ORIGINAL ARTICLES

Upregulation of a disintegrin and metalloprotease 8 is associated with progression and prognosis of patients with gastric cancer

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A disintegrin and metalloprotease 8 (ADAM8) is involved in the tumorigenesis of several types of solid tumors. However, its exact role in gastric cancer (GC) remains unclear. The aim of this study was to evaluate the clinical significance of ADAM8 in GC and to explore its biological effects on gastric carcinogenesis. In this study, quantitative reverse transcription–polymerase chain reaction, Western blotting, and immunohistochemical staining analysis revealed that ADAM8 messenger RNA expression was significantly upregulated in GC tissues compared with noncancerous tissues ($P = 0.004$), and that positive ADAM8 expression is much more common in tumor tissues compared with normal tissues ($P < 0.001$) and is correlated with T stage ($P = 0.036$), N stage ($P = 0.048$), vessel invasion ($P = 0.002$), and a shorter patient overall survival ($P = 0.024$). In vitro assay indicated that ADAM8 overexpression promoted cell growth and increased migration and invasion abilities by decreasing the p-p38/p-extracellular regulated protein kinases (p-ERK) ratio. In conclusion, ADAM8 promotes GC cell proliferation and invasion, and its expression is positively correlated with poor survival, indicating that it might be a promising target in GC therapy. (Translational Research 2015;166:602–613)

Abbreviations: ADAM8 = a disintegrin and metalloprotease 8; GC = gastric cancer; OS = overall survival

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AT A GLANCE COMMENTARY

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Background

A disintegrin and metalloprotease 8 (ADAM8) has been involved in the tumorgenesis of several types of solid tumor. Meanwhile, ADAM8 can be acted as an independent factor for lymph node metastasis in gastric cancer (GC). However, its exact role in GC remains unclear.

Translational Significance

ADAM8 promotes GC cells proliferation and invasion, and correlates with poor survival of patients with GC, indicating that ADAM8 might be a promising target in GC therapy.

INTRODUCTION

Gastric cancer (GC) is the fourth most common alimentary tract malignancy and the second leading cause of cancer-related deaths after lung cancer.[1] Although the incidence of GC has been declining, it is estimated that approximately 400,000 new cases are diagnosed in China annually, comprising approximately 43% of the total cases worldwide.[2] Most GC patients are diagnosed at an advanced stage, with 50%–75% presenting with regional lymph node metastasis.[3] Lymph node status determines the tumor node metastasis (TNM) stage and affects the postoperative prognosis of GC patients. Although preoperative examinations, such as computerized tomography and magnetic resonance imaging, can predict lymph node status, they are not reliable indicators of lymph node metastasis.[4,5] Therefore, there is great interest in identifying biomarkers to predict regional and or distant metastasis. Several genes seem to contribute to lymph node metastasis, including cell proliferation, cell to cell interactions, and cell invasion and migration.[6-9] Of them, members of a disintegrin and metalloproteinases (ADAMs) family are involved in invasion and metastasis in GC.[10-12]

ADAM8, a member of the ADAMs family, was initially reported to play potential roles in inflammatory and allergic processes,[13] and further studies have shown that its overexpression is associated with progression and poor survival in various solid tumors.[12,14,15] ADAM8 has been reported to be an independent indicator of lymph node metastasis in human GC[12]; however, its precise effects on GC progression and prognosis remain unclear. In this study, we focused on the correlation of ADAM8 expression with clinicopathologic features and overall survival (OS); in addition, the mechanisms implicating this protein in GC progression were studied at the molecular level by in vitro assays.

MATERIALS AND METHODS

Patients and tissue samples. Two hundred three consecutive patients undergoing gastrectomy for GC at the Sixth Affiliated Hospital of Sun Yat-Sen University, China, from January 2007 to December 2008 were included in this study. These patients had not received preoperative chemotherapy or radiotherapy. All patients were diagnosed by a clinician, and the diagnosis was confirmed by a pathologist. Tissue samples, including tumor and nontumor tissue samples, were obtained from the resected specimens and were snap-frozen in liquid nitrogen and stored at −80°C until use.

This study was carried out according to the Code of Ethics of the World Medical Association (Declaration of Helsinki) and was approved by the research ethics committee of Sun Yat-Sen University, China. Informed consent was obtained from all patients in this study.

Tissue microarrays. Tissue microarrays (TMAs) were performed to assess 203 GC specimens and 20 normal tissues obtained from the Tissue Bank of the Sixth Affiliated Hospital. The TMAs were constructed using an automated TMA instrument (ALPHELYS, Plaisir, France). The method for constructing the TMAs was similar to a previously described method.[16]

Immunohistochemistry and evaluation of immunohistochemical (IHC) staining. IHC staining was performed on the TMA slides using a Polink-2 plus Polymer Horseradish Peroxidase Detection System (GBI, Bothell, WA, USA) according to the manufacturer's instructions, as described previously.[17]

IHC staining was analyzed using Image Pro-Plus (version 6.0; Media Cybernetics, Silver Spring). Briefly, the tumor area was selected as the area of interest (AOI), and the area sum and integrated optical density of the AOI were selected as the measurement parameters. The ADAM8 expression index equaled the quotient between the integrated optical density and the total AOI. Finally, statistical analysis of the mean expression index for each duplicate was performed. Receiver operating characteristic curve analysis was conducted to select cutoff scores for ADAM8. The cutoff value was determined using the log-rank test with respect to OS. The expression of ADAM8 was considered negative if the cutoff value was 4.2 or less and positive if the value was more than 4.2.

Cell culture and establishment of stably transfected cell lines. Five GC cell lines (SGC7901, AGS, MGC803, BGC823, and MKN45) and an immortalized gastric

mucosa cell line (GES1) were obtained from the Culture Collection of the Chinese Academy of Science (Shanghai, China). These cells were cultured in the recommended medium supplemented with 10% fetal bovine serum and incubated in 5% CO_2 at 37°C.

