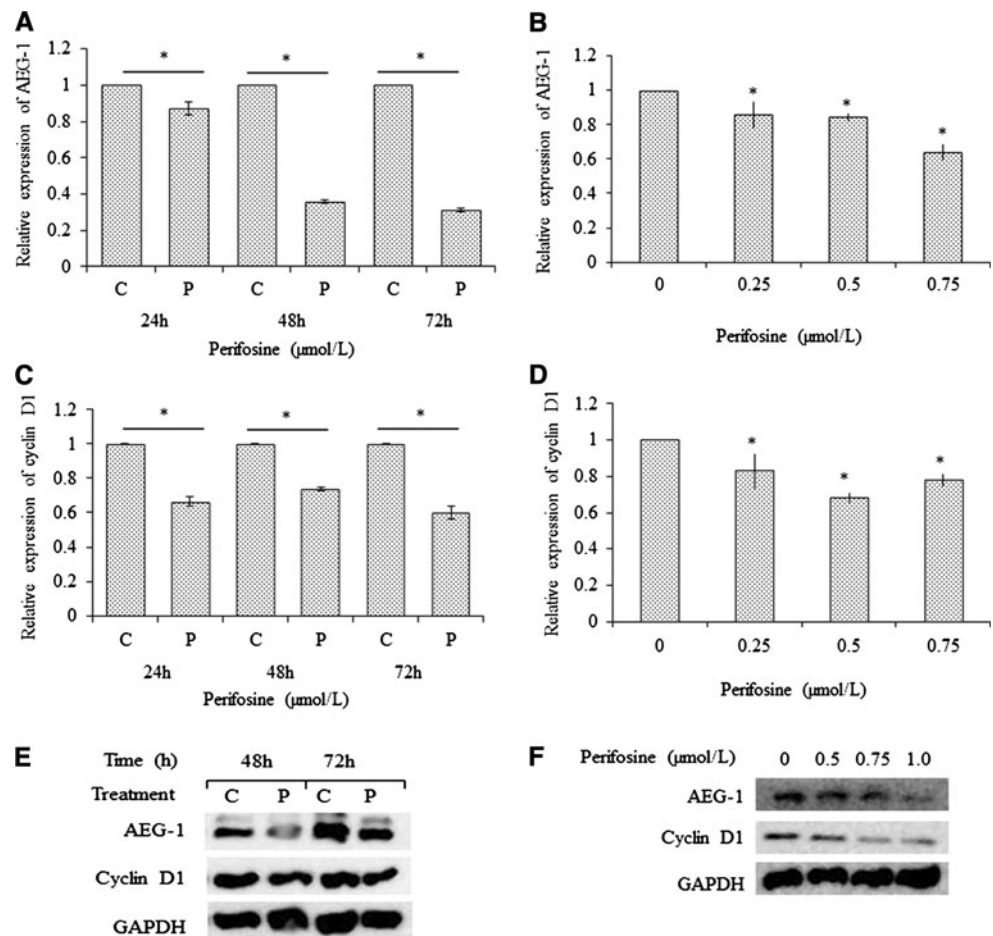
Fig. 3 Knockdown of AEG-1 inhibited expression of cyclin D1 but not C-MYC in gastric cancer cells. SGC7901 and MGC-803 cells were transfected with 100 nmol/L AEG-1 siRNA or control siRNA for 24 h; cells were seeded into 6-well plates. qRT-PCR assay was performed to detect mRNA expression of AEG-1 (**a**, **b**), cyclin D1 (**c**, **d**), and C-MYC (**e**, **f**). Data are expressed as mean ± SD of three replicate determinations; * $P < 0.05$. **g** Whole-cell protein lysates were purified and western blot analysis was performed to detect AEG-1, cyclin D1, and C-MYC protein expression. GAPDH was used as loading controls. *Ctrl* control siRNA, *AEG-1* AEG-1 siRNA
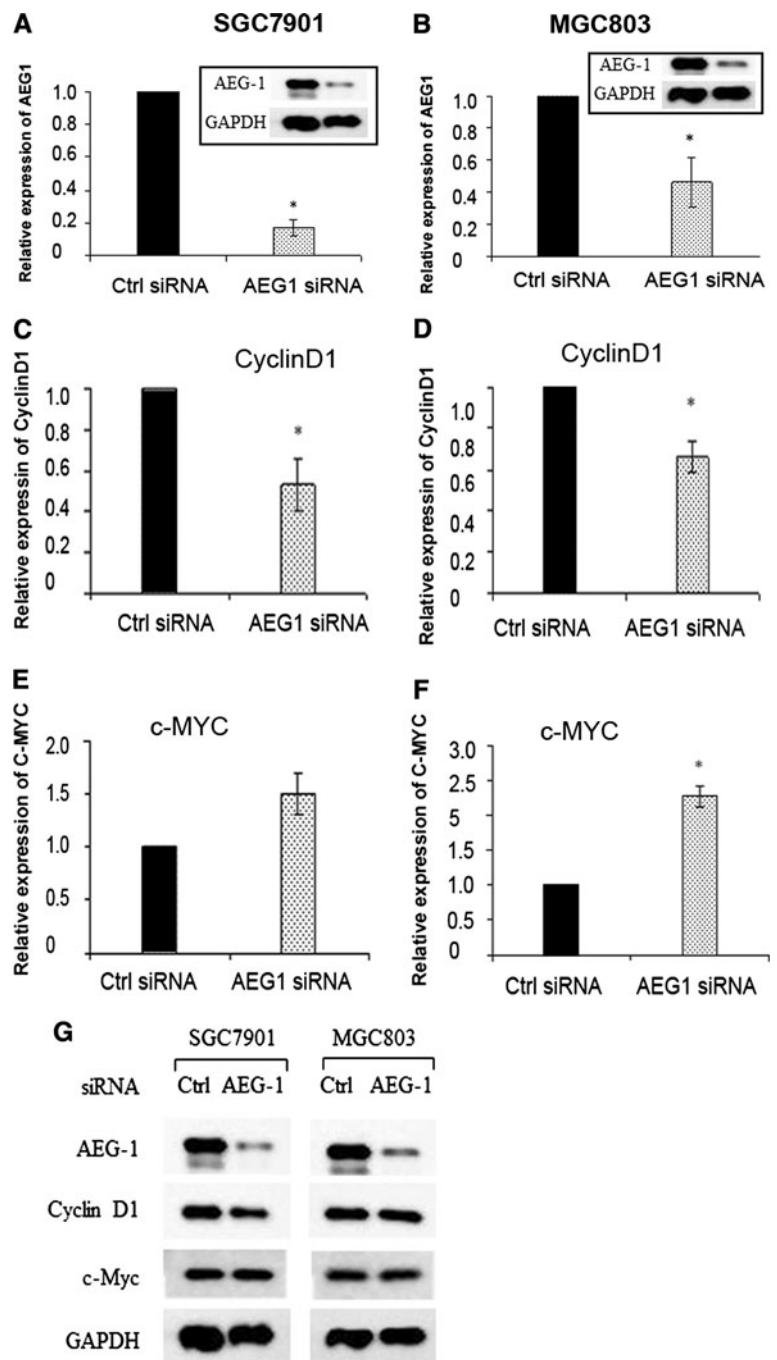

