ADAM8 promotes gastric cancer cell invasiveness via EGFR/ERK signaling pathway to upregulate matrix metalloproteinases 2 and 9 expression

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Abstract: This study aimed to investigate the role of A disintegrin and metalloprotease 8 (ADAM8) in the invasion of gastric cancer (GC) and the signaling pathway. Small interfering RNA (siRNA) oligonucleotides and overexpression plasmid specific to ADAM8 were constructed to silence and upregulate expression of ADAM8, and the expression of ADAM8 was monitored by quantitative reverse transcriptase polymerase chain reaction and western blotting. The invasiveness of gastric cancer cells was determined by matrigel invasion assay. The results revealed that ADAM8 expression was significantly upregulated in gastric cancer tissues compared with noncancerous tissues, and in tumor tissues from patients with lymph node metastasis and advanced T stages compared with those with no lymph node metastasis and early stages. The in vitro assay indicated that ADAM8 overexpression promoted cell invasiveness, and upregulated the expression of MMP-2, MMP-9 and the phosphorylation of EGFR and ERK. ERK inhibitor (U0126) and EGFR inhibitor (AG1478) decreased the invasiveness of GC cells, and downregulated the expression of MMP-2 and MMP-9. In conclusion, our findings illustrated that ADAM8 could promote the invasion of GC cells by activating EGFR/ERK pathway to increase MMPs activation.

Keywords: Gastric cancer, A disintegrin and metalloprotease 8, invasion, extracellular signal-regulated kinase, matrix metalloproteinases, epidermal growth factor receptor

Introduction

As one of the top causes of cancer-related death worldwide, Gastric cancer (GC) is responsible for over 700,000 deaths per year [1]. It is the second most common cancer and the third leading cause of death among cancer patients in China [2]. The potent invasion and metastasis ability of GC are important factors that lead to poor prognosis [3]. Thus, a better understanding of the pathogenesis and molecular events of gastric cancer may lead to new diagnostic, therapeutic and preventive strategies to combat with this disease.

ADAM8, a member of the ADAMs family, was initially reported to play potential roles in inflammatory and allergic processes [4-8], and further studies have shown that its expression was associated with invasion and metastasis in various solid tumors, and affected the prognosis [9-11]. However, its precise biologic mechanisms on GC invasion and metastasis remain unclear. In this study, we focused on to elucidate the correlation of ADAM8 expression with GC cell invasion and its mechanisms.

Materials and methods

Tissue specimens and cell culture

All tissue samples were obtained from Union Hospital, Tongji Medical College, Huazhong University of Science and Technology, China. All subjects gave their informed consent for inclusion before they participated in the study. The study was conducted in accordance with the Declaration of Helsinki, and the protocol was approved by the research ethics committee of Tongji Medical College. 5 Cell lines (SGC-7901, MKN-45, MKN-28, AGS, GES1) were obtained from the Cell Bank of Chinese Academy of
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Sciences. These cells were cultured in the recommended medium supplemented with 10% fetal bovine serum (Gibco, USA) and 1× penicillin/streptomycin (Invitrogen, USA) at 37°C in a humidified atmosphere (5% CO₂/95% air).

RNA isolation and quantitative polymerase chain reaction

Total RNA was extracted with the TRizol reagent (Invitrogen, USA) according to the manufacturer’s protocol. Complementary DNA was reverse transcribed using a reverse transcription kit (Takara, Japan). Quantitative reverse transcription polymerase chain reaction (qRT-PCR) was performed to assess ADAM8 and GAPDH expression with a SYBR Green Master Mix Kit and ABI 7500 System (Applied Biosystems, USA). GAPDH was used as endogenous control for the normalization of gene expression. The PCR primers used were as follows: ADAM8 (forward: 5’-GAGCAGTATGAGGTCGTGTT-3’, reverse 5’-GTGGAGGGTGAAGTTGTG-3’) and GAPDH (forward: 5’-AGGTCGGAGTCAACGGATTTG-3’, reverse 5’-GTGATGCGATGGAATGTG-3’). The PCR cycle conditions consisted of an initial denaturation step at 95°C for 30 sec, followed by 40 cycles at 95°C for 30 sec, 60°C for 30 sec, and 72°C for 30 sec. Relative mRNA expression levels were determined by the 2-∆∆CT method in comparison with control cells. All the primers used in this research were synthesized by Shanghai Sangon Biotechnology Co Ltd.

