ADAM9 functions as a promoter of gastric cancer growth which is negatively and post-transcriptionally regulated by miR-126

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Abstract. A disintegrin and metalloproteinase domain 9 (ADAM9) is a membrane-anchored protein implicated in cell-cell and cell-matrix interactions, including the process of tumorigenesis. However, the role of ADAM9 in gastric cancer (GC) has not been clearly illustrated. In the present study, we found aberrant overexpression of ADAM9 in both GC tissues and cell lines. The expression of ADAM9 was significantly correlated with patient clinicopathological features including tumor size, local invasion, lymph node metastasis and tumor-node-metastasis (TNM) stage. Knockdown of ADAM9 in GC SGC-7901 cells, which presented the highest ADAM9 expression among the cell lines, induced a dramatic suppression of cell proliferation along with the arrest of the cell cycle in the G0/G1 phase. Furthermore, we validated that the 3' untranslated region of ADAM9 mRNA could be bound by miR-126, a suppressor in GC, and overexpression of miR-126 significantly downregulated ADAM9 in the GC cells. In conclusion, ADAM9 functions as a tumor promoter in GC by modulating GC cell proliferation. ADAM9 could possibly be regarded as a biomarker for GC diagnosis and prevention. Moreover, as directly targeted by miR-126 in GC, ADAM9 may be a potential target for GC therapeutic treatment which warrants intensive study.

Introduction

As one of the most common digestive malignancies in humans worldwide, gastric cancer (GC) exhibits aggressive malignant behavior. GC patients have a current 5-year survival rate of only ~24% (1). At present, most GC patients are clinically diagnosed in the advanced stages and the median survival time of GC patients with local invasion or metastasis is less than 12 months (2). The challenges of discovering associated tumor markers and understanding the mechanisms of GC initiation, progression and metastasis, are the keys to diagnose, prevent and treat GC appropriately in the early stages as well as develop targeted treatment (3)

A disintegrin and metalloproteinases (ADAMs), are a family of proteins which play a pivotal role in the proteolytic process implicated in cell-cell and cell-matrix interactions (4). Members of the ADAM family are divided into two groups: membrane-anchored and the secreted-type proteins (ADAMST) (5). Noteworthy, membrane-anchored ADAMs belong to type I *trans*-membrane proteins, which consist of a disintegrin-containing extracellular domain and a metalloproteinase domain (6). The functions of ADAMs are multiple and they are mainly involved in the proteolytic processing of *trans*-membrane proteins, contributing to various pathologies, including cell adhesion, cell signaling pathways and human tumors (7). Ectodomain shedding is a critical process conducted through proteolytic cleavage of membrane-anchored molecules into the extracellular microenvironment, and is related with tumorigenesis (8). ADAMs also participate in the ectodomain shedding process by undergoing cleavage close to the *trans*-membrane domains (9).

A disintegrin and metalloproteinase domain 9 (ADAM9), one of the ADAM family members, has been found and described in a variety of solid tumors with overexpression and dysregulation, in glioma, prostate, colon and breast cancer, which suggest ADAM9 as an important molecule involved in tumorigenesis (10-13). However, in GC, the role of ADAM9 is still elusive and deserves to be elucidated.

MicroRNAs (miRs) are a class of non-coding RNAs consisting of 22 nucleotides, which recognize a specific sequence of messenger RNAs (mRNAs) on the 3' untranslated region (3'UTR) as targets, and consequentially induce either inhibition of mRNA translation or degeneration of the targeted mRNAs (14). miRs functionally regulate and control various pivotal pathophysiological processes post-transcriptionally, including tumor initiation and progression (15,16). For instance, miR-146 suppresses gallbladder cancer cell proliferation

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by targeting epidermal growth factor (EGF) (17); miR-99a inhibits cell growth in osteosarcoma by negatively regulating TNFAIP8 (18). In GC, numerous miRs exhibit complex and marked effects either by suppressing or promoting tumor progression. For example, miR-30a reportedly targets RPA1 in GC cells and consequently suppresses the growth of GC cells with cell cycle arrest (19); on the contrary, by decreasing the expression of FBW7 through direct post-transcriptional regulation, miR-363 significantly promotes the cell proliferation and chemotherapy resistance in GC (20). Certainly, miRs provide us with enormous possibilities to discover new targets in GC prevention, diagnosis and therapeutic treatment.

Based on our previous research and studies from other authors, microRNA-126 (miR-126) appears to be an extremely important microRNA that functions as a suppressor in GC progression and which is frequently downregulated in both GC tissues and cell lines (21-24). As interactions between miRs and their targets show complex networking, an individual miR could target various mRNAs in multiple pathophysiological processes and an individual mRNA could possibly be targeted by different miRs simultaneously. Intensive detection of miR-126-targeted molecules in GC is valuable to provide us with a clear sense of how this suppressor functions in GC and also to provide us with sufficient evidence in order to find new antitumor targets.