Fig. 4 Knockdown of AEG-1 inhibited the growth of gastric cancer cells. SGC7901 and MGC-803 cells were transfected with 100 nmol/L AEG-1 siRNA or control siRNA for 24 h; cells were seeded into 96-well plates. Sulforhodamine B assay was used to determine cell numbers from day 1 to day 5. Data are expressed as mean ± SD of three replicate determinations
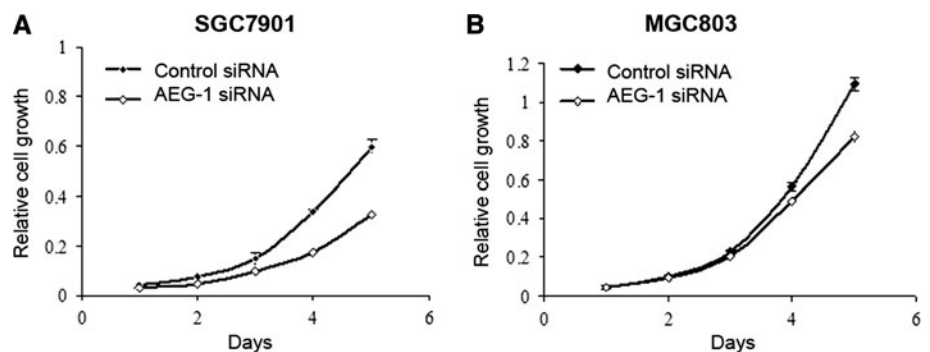
Fig. 5 AEG-1 expression was elevated in both human gastric cancer tissues and cancer cell lines. **a** Total-mRNA were purified from GES-1, SGC7901, MGC803, AGS, and MKN45 cells after 24 h culturing, and then subjected to qRT-PCR assay. Relative AEG-1 expression was determined by $2^{-\Delta\Delta CT}$ method. Data are expressed as mean ± SD of three replicate determinations; * $P < 0.05$. **b** Whole-cell protein lysates were purified and western blot analysis was used to detect AEG-1 protein expression. **c–f** Immunohistochemical staining of AEG-1 in normal gastric mucosa, dysplasia and gastric adenocarcinoma. **c** Weak staining in a normal gastric mucosa. **d** Strong staining in a low-grade dysplasia. **e** Strong staining in a high-grade dysplasia. **f** Strong staining in a gastric adenocarcinoma. Representatives of IHC staining. Magnification, ×200
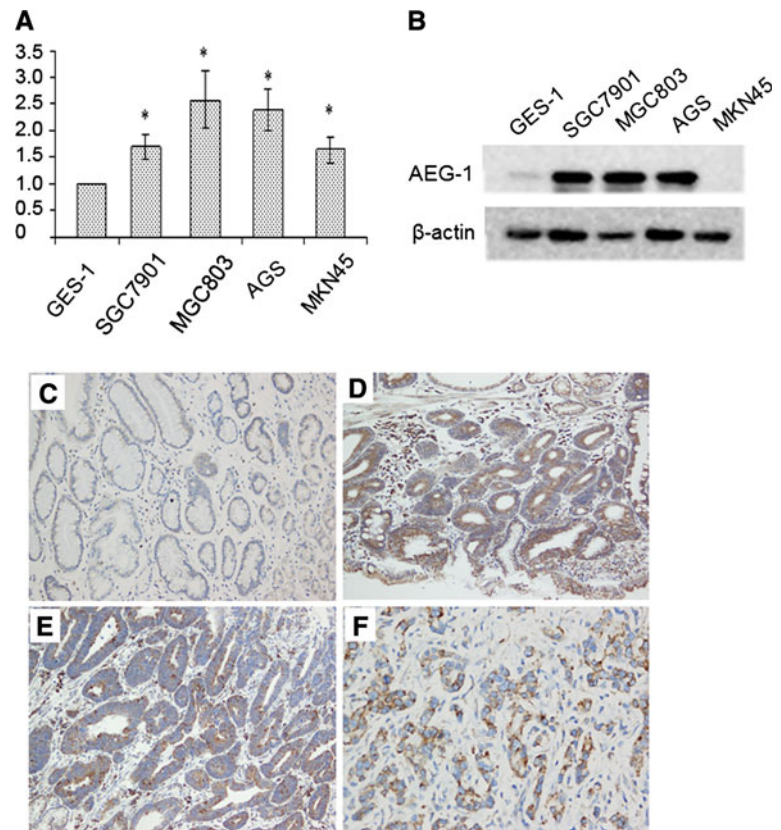
lines (SGC7901, MGC803 and AGS cells), as compared with that in GES-1 cells (b).