BGC823 and AGS cells, both of which have a low level of endogenous ADAM8 expression, were transfected with an ADAM8 overexpression plasmid (pCDNA3.1(+)-ADAM8) or an empty vector (pCDNA3.1(+)) using Lipofectamine 2000 (Invitrogen, Carlsbad, California) according to the manufacturer's protocols. Transfected BGC823 and AGS cells were selected with 600 and 800 μg/mL G418 (Invitrogen), respectively. Transfectants with ADAM8 overexpression or empty vector were confirmed by Western blotting for use in assessment of its expression status.

RNA interference. Small interfering RNA (siRNA) oligonucleotides specific to ADAM8 and siRNA control oligonucleotides were obtained from RiboBio Co Ltd (Guangzhou, China). MKN45 cells (1×10^5) were cultured in 6-well plates until 50% confluence was reached, and then they were transfected with 100 nM of the indicated siRNA using Lipofectamine Imax (Invitrogen) according to the manufacturer's instructions. The effects of ADAM8 knockdown were analyzed at 48 hours after transfection.

RNA isolation and quantitative real-time polymerase chain reaction. Total RNA was isolated from GC cell lines and tumor and normal tissues using an RNeasy Mini Kit (Qiagen, Valencia, California) according to the manufacturer's instructions. RNA was subsequently reverse transcribed to generate complementary DNA (cDNA) with a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, California). Quantitative reverse transcription–polymerase chain reaction (qRT-PCR) was performed to assess ADAM8 and β-actin expression with a SYBR Green Master Mix Kit and ABI 7500 System (Applied Biosystems). β-Actin levels were used for normalization. The PCR primers used were as follows: ADAM8 (sense: ACAATGCAGAGTTCCAG ATGC, antisense: GGACCACACGGAAGTTGAGTT) and β-actin (sense: CAATGAGCTGCGTGTGGCT, antisense: TAGCACAGCCTGGATAGCAA).

Western blot. Total proteins were extracted from transfected GC cells and control cells. Thirty micrograms of protein were separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to an equilibrated nitrocellulose membrane (Amersham Biosciences, Buckinghamshire, UK). The target proteins were immunoblotted with specific antibodies against ADAM8, cleaved-poly ADP-ribose polymerase, total and phosphorylated ERK/p38/protein kinase B (AKT)/mammalian target of rapamycin (mTOR), and actin, respectively. After incubation with primary antibodies overnight at 4°C and then with species-specific fluorescently conjugated

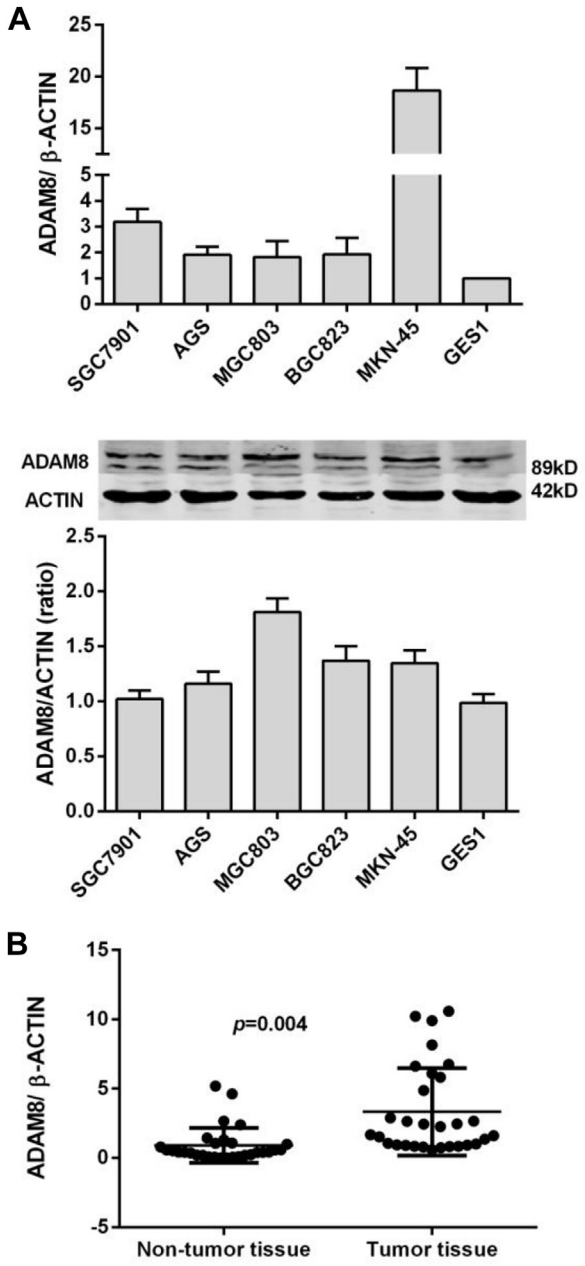

Fig 1. Expression of ADAM8 in gastric cancer. (**A**) Expression of ADAM8 at the mRNA and protein levels in 5 gastric cancer cell lines (SGC7901, AGS, MGC803, BGC823, and MKN45) and 1 immortalized gastric mucosa cell, GES1, were analyzed by qRT-PCR (top panel) and Western blotting (bottom panel), respectively. (**B**) ADAM8 mRNA expression levels in gastric cancer samples (n = 30) paired with adjacent nontumor tissues were also measured by qRT-PCR. The expression levels of ADAM8 mRNA were normalized to that of β-actin mRNA, which served as a control for the input complementary DNA. The data are expressed as the mean ± standard deviation. ADAM8, a disintegrin and metalloprotease 8; mRNA, messenger RNA; qRT-PCR, quantitative reverse transcription–polymerase chain reaction.