Western blotting analysis

Total proteins were extracted from gastric cancer tissues and corresponding adjacent non-tumor tissues, transfected GC cells and control cells, respectively. The protein concentration of extracts was determined with BCA reagent (Beyotime Biotechnology, China). Equal amounts of protein (40 µg/lane) were loaded, separated by 12% SDS-PAGE and transferred onto polyvinylidene difluoride membranes. After blocked with 5% non-fat milk, the membranes were incubated with different primary antibody at 4°C overnight, then incubated with horseradish peroxidase-labeled secondary antibody (Beyotime Biotechnology, China) for 1 hour at room temperature, the blots were observed using an Odyssey infrared imaging system. Immunoreactive bands were visualized using ECL Western blot detection reagents (Biosharp Technologies, China). The density of each band was quantified by scanning densitometry. The expression levels of GAPDH were monitored as an internal control and the band intensities were normalized to that of GAPDH. Primary antibodies against ADAM8, EGFR and p-EGFR were purchased from Abcam; antibodies against ERK, p-ERK, MMP-2, MMP-9, Akt, p-Akt and GAPDH were from Cell Signaling Technology.

Matrigel invasion assay

Matrigel invasion assay were performed using matrigel-coated transwell inserts containing polycarbonate filters with 8 µm pores (BD Biosciences, USA) in accordance with manufacturer’s instructions. Briefly, the inserts were pre-hydrated with serum-free RPMI-1640 medium (500 µl/well) for 2 h at 37°C in a humidified atmosphere (5% CO₂/95% air). Cells (1×10⁵) in 200 µl serum free RPMI-1640 medium were plated onto the upper chambers, whereas 600 µl complete medium was added to the lower wells. After 24 h, cells on the upper surface of the filter removed by a cotton swab, and the invaded cells below the filter were fixed in methanol for 20 mins, subsequently stained with 0.1% crystal violet for 30 mins. The numbers of invading cells were then counted in each of ten random fields for a given well under an inverted microscope and photographed. Ten random fields were numerically averaged and counted for each assay.

Statistical analysis

Statistical Product and Service Solutions (SPSS) version 17.0 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 or one-way ANOVA. P<0.05 was considered statistically significant.

Results

Establishment of stably transfected and RNA interference cell lines

To establish cell lines for analysis of GC in vitro, we selected AGS cell line (low endogenous levels of ADAM8) and MKN-45 cell line (high endogenous levels of ADAM8) as determined by qRT-PCR and western blotting in 4 GC cell lines (Figure 1A, 1B). AGS cell line with ADAM8 overexpression plasmid and MKN-45 cell line with ADAM8 RNA knockdown were generated.
The cDNA encoding human ADAM8 was obtained from human placenta and the pcDNA-3.1-vector were purchased from Invitrogen. pcDNA3.1-ADAM8 was constructed by our laboratory. Stable transfection of pcDNA3.1-ADAM8, namely AGS-OVADAM8, were done using Lipofectamine 2000 (Invitrogen, USA) according to manufacturer’s instructions. Forty-eight hours after transfection, 800 µg/ml G418 (Invitrogen, USA) was applied to select G418 resistant cells. Transfectants with ADAM8 overexpression or empty vector were confirmed by Western blotting for use in assessment of its expression status (Figure 1C).
onucleotides were obtained from RiboBio Co Ltd (Guangzhou, China). 1x10^5 cells were cultured in 6-well plates until 60% confluence was reached, and then they were transfected with 50 nM of the indicated siRNA using riboFECT™ CP Reagent (RiboBio, China) according to the manufacturer’s instructions. The effects of ADAM8 knockdown were analyzed at 48 hours after transfection by western blotting (Figure 1D).

**ADAM8 is upregulated in the primary GC tissue**

Expression of ADAM8 at the messenger RNA (mRNA) levels in GC tissues (20 tumor tissues and corresponding adjacent non-tumor tissues) was analyzed by qRT-PCR. The ADAM8 mRNA levels were found to be significantly higher in the primary GC tissues compared with the adjacent non-tumor tissues (P<0.0001) (Figure 2A). Moreover, combined with clinicopathologic characteristics (data unpublished), the ADAM8 protein expressions were found to be significantly higher in tumor tissues from patients with lymph node metastasis and advanced T stages compared to those with no lymph node metastasis and early stages (P=0.0082) (Figure 2B).