To confirm the above results, we further examined AEG-1 expression in gastric cancers and precancerous lesions by immunohistochemistry. The representative immunohistochemistry staining images are shown in Fig. 5c–f. Table 1 summarizes the AEG-1 expression in gastric cancer and precancerous lesions. AEG-1 expression was higher in gastric cancer tissues and precancerous lesions (low grade and high grade dysplasia) than that in normal mucosa ($P = 0.000$). However, no significant differences was seen among cancers, low-grade dysplasia and high-grade dysplasia ($P > 0.05$). Table 2 summarizes the correlation between AEG-1 expression and clinical pathological parameters in gastric carcinoma. AEG-1 high expression significantly correlated with Lauren classification ($P = 0.027$), T classification ($P = 0.001$), N classification ($P = 0.002$), and pTNM staging ($P = 0.043$). Overexpression of AEG-1 was more frequent in diffuse types of cancer than in intestinal and mixed types of cancers, and in more advanced tumors.

Discussion

The Akt inhibitor, perifosine, is an oral alkylphospholipid, which exhibits antitumor activity both in preclinical and clinical studies. It showed an inhibition of tumor growth and induced apoptosis in a variety of types of cancers, such as lung cancer, head and neck cancer, prostate cancer, multiple myeloma, and leukemia [21]. It has been tested in clinical trials both as a single agent or in combination with other anticancer reagents [21]. We recently reported that it inhibits the growth of gastric cancer cells [19, 20]. To date, its mechanisms on gastric cancer are still unclear. In this study, we observed that perifosine inhibited the growth of gastric cancer cells. Administration of perifosine in gastric cancers inhibited expression levels of p-AKT, p-GSK3β, cyclin D1, C-MYC and AEG-1. Furthermore, we demonstrated that cyclin D1 (but not C-MYC) is a downstream target of AEG-1 that is over-expressed in gastric cancer cell lines, gastric dysplasia and primary cancer tissues.