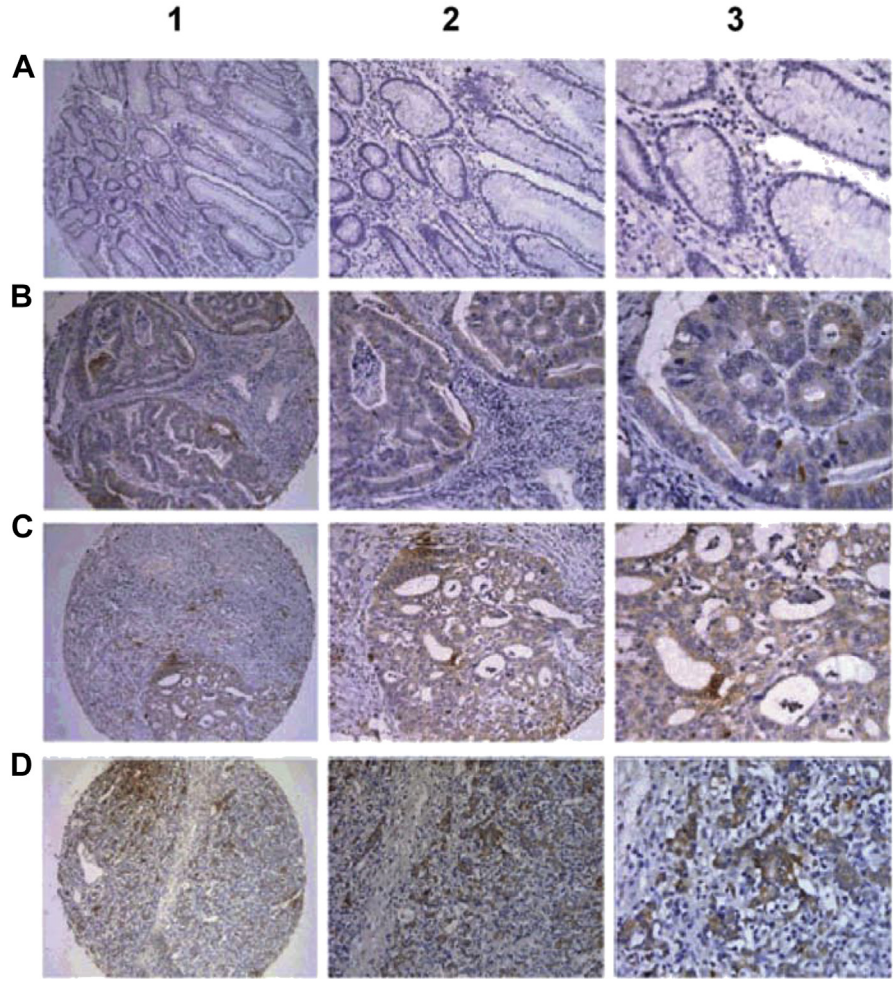

Fig 2. IHC staining of ADAM8 protein in gastric cancer. Specimens of GC tissues and matched corresponding nontumor tissues were stained with an antibody specific to the human ADAM8 protein. Representative IHC images of ADAM8 are shown. (**A**1–3) negative expression of ADAM8 in nontumor tissues and its positive expression in well-differentiated (**B**1–3), moderately differentiated (**C**1–3) and poorly differentiated adenocarcinoma (**D**1–3). Magnification: original magnifications of ×100 (**A**1–**D**1), ×200 (**A**2–**D**2), and ×400 (**A**3–**D**3). ADAM8, a disintegrin and metalloprotease 8; GC, gastric cancer; IHC, immunohistochemical.

secondary antibodies (Santa Cruz Biotechnology, California) for 1 hour at room temperature, the blots were observed using an Odyssey infrared imaging system. The density of each band was quantified by scanning densitometry.

Cell proliferation and viability. Cell viability was examined using a Vybrant 3-(4,5-dimethyl-2-thiazolyl)-2, 5-diphenyl-2-H-tetrazolium bromide (MTT) Cell Proliferation Assay Kit (Invitrogen) according to the manufacturer's instructions. Briefly, transfected and control cells were plated in 96-well plates (2000 cells per well) in triplicate, and cell viability was determined after 24, 48, 72, and 96 hours.

Cell proliferation was detected using a Click-iT 5-Ethynyl-2'-deoxyuridine (EdU) Cell Proliferation Assay Kit (Invitrogen, Camarillo, California) following the manufacturer's instructions. Briefly, cells were cultured in triplicate in 96-well plates at a density of 1×10^4 cells per well for 24 hours. Subsequently, 20 μM EdU labeling solution was added to the treated cells and incubated for 2 hours at 37°C. After incubation at room temperature, the cells were fixed with 3.7% formaldehyde for 15 minutes and treated with 0.5% Triton X-100 for 20 minutes. Then, they were treated with 100 μL Click-iT reaction cocktail for 30 minutes after 2 washes with phosphate-buffered saline. Subsequently, the cells were stained with 100 μL of 1× Hoechst 33342 for 30 minutes, and a digital image was captured using a Leica Digital Microscopy Intelligence (DMI) 4000B inverted microscope (Leica Microsystems, Wetzlar, Germany).

Table I. Relationship between a disintegrin and metalloprotease 8 (ADAM8) expression and clinicopathologic characteristics in gastric cancer (n = 203)

Indicator	ADAM8 expression		*P* value
	Negative (n = 73)	Positive (n = 130)	
Gender			0.459
Male	48 (65.8%)	92 (70.8%)	
Female	25 (34.2%)	38 (29.2%)	
Age			0.228
≤60 y	39 (53.4%)	58 (44.6%)	
>60 y	34 (46.5%)	72 (55.4%)	
Location			0.160
Proximal	12 (16.4%)	14 (10.8%)	
Middle	31 (42.5%)	45 (34.6%)	
Distal	30 (41.1%)	71 (54.6%)	
Tumor size			0.077
<5 cm	42 (57.5%)	58 (44.6%)	
≥5 cm	31 (42.5%)	72 (55.4%)	
Lauren's classification			0.361
Intestinal	38 (52.1%)	59 (45.4%)	
Diffuse	35 (47.9%)	71 (54.6%)	
Histologic type			0.677
Adenocarcinoma	58 (79.5%)	100 (76.9%)	
Mucinous/signet-ring adenocarcinoma	15 (20.5%)	30 (23.1%)	
Differentiation grade			0.253
Well	8 (11.0%)	16 (12.3%)	
Moderately	11 (15.1%)	10 (7.7%)	
Poorly	54 (74.0%)	104 (80.0%)	
T stage			0.036
T1	8 (11.0%)	12 (9.2%)	
T2	12 (16.4%)	8 (6.2%)	
T3	47 (64.4%)	89 (68.5%)	
T4	6 (8.2%)	21 (16.1%)	
N stage			0.048
N0	25 (34.2%)	28 (21.5%)	
N+	48 (65.8%)	102 (78.5%)	
M stage			0.555
M0	65 (89.0%)	112 (86.2%)	
M1	8 (11.0%)	18 (13.8%)	
TNM stage			0.304
I	14 (19.2%)	13 (10.2%)	
II	21 (28.8%)	37 (28.1%)	
III	32 (43.8%)	66 (50.8%)	
IV	6 (8.2%)	14 (10.9%)	
Vessel invasion			0.002
No	46 (63.0%)	52 (40.0%)	
Yes	27 (37.0%)	78 (60.0%)	
C-erbB-2 expression			0.162
Negative	56 (76.7%)	110 (84.6%)	
Positive	17 (23.3%)	20 (15.4%)	