In the present study, by evaluating 76 pairs of GC tissues compared with the adjacent non-cancerous tissues and 4 GC cell lines (SGC-7901, MKN-45, MKN-28 and SUN-16), we ascertained that ADAM9 was aberrantly overexpressed in GC. High levels of ADAM9 were significantly correlated with GC clinicopathological features, such as tumor size, local invasion, lymph node metastasis and tumor stage, which suggest a poorer prognosis for patients with a high ADAM9 level. Knockdown of ADAM9 expression in SGC-7901 cells significantly suppressed cell proliferation and arrested the cell cycle at the G0/G1 phase. Moreover, by applying a dual-luciferase reporter assay, we discovered that miR-126 could directly bind to the 3'UTR of ADAM9 mRNA and markedly downregulate ADAM9 expression. The promotional effect of ADAM9 on GC cell proliferation was revealed through overexpression of miR-126. All the aforementioned findings illustrate that ADAM9 functions as a tumor promoter in GC and exerts a tumor-suppressive function.

Materials and methods

Cell culture and surgical specimens. The immortalized gastric epithelium cell line and 4 GC cell lines (SGC-7901, MKN-45, MKN-28 and SUN-16) were purchased from the Shanghai Institutes for Biological Sciences, Chinese Academy of Science (Shanghai, China). SGC-7901 cells overexpressing miR-126 (SGC-7901/miR-126) and the negative control (NigmiR) were constructed in our previous study (25). All cells were cultured in RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 μg/ml streptomycin and 100 U/ml penicillin in a humidified cell incubator at 37˚C with an atmosphere of 5% CO_2.

Seventy-six pairs of GC specimens and adjacent non-cancerous tissues were collected from GC patients who had undergone a radical gastrectomy without preoperative therapy at the Department of Surgery, Ruijin Hospital, Shanghai Jiao Tong University School of Medicine during 2012-2014. Ethical approval was obtained from the Research Medical Ethics Committee of Rujin Hospital, Shanghai Jiao Tong University School of Medicine.

Immunohistochemistry and western blot analyses. Antibodies against ADAM9 and GAPDH (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), and horseradish peroxidase-conjugated secondary antibody (Abcam, Cambridge, MA, USA) were prepared. Immunohistochemical analysis was carried out using antibody against ADAM9 following the manufacturer's instructions (1:50), and the tissues were individually examined by two professional pathologists. GAPDH was used as a loading control.

RIPA buffer containing a protease inhibitor cocktail was used to lyse the cells, and the protein concentration was measured by BCA Protein Assay kit (both from Pierce, Rockford, IL, USA). Proteins were electrophoresed and electrotransferred. Antibodies against ADAM9 (1:1,000) and GAPDH (1:5,000) were probed, and a horseradish peroxidase-conjugated secondary antibody was used for further probing. The protein quantity was detected using GAPDH as a loading control.

RNA isolation and real-time qPCR assay. Total RNA was extracted from the cell lines using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. The first-strand cDNA was synthesized using a HighCapacity cDNA Reverse Transcription kit (ABI, Foster City CA, USA). RT-primers of ADAM9 mRNAs were synthesized as follows: forward, 5'-GGAAGAGTGTGACTGTGGTAC-3' and reverse, 5'-CCTCGGCATAAAGTACCTCC-3' by Sangon Biotech Co. (Shanghai, China). Real-time quantitative polymerase chain reaction (qRT-PCR) was performed according to TaqMan Gene Expression Assays protocol (ABI).

Cell transfection. SGC-7901 cells were transfected with pGU6/Neo vectors (GenePharma, Shanghai, China) containing shRNA suppressing ADAM9 translation or non-containing ones. Cells were cultured and selected in medium containing 400 μg/ml G418 (Santa Cruz Biotechnology, Inc.). The stable transfected cells aforementioned were assessed by qRT-PCR and western blot analysis compared with the negative control cells. All cells were cultured and maintained in medium containing 200 μg/ml G418.

Recombinant adenovirus Ad5/F35 (Ad5/F35-ADAM9) was constructed for overexpressing ADAM9 and Ad5/F35-Null was used as a negative control (GenePharma). SGC-7901 cells overexpressing miR-126 (SGC-7901/miR-126) and the negative control (NigmiR) were further transfected with Ad5/F35-ADAM9 or Ad5/F35-Null, and were assessed.

Cell proliferation assay and cell cycle analysis. SGC-7901 cells ($1x10^6$) stably transfected or the negative control cells were cultured in 96-well microtiter plates in triplicate and incubated for 5 days at 37˚C with an atmosphere of 5% CO_2. The OD was measured using microplate computer software (Bio-Rad Laboratories, Inc., Hercules, CA, USA) according to the protocol from the Cell Counting Kit-8 (CCK-8) assay kit (Dojindo, Tokyo, Japan). The curves for cell proliferation were plotted.