Unlike other kinase inhibitors which compete for the ATP binding sites, perifosine inhibits Akt through targeting its pleckstrin homology (PH) domain and interferes with its recruitment to the plasma membrane and subsequent phosphoylation and activation [22]. In addition to Akt, perifosine also regulates MAPK, JNK and DR5 [5–7]. Furthermore, perifosine interferes with the structure, metabolism, and permeability of the cell membranes [21]. Currently, the mechanism of perifosine has not been fully elucidated. In the present study, for the first time, we found that perifosine down-regulated AEG-1 expression. We also observed that it inhibited the Akt/GSK3β/C-MYC

Table 1 AEG-1 expression in normal mucosa, precancerous lesions, and gastric carcinoma

Group	n	AEG-1 expression	
		High expression	Low expression
Normal mucosa	47	6	41
Low-grade dysplasia	30	18	12
High-grade dysplasia	30	22	8
Cancer	87	53	34

Compared to normal mucosa, low-grade dysplasia, $\chi^2 = 19.043$, $P = 0.000$; high-grade dysplasia, $\chi^2 = 29.029$, $P = 0.000$; cancer, $\chi^2 = 28.712$, $P = 0.000$. Cancer versus high-grade dysplasia, $\chi^2 = 1.494$, $P = 0.222$; cancer versus low-grade dysplasia, $\chi^2 = 0.008$, $P = 0.929$; low-grade versus high-grade dysplasia, $\chi^2 = 1.200$, $P = 0.273$

pathway. Even though C-MYC has been suggested to be located both upstream and downstream of AEG-1 [14, 23], our findings that knockdown of AEG-1 does not lead to a decrease of C-MYC levels suggest that C-MYC is not a direct downstream target of AEG-1 in the gastric cancer cell lines we examined. On the other hand, it may locate at upstream of AEG-1 and be negatively regulated by AEG-1, because knockdown of AEG-1 led to an increase of C-MYC transcription. Interestingly, we found that cyclin D1 is significantly down-regulated after AEG-1 knock-down, indicating that cyclin D1 is a downstream target of AEG-1. Cyclin D1 is a major regulator of cell cycle progression and our findings suggest that the inhibition of tumor cell growth observed in perifosine treatment may be through down-regulation of the AEG-1-cyclin D1 pathway. We also showed that perifosine decreased the phosphorylation of GSK3β at serine 9, an indicator of inhibition of GSK3β. These findings indicate that a GSK3β-involved, ubiquitination-mediated protein degradation mechanism may contribute to perifosine's effects on AEG-1 [24]. More studies are needed to fully elucidate the regulatory mechanisms of perifosine on AEG-1.

Overexpression of AEG-1 has been reported in liver cancer, breast cancer, prostate cancer, lung cancer, and melanoma. In this study, we found that AEG-1 expression was significantly higher in low-grade dysplasia, high-grade dysplasia and gastric cancers than that in normal gastric tissues, but no differences among low-grade dysplasia, high-grade dysplasia and gastric cancers. These results suggest that over-expression of AEG-1 is an early event and may be involved in early carcinogenesis of stomach. Our results are similar to the report of Xu et al. [25]. In line with these findings, we also found that manipulating AEG-1 expression by siRNA inhibited the growth of gastric cancer cells, supporting that it may be an oncogene in gastric cancer.

Table 2 Correlation between expression of AEG-1 and clinicopathologic parameters of gastric carcinoma

Clinicopathologic parameters	n	AEG-1 expression		χ^2	P value
		High expression	Low expression		
Normal mucosa	47	6	41	28.712	0.000
Cancer	87	53	34		
Sex					
Male	50	31	19	0.058	0.810
Female	37	22	15		
Age					
>55	59	39	20	2.068	0.150
≤55	28	14	14		
Location					
Cardia	23	14	9		
Body	23	13	10	0.294	0.863
Antrum	41	26	15		
Lauren classification					
Intestine	19	9	10		
Mixed	35	18	17	7.215	0.027
Diffuse	33	26	7		
T stage					
T1	7	1	6		
T2	20	8	12	15.448	0.001
T3	45	31	14		
T4	15	13	2		
N stage					
N0	33	12	21		
N1	12	8	4	14.534	0.002
N2	33	25	8		
N3	9	8	1		
pTNM stage					
I	14	6	8		
II	18	8	10	8.147	0.043
III	45	30	15		
IV	10	9	1		

In summary, perifosine inhibited the growth of gastric cancer cells, likely through down-regualtion of AEG-1-cyclin D1 expression. AEG-1 was elevated both in the gastric cancer tissues and in some gastric cancer cell lines, and down-regulation of AEG-1 expression inhibited the growth of gastric cancer cells. Our results suggest that AEG-1 may be important in gastric cancer development and progression and could be a therapeutic target by small molecules like perifosine.

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