Invasion and migration assay. Cell invasion and migration abilities were assessed with a BioCoat Growth Factor Reduced Matrigel Invasion Chamber and Transwell Migration Chamber (BD Biosciences, Franklin Lakes, New Jersey) according to the manufacturer's protocols. Medium containing 20% fetal bovine serum was used as a chemoattractant. The insert membranes were stained with 4',6-diamidino-2-phenylindole (DAPI), and the permeating cells were counted under an inverted microscope and photographed.

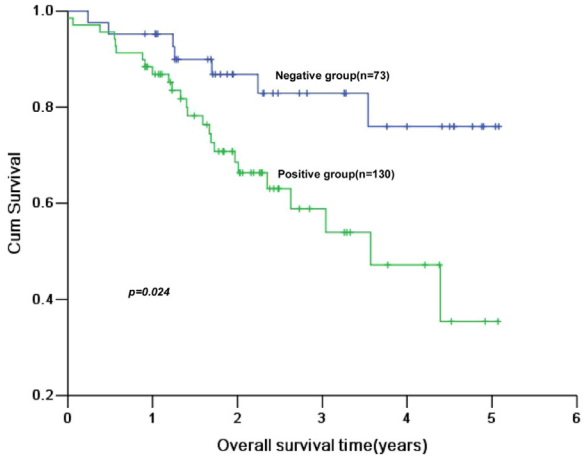

Fig 3. Kaplan-Meier survival curves of gastric cancer patients according to ADAM8 expression levels. The cumulative 5-year survival rate was higher for the patients with negative ADAM8 expression compared with those with positive expression (68% vs 36%, $P = 0.024$). ADAM8, a disintegrin and metalloprotease 8; cum, cumulative.

Cell cycle and apoptosis assay. To assess the effects of ADAM8 on the cell cycle and apoptosis, the transfected and control cells were cultured in 12-well plates at a density of 1×10^6 cells per well. After 48 hours of culturing, the cells were harvested and fixed in 70% ethanol for 24 hours, followed by staining with 50 mg/mL propidium iodide (BD Pharmingen, San Jose, California). They were then sorted by fluorescence activated cell sorting (FACS) Calibur (BD Biosciences), and the cell cycle profiles were analyzed by ModFit 3.0 software (Verity Software House, Topsham, Maine). Apoptosis was determined by dual staining with annexin V-fluorescein isothiocyanate (FITC) and propidium iodide (Invitrogen). Annexin V-positive cells were counted as apoptotic cells.

Statistical analysis. Statistical Product and Service Solutions (SPSS) version 16.0 for Windows (SPSS, Inc, Chicago, Illinois) was used to perform statistical analysis. Continuous variables were presented as the mean ± standard deviation and analyzed by Student's *t* test. Categorical variables were compared with the chi-square test or Fisher's exact test. Survival time was calculated from the date of surgery to the date of death from any cause or the last day of follow-up. Survival analysis was performed using the Kaplan-Meier method and compared using the log-rank test.