**Expression of ADAM8 increase the invasiveness of GC cells**

To evaluate the effect of ADAM8 on the invasiveness of cancer cells, matrigel invasion...
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Figure 4. Effect of upregulating and silencing ADAM8 expression on the protein expression of MMP-2 and MMP-9 of gastric cancer cells. A. The expression of MMP-2 and MMP-9 protein were found to be significantly higher in AGS-OVADAM8 compared with AGS-CTRL. B. The expression of MMP-2 and MMP-9 protein were found to be significantly lower in MKN-45 siADAM8 compared with MKN-45. C. AGS-OVADAM8 cells transfected with MMP-2 or MMP-9 siRNA suppressed the expression of MMP-2 or MMP-9, respectively. D. AGS-OVADAM8 cells transfected with MMP-2 or MMP-9 siRNA suppressed the invasion of gastric cancer cell, compared with AGS-CTRL. The data were obtained from three independent experiments. The data are presented as the mean ± standard deviation and one-way ANOVA. ADAM8, a disintegrin and metalloprotease 8; AGS-CTRL, untreated AGS cells, control cells; AGS-OVADAM8, AGS cells transfected with an ADAM8 overexpression plasmid; MKN-45 siADAM8, MKN-45 cell transfected with ADAM8 siRNA; AGS-OVADAM8 siMMP-2, AGS-OVADAM8 cell transfected with MMP-2 siRNA; AGS-OVADAM8 siMMP-9, AGS-OVADAM8 cell transfected with MMP-9 siRNA; GAPDH, used as a loading control. *P<0.05; **P<0.001.

assays were performed. Ectopic expression of ADAM8 dramatically increased cell invasiveness for AGS-OVADAM8 compared with empty vector control cells (AGS-CTRL) (average number of cells 22.7 vs 11.6, P<0.0001) (Figure 3A). Further validated invasiveness of GC cells, siRNAs specific to ADAM8 were used to knockdown its expression in MKN-45 cells, which
normally expressed this protein at relatively high levels. ADAM8 knockdown in MKN-45 cells (MKN-45 siADAM8) was also found to significantly inhibited cell invasiveness (average number of cells 26.4 vs 13.1, \( P<0.0001 \)) (Figure 3B).

**ADAM8 increased MMP-2 and MMP-9 expression in GC cells**

Extensive data showed that metalloproteases (M-MPs), in particular MMP-2 and MMP-9, play important roles in cancer cell invasion and metastasis. Thereby, Western blot analysis was employed to determine MMP-2 and MMP-9 expression levels in GC cells. Results demonstrated that upregulated expression of ADAM8 in AGS cells led to increased expression levels of MMP-2 and MMP-9 \( (P<0.05) \) (Figure 4A). Meanwhile, knockdown of ADAM8 in MKN-45 cells inhibited the expression of MMP-2 and MMP-9 \( (P<0.05) \) (Figure 4B).

To determine the contribution of MMP-2 or MMP-9 to ADAM8 regulated cell invasiveness, two independent small interfering RNA sequences targeting MMP-2 and MMP-9 were used to silence MMP-2 and MMP-9 in AGS-OVADAM8 cells, respectively. Results demonstrated that the two siRNA inhibited MMP-2 and MMP-9 expression significantly at protein levels, respectively, compared to AGS-OVADAM8 cells.
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Moreover, the siRNA mediated knockdown of MMP-2 or MMP-9 of AGS-OVADAM8 cells resulted in decreased GC cell invasive ability to penetrate the extracellular matrix in vitro (P<0.0001) (Figure 4D). These results revealed the involvement of MMP-2 and MMP-9 in ADAM8 mediated cell invasion in AGS-OVADAM8 cells.

**EGFR-ERK pathway was involved in regulating the ADAM8-induced cell invasion and MMP expression**

To explore the underlying mechanism involved in ADAM8-regulated cell invasiveness, we assessed the downstream signaling pathways modulated by this protein. First of all, we measured the effects of ADAM8 upregulating and silencing on EGFR expression by Western blotting (Figure 5A, 5B). ADAM8 overexpression caused a significantly increase in EGFR phosphorylation compared to AGS-CTRL (P=0.024), while total EGFR was not significantly affected by the expression level of ADAM8 (P=0.154). As EGFR activation can initiate a mitogenic cascade through ERK and Akt [38], we therefore investigate whether ADAM8 can activate Akt and/or ERK signaling pathway. The levels of Akt and ERK were measured to evaluate the activation of PI3K-Akt and MAPK-ERK pathways after upregulation or knockdown of ADAM8. There were no significant changes in the level of the ERK, AKT or phos-