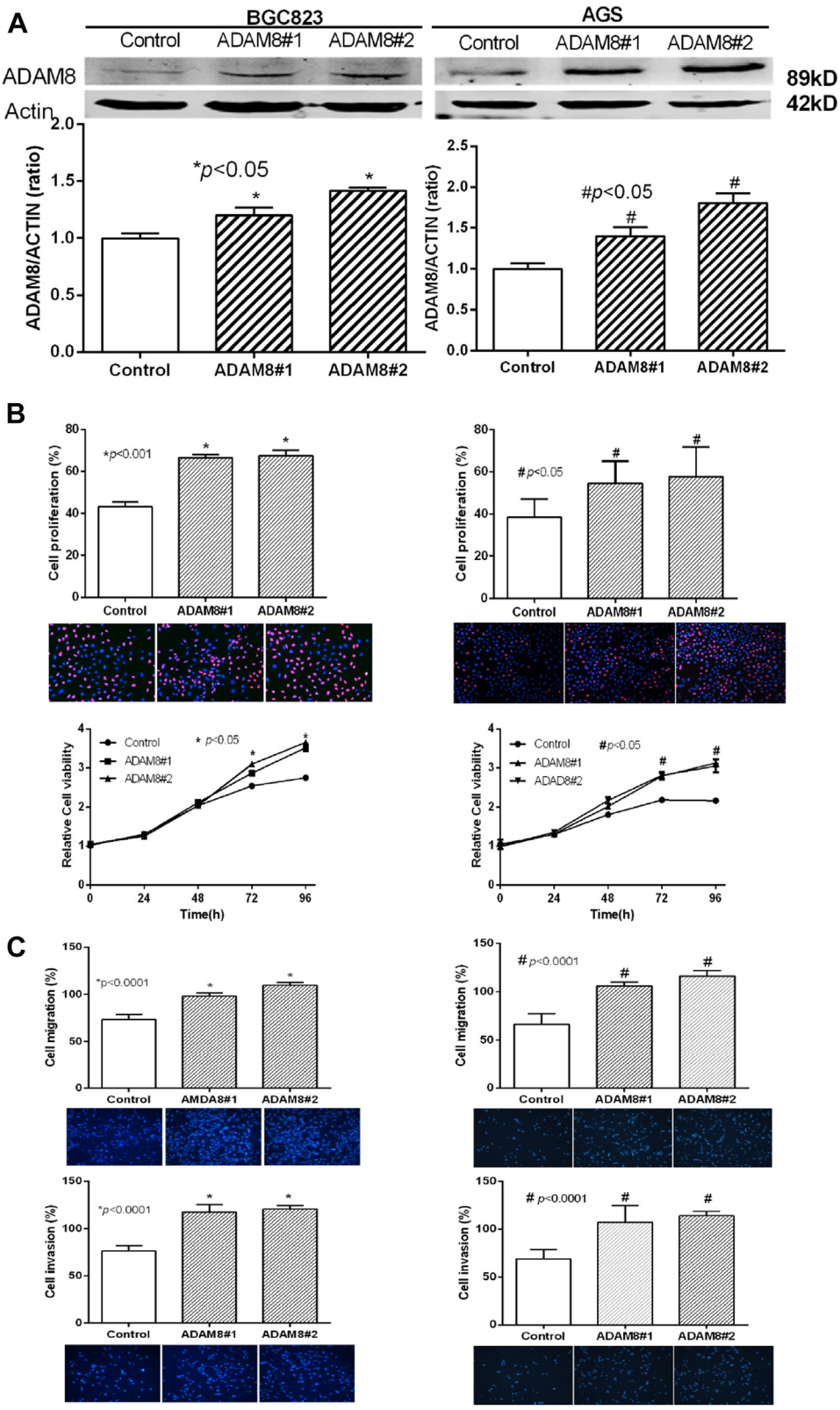

Fig 4. Effect of ectopic ADAM8 expression on proliferation and aggression of gastric cancer cells. (**A**) Expression of ADAM8 protein in MGC823 and AGS cells transfected with ADAM8 mRNA was determined by Western blotting. Compared with control groups, the ADAM8 protein levels increased by 1.4- to 1.8-folds for AGS cells and

The Cox proportional hazards model was used in multivariate survival analysis. A $P < 0.05$ was considered statistically significant.

RESULTS

ADAM8 is upregulated in primary GC. Expression of ADAM8 at the messenger RNA (mRNA) and protein levels in GC tissues (tumor tissues and corresponding adjacent nontumor tissues) and cell lines was analyzed by qRT-PCR and Western blotting, respectively. Compared with the immortalized gastric mucosa cell line GES1, the relative mRNA expression levels in 5 GC cell lines were increased by 1.81 ± 0.78- to 18.67 ± 3.52-fold (Fig 1, *A* top panel). Meanwhile, the ADAM8 protein expression status was also determined in these cells by Western blotting (Fig 1, *A* bottom panel). Furthermore, the ADAM8 mRNA levels were also examined in an independent set of 30 GC samples paired with adjacent corresponding nontumor tissues and were found to be significantly higher in the primary GC tissues compared with the nontumor tissues (2.72 ± 0.56 vs 0.91 ± 0.23, $P = 0.004$). The representative data are shown in Fig 1, *B*. The expression level of ADAM8 mRNA was normalized to that of β-actin mRNA, which served as a control for the input cDNA.

Association of ADAM8 protein expression with clinicopathologic factors of patients with GC. Immunohistochemistry analysis was performed to detect ADAM8 protein expression in 203 GC tissue samples matched with adjacent nontumor tissues. Positive ADAM8 protein expression was detected in 130 (64.0%) of the 203 GC patients, and negative expression was found in 73 (36.0%). In the matched nontumor tissues, negative expression was found in 178 cases (87.7%) and positive expression in 25 (12.3%). The percentage of gastric tumor tissues with a high level of ADAM8 protein expression was much greater than that of the matched nontumor tissues ($P < 0.0001$). ADAM8 was predominantly expressed in the cytoplasm and membranes of tumor cells (Fig 2).

ADAM8 expression in GC was significantly correlated with T stage ($P = 0.036$), N stage ($P = 0.048$), and vessel invasion ($P = 0.002$) and was not associated with other clinicopathologic factors or with C-erbB-2 expression status (Table I). The Spearman correlation coefficients of ADAM8 expression with T stage, N stage, and tumor vessel invasion were 0.145 ($P = 0.039$), 0.139 ($P = 0.048$), and 0.221 ($P = 0.002$), respectively.

Correlation between ADAM8 protein expression and prognosis of GC patients. In the present study, the effect of ADAM8 protein expression status on the OS of GC patients was evaluated. For all patients in this study, the mean survival time was 5.48 ± 0.42 years, and the 3- and 5-year OS rates were 68% and 43%, respectively. The 3- and 5-year OS rates for the patients with positive ADAM8 expression were 52% and 31%, respectively, and they were 82% and 67%, respectively, for those with negative expression. The GC patients with positive ADAM8 expression had a shorter survival time compared with those with negative expression ($P = 0.024$, Fig 3). Univariate analysis indicated that T stage ($P < 0.0001$), N stage ($P < 0.0001$), M stage ($P = 0.003$), TNM stage ($P < 0.0001$), vessel invasion ($P = 0.018$), and ADAM8 expression status ($P = 0.024$) were significantly associated with prognosis, whereas gender, age, location, tumor size, Lauren's classification, histologic type, differentiation grade, and C-erbB-2 expression status were not found to be related to patient survival. Furthermore, multivariate analyses demonstrated that TNM stage ($P = 0.026$), vessel invasion ($P = 0.041$), and ADAM8 expression status ($P = 0.024$) were independent prognostic predictors for GC patients.

ADAM8 promotes GC cell growth and proliferation. The finding of the upregulation of ADAM8 in GC indicates that it may be a potential tumor promoter gene involved in the progression of GC. To confirm this hypothesis, BGC823 and AGS cells transfected with full-length ADAM8 RNA were established. The ADAM8 protein expression levels in both transfected and control cells were determined by Western blot analysis (Fig 4, *A*). To assess the potential effects of ectopic ADAM8 expression on cell proliferation and survival, EdU cell proliferation, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide (MTT) cell viability, and cell apoptosis assays were performed. EdU cell proliferation assay indicated that proliferative ability was significantly increased by approximately 1.5-fold for both BGC823 ($P < 0.001$) and AGS ($P < 0.05$) cells compared with control cells (Fig 4, *B*). Cell viability was assessed by MTT assay

1.2- to 1.4-folds for BGC823 cells. (**B**) Cell proliferation was determined by EdU proliferation assay at 24 hours after culturing (top panel), and cell viability was evaluated by MTT assay at 0, 24, 48, 72, and 96 hours (bottom panel). (**C**) The migration and invasion of MGC823 and AGS cells were evaluated by migration and invasion assays. The data are presented as the mean ± standard deviation. The experiment was repeated 3 times in triplicate. ADAM8, a disintegrin and metalloprotease 8; mRNA, messenger RNA. $^{*}P < 0.001$, $^{\#}P < 0.05$.

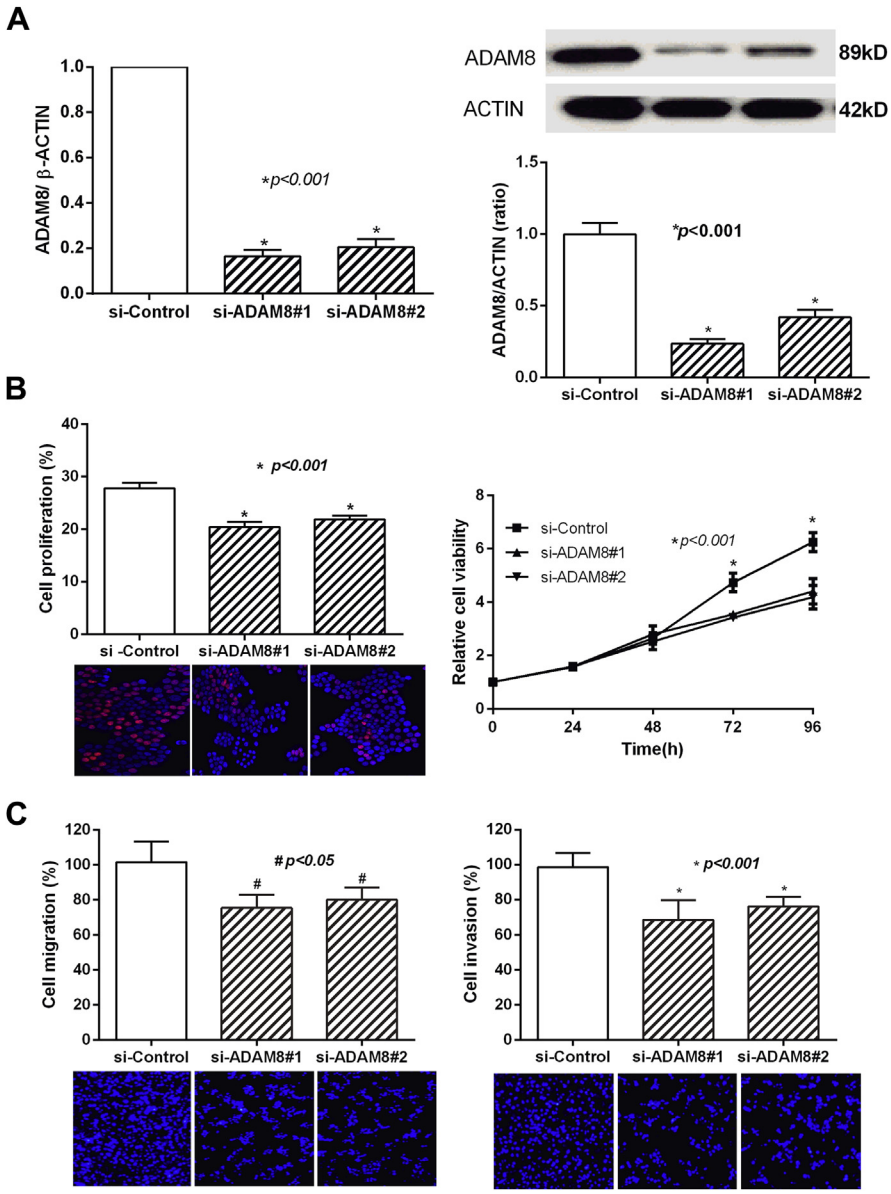

Fig 5. Downregulation of ADAM8 inhibits proliferation and aggression of gastric cancer cells. (**A**) Expressions of ADAM8 at the mRNA and protein levels in MKN45 cells transfected with siRNAs specific to ADAM8 were determined by qRT-PCR and Western blotting, respectively. (**B**) Cell proliferation was determined by EdU proliferation assay at 24 hours after culturing (top panel), and cell viability was evaluated by MTT assay at 0, 24, 48, 72, and 96 hours (bottom panel). (**C**) The migration and invasion of MKN45 cells were evaluated by migration and invasion assays. The data are presented as the mean ± standard deviation. The experiment was repeated 3 times in triplicate. ADAM8, a disintegrin and metalloprotease 8; mRNA, messenger RNA; qRT-PCR, quantitative reverse transcription–polymerase chain reaction; siRNA, small interfering RNA. $^{*}P < 0.001$, $^{\#}P < 0.05$.

at 24, 48, 72, and 96 hours after culture. As shown in Fig 4, *B*, significant increases in cell number were detected for both BGC823 and AGS cells (both $P < 0.05$ vs control at 72 and 96 hours) compared with control cells. However, no significant increases in growth at 24 and 48 hours were found. To examine the effect of ADAM8 on cell survival, flow cytometry with annexin V-FITC and propidium iodide staining was performed. Compared with control cells, the percentage of apoptotic cells was not found to be significantly increased for either BGC823 or AGS cells transfected with ADAM8. Taken together, these results suggest