![Figure 5. Upregulating and silencing ADAM8 expression influences the EGFR/ERK signaling pathway in gastric cancer cells. A. The effect of ADAM8 overexpression on targets in the EGFR/ERK signaling pathways was assessed by Western blotting. B. The effect of ADAM8 knockdown on targets in the EGFR/ERK signaling pathways was assessed by Western blotting. C. AGS-OVADAM8 cells treated with ERK inhibitor (U0126) suppressed the expression of MMP-2 and MMP-9. D. AGS-OVADAM8 cells treated with EGFR inhibitor (AG1478) suppressed the expression of MMP-2 and MMP-9. E. AGS-OVADAM8 cells treated with inhibitors ERK inhibitor (U0126) or EGFR inhibitor (AG1478) suppressed the invasion of gastric cancer cell. The average number of cells that invaded through the filter was counted. The data were obtained from three independent experiments. The data are presented as the mean ± standard deviation and one-way ANOVA. ADAM8, a disintegrin and metalloprotease 8; AGS-OVADAM8, AGS cells transfected with an ADAM8 overexpression plasmid; MKN-45 siADAM8, MKN-45 cell transfected with ADAM8 siRNA; U0126, ERK inhibitor; AG1478, EGFR inhibitor. *P<0.05; **P<0.001.](image-url)
Importance of membrane-associated proteins may be infiltration, and metastasis by the shedd-
tation and cell signalling functions [11, 12, 23, 24]. In particular, the contribution of ADAM8 to extracellular remodelling and tumor growth, infiltration, and metastasis by the shedding of membrane-associated proteins may be important [9, 10, 25].

In this study, The ADAM8 mRNA levels were found to be significantly higher in the primary GC tissues compared with the adjacent non-
tumor tissues (P<0.001). Moreover, It has been shown that the expressions of ADAM8 protein were found to be significantly higher in tumor tissues from patients with lymph node metastasis and advanced T stages compared to those with no lymph node metastasis and early stages (P=0.0082). We further established sta-
ble AGS cells with ADAM8 overexpression plasmid, and MKN-45 cells with ADAM8 RNA knock-
down. In the present study, ectopic expression of ADAM8 was found to significantly promote cell invasion of GC cells (P<0.0001). In con-
trast, ADAM8 downregulation significantly suppressed its stimulation of invasion (P<0.0001). Similar results have been obtained from other studies [17, 18, 25].

To clarify the mechanism of action of ADAM8, we investigated whether the promoting effect of ADAM8 on cell invasion was through upregu-
lated the expressions level of MMP-2 and MMP-9, which further explained the cause that ADAM8 promoted cell invasion in the matrigel invasion assay (P<0.05). As we all know, the invasion of tumor cells depends critically on extracellular matrix (ECM) remodelling, because degrada-
tion of the ECM allows cancer cells to invade from the blood or lymphatic system and spread to other tissues and organs [26-28]. Given the importance of the ECM in GC, the proteolytic release of membrane proteins as well as ECM degradation has previously been postulated to have a pivotal role in promoting the invasion of tumor cells in the tumor microenvironment [25, 29]. Matrix metalloproteases (MMPs) and/or ADAM (A disintegrin and metalloproteinase) proteases have been described in these pro-
cesses [30-32]. And especially MMP-2 and MMP-9 are responsible for degrading the ECM [28, 33]. Therefore, the pro-metastasis effect of ADAM8 on GC might be through regulating MMPs activities.

ERK signalling is considered a major pathway in tumor cell proliferation and metastasis [34, 35], and has been shown to regulate MMPs release [36, 37]. Moreover, the EGFR pathway has been established as an upstream effector of ERK [38], so that we investigated whether
the upregulation of MMP-2 and MMP-9 induced by ADAM8 is through activation of the EGFR/ERK signaling pathway. The results showed that the expression levels of pERK and pEGFR were significantly increased in AGS-OVADAM8 cells compared with empty vector control cells (P<0.05). Meanwhile, it was found that down-regulated the expression of ADAM8 in MKN-45 cells can reduced the expression of pERK and pEGFR (P<0.05). Inhibition of ERK and EGFR suppressed the invasiveness of AGS-OVADAM8 cells, and reduced the expression of MMP-2 and MMP-9 in AGS-OVADAM8 cells (P<0.05). On the basis of these results, we consider that ADAM8 promotes the invasion of tumor cells and increases MMP-2 and MMP-9 expression through modulation of the EGFR/ERK signaling pathway.

In conclusion, we firstly identify ADAM8 as a novel regulator contributes to gastric cancer cells invasion by activated the EGFR/ERK signaling pathway which consequentially increased the expression of MMP-2 and MMP-9. The present study strengthened the proposal that ADAM8 could be made as an attractive target for anticancer therapies of gastric cancer.

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Disclosure of conflict of interest

None.

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