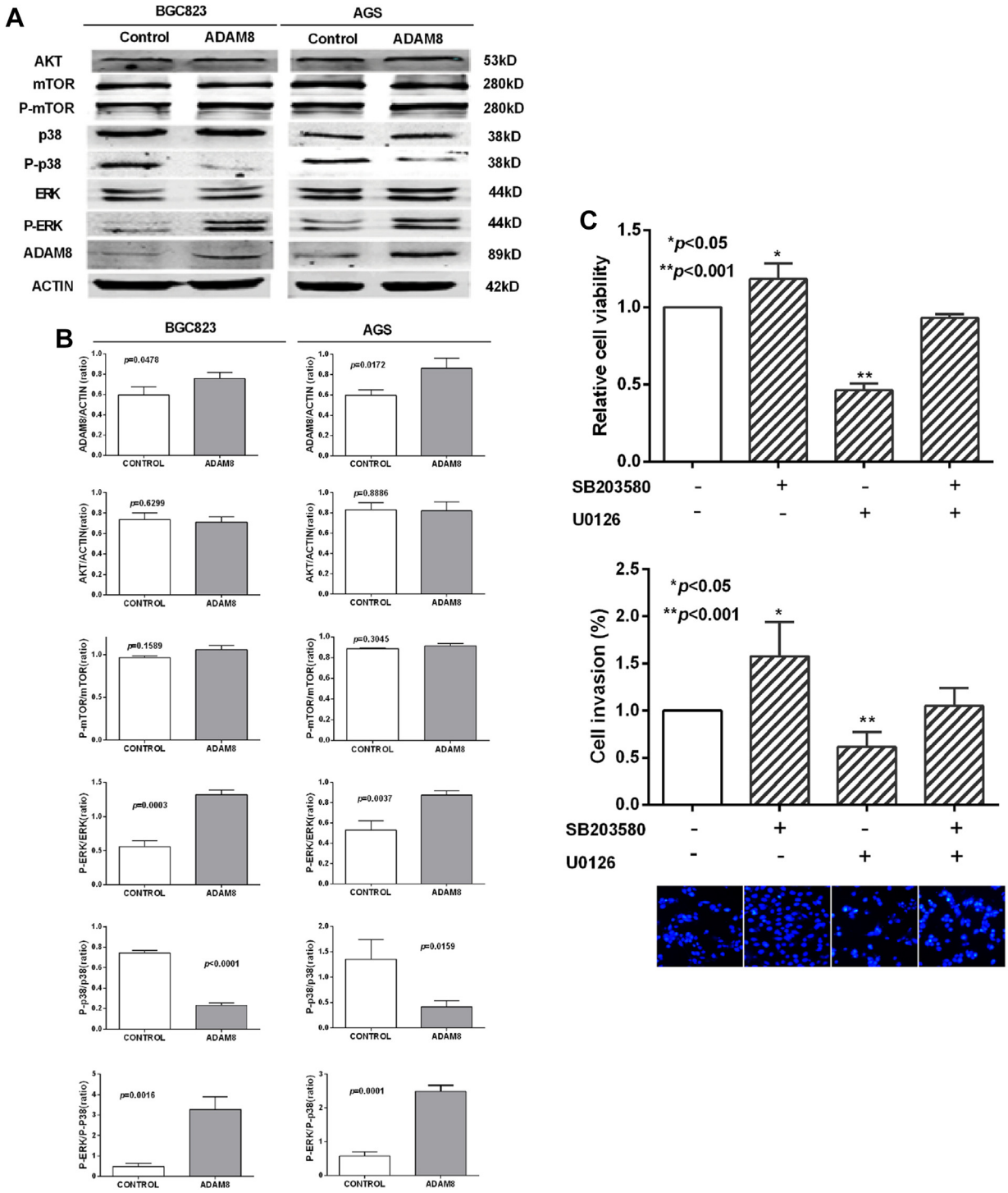

Fig 6. Overexpression of ADAM8 influences the p38/ERK MAPK signaling pathway in gastric cancer cells. (**A**) The effect of ADAM8 overexpression on targets in the AKT/mTOR, ERK, and p38 signaling pathways was assessed by Western blotting. Actin was used as a loading control. (**B**) The bands on Western blot for BGC823 and AGS cells were scanned by densitometry. The ratios of phosphor-mTOR to mTOR, phosphor-p38 to p38, phosphor-ERK to ERK, and phosphor-ERK to phosphor-p38 were calculated. (**C**) AGS cells were treated with inhibitors. p38 inhibitor (SB203580) suppressed cell proliferation and aggression, whereas Erk inhibitor (U0126) had the opposite effect. The results are presented as the mean ± standard deviation. The experiment was repeated 3 times in triplicate. ADAM8, a disintegrin and metalloprotease 8. $^{*}P < 0.05$, $^{**}P < 0.001$.

that ADAM8 upregulation stimulates cell growth by promoting cell proliferation and not by inducing apoptosis.

ADAM8 enhances the invasion and migration abilities of GC cells. To evaluate the effect of ADAM8 on the migration and invasion abilities of cancer cells, migration and invasion assays were performed. As shown in Fig 4, *C*, ADAM8 dramatically increased cell migration by 1.34- to 1.49-fold for BGS823 ($P < 0.0001$) and by 1.60- to 1.76-fold for AGS ($P < 0.0001$) on average compared with control cells. Moreover, ADAM8 remarkably enhanced cell invasion abilities by 1.54- to 1.58-fold for BGC823 ($P < 0.0001$) and by 1.55- to 1.65-fold for AGS ($P < 0.0001$) relative to empty vector control cells (Fig 4, *C*). These results indicate that ADAM8 plays an important role in regulating the migration and invasion of GC cells.

To further validate the stimulatory effect of ADAM8 on cell proliferation and invasion, siRNAs specific to ADAM8 were used to knockdown its expression in MKN45 cells, which normally express this protein at high levels, and the effects were analyzed by qRT-PCR and Western blotting (Fig 5, *A*). ADAM8 knockdown was also found to significantly inhibit cell growth ($P < 0.001$ at 72 and 96 hours) and proliferation (with a decrease to 82.3% for si-ADAM8#1 and 78.6% for si-ADAM8#2, respectively, $P < 0.001$; Fig 5, *B*) compared with control cells. Similarly, downregulation of ADAM8 expression significantly inhibited cell migration (with a decrease to 74.2% for si-ADAM8#1 and 78.7% for si-ADAM8#2, $P < 0.05$) and invasion (with a decrease to 65.9% for si-ADAM8#1 and 77.1% for si-ADAM8#2, $P < 0.001$; Fig 5, *C*). These results demonstrate that ADAM8 acts as a potential oncogene in GC.

Overexpression of ADAM8 affects p38/ERK mitogen-activated protein kinase signaling pathway in GC. To explore the underlying mechanism of ADAM8 in gastric carcinogenesis, we assessed the downstream signaling pathways modulated by this protein by examining 3 common cancer-related signaling pathways (AKT/mTOR, p38, and ERK) by Western blotting (Fig 6, *A*). Total and phosphorylated p38, mTOR, and ERK expression levels were quantified by scanning the representative bands on the blot and calculating the ratios of the phosphorylated forms to the total protein levels. There were no significant changes in the total amounts of AKT, mTOR, p38, ERK, or phosphor-mTOR in either BGC823 or AGS cells after ectopic ADAM8 expression. However, the levels of phosphor-p38 were dramatically decreased in both BGC823 (0.214 vs 0.658, $P = 0.0012$) and AGS (0.250 vs 0.661, $P = 0.0005$) cells, and those of phosphor-ERK were greatly increased in both BGC823 (0.682 vs 0.315, $P = 0.0033$) and AGS (0.623 vs 0.380, $P = 0.0185$) cells compared with control cells. In addition, the p-extracellular regulated protein kinases (p-ERK) to p-p38 ratios were significantly increased in both BGC823 (3.282 vs 0.492, $P = 0.0016$) and AGS (2.493 vs 0.578, $P = 0.0001$) cells compared with control cells (Fig 6, *B*). To confirm that the p38/ERK mitogen-activated protein kinase (MAPK) signaling pathway plays a role in cell proliferation and contributed to aggressive phenotype, MTT and invasion assays were performed after treatment of ADAM8-transfected BGC823 cells with a p38 inhibitor (SB203580) and ERK inhibitor (U0126). After 72 hours of exposure to SB203580, U0126 or both, SB203580 was found to significantly promote cell proliferation. On the contrary, U0126 blocked cell proliferation (Fig 6, *C*). Similarly, cell invasion abilities were increased by SB203580 and decreased by U0126 (Fig 6, *C*). Taken together, these results indicate that ADAM8 overexpression is involved in cell proliferation and aggressive phenotype by downregulating p38 expression and upregulating ERK expression in GC cells.

DISCUSSION

The ADAMs are a family of multidomain transmembrane glycoproteins that are involved in extracellular matrix remodeling and influence cell adhesion and cell migration.[18,19] Abnormal expression of some members of the ADAM family, including ADAM9,[20,21] ADAM10,[20] ADAM12,[21,22] ADAM15,[21] ADAM17,[20] ADAM20,[23] and ADAM33,[24] has been observed in GC, and overexpression of these proteins is correlated with its progression and prognosis.

Although ADAM8 is upregulated in various types of human solid tumors,[25,26] this is the first study to explore its precise effect on gastric carcinogenesis. Both the significant increase in the ADAM8 mRNA level and the greatly increased percentage of ADAM8 protein expression in GC tissues observed in this study indicate that this protein is involved in the initiation and progression of GC. Hence, we consider that it is a tumor promoter gene that plays a potential role in regulating the pathogenesis of GC.

It has been reported that ADAM8 overexpression is correlated with the clinicopathologic characteristics and worse prognosis of patients with certain cancers. In this study, ADAM8 protein expression in the tumor tissues was significantly correlated with invasion depth (pT stage), lymph node involvement (pN stage), and vessel invasion, which is in agreement with recent published studies of gastric and prostate cancer.[12,26] Furthermore, no correlation was found with TNM

stage, although a previous study has revealed differing results.[25] Survival analysis demonstrated that patients with positive ADAM8 expression have shorter survival times than those with negative expression and that this protein is an independent predictor of poor survival of GC patients, which is consistent with the results of studies on lung, renal, and brain cancers.[27-29]

Although ADAM8 is involved in several types of cancers, few data have been obtained with regard to its mechanism of action. To explore the potential roles and mechanism of ADAM8 in the initiation and progression of GC, we further established stable BGC823 and AGS cells overexpressing ADAM8 and MKN45 cells with knockdown of ADAM8. In the present study, ectopic expression of ADAM8 was found to significantly promote cell growth by stimulating proliferation and not by inducing apoptosis and to enhance the aggressive phenotype of GC cells. In contrast, ADAM8 downregulation markedly suppressed its stimulation of cell growth, invasion, and migration. Similar results have been obtained for brain and prostate cancers.[9,13] These data demonstrate that ADAM8 can promote the rapid growth and aggressiveness of GC cells. To explore the underlying mechanism of the influence of ADAM8 on GC cell proliferation and aggression, we detected the expression levels of phosphor-mTOR, phosphor-p38, and phosphor-ERK in both transfected BGC823/AGS cells and control cells. Western blot analysis revealed that ADAM8 overexpression markedly decreased p38 activity and increased ERK activity. A previous study has shown that a high p38/ERK ratio leads to cancer cell quiescence and a low ratio promotes cell growth in human head and neck carcinoma.[30] In this study, further analysis indicated that p38 inhibitor promoted and ERK inhibitor suppressed cell proliferation and aggression in ADAM8-transfected BGC823 cells. On the basis of these results, we consider that ADAM8 overexpression is involved in the initiation and progression of GC through modulation of the p38/ERK MAPK signaling pathway, which is supported by the finding that a high ERK/p38 ratio favors tumor growth and that a high p38/ERK ratio induces tumor growth arrest.[30]

CONCLUSIONS

Our study has revealed that the dysregulation of ADAM8 in GC promotes cell growth and invasion via the p38/ERK MAPK signaling pathway. ADAM8 expression is positively correlated with the aggressive phenotype and poor survival of patients with GC, demonstrating that it may be a potential therapeutic target.

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Conflicts of Interest: All authors have read the journal's policy on the disclosure of potential conflicts of interest and have none to declare.

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