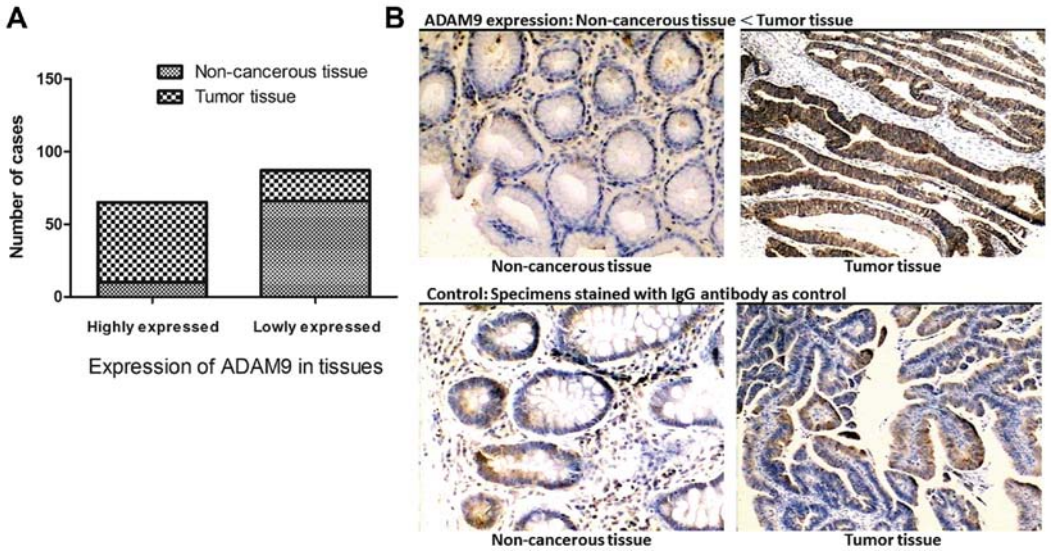

Figure 1. Expression of a disintegrin and metalloproteinase domain 9 (ADAM9) in gastric cancer (GC) and adjacent non-cancerous tissues. (A) Statistics from the number of cases with high or low expression of ADAM9 in specimens. The expression of ADAM9 was upregulated in most of the tumor tissues (55/76), and was expressed at a lower level in most of the adjacent non-cancerous tissues (66/76). The expression of ADAM9 in the tumor tissues was frequently and significantly higher than that in the adjacent non-cancerous tissues (P<0.01). (B) Representative immunohistochemistry analysis images carried out on tissue microarray (magnification, x100). Specimens stained with an IgG antibody were regarded as the control. The ADAM9 expression in GC tumor specimens was significantly higher than that in the adjacent non-cancerous tissues.

The aforementioned cells were treated with ethanol fixation, RNase A treatment and propidium iodide staining, and were then detected under flow cytometry by FACSCalibur (Becton-Dickinson, Franklin Lakes, NJ, USA). Cell populations at the G0/G1, S and G2/M phases were quantified by ModFit software (Becton-Dickinson) excluding a calculation of cell debris and fixation artifacts.

Dual-luciferase reporter assay. ADAM9 was predicted as a potential target of miR-126 by bioinformatics analysis (microcosm, http://mirecords.biolead.org). A 206 bp sequence from the 3'UTR of ADAM9 mRNA including the putative miR-126 binding site was selected as follows: 5'-uagagaaauuaauuuaaauuagaauuucuauuaugaaucaugugaaagcaugacauucguucacaauagcacuauuuuaaauaaauuauaagcuuuaagguacgaaguauuuaaugaucuaaucaaauauguugauucauggcuauaauaaagcaggagcaauuauaaaaucuucaaucaauugaacuuuuacaaaaccacuug-3'. The corresponding mutant sequence was constructed by Sangon Biotech Co., as follows: 5'-aacacauaauuaauaauuuuaacaauuacaaauuucauugaagaguauggaagucuuaccuacucuaaaccucaaauauuauuuauuaaaaugguauuacgaagguacuuuauuaaacaacaauugauaaaagaucaaugaagccaaaauuuauggacguggauuaaaauauuguacuaacuaaucaucauauucuauagcucauc-3'. These sequences were cloned into pMIR-REPORT luciferase vectors (Promega, Madison, WI, USA), containing Firefly luciferase, and pRL-TK vectors containg *Renilla* luciferase which were used as a control. SGC-7901 cells overexpressing miR-126 or the negative control were co-transfected with the aforementioned vectors and the luciferase activity was measured using a Dual-Glo Luciferase assay system (Promega) 48 h post-transfection.

Statistical analysis. Statistical analysis was carried out using SPSS 18.0. P-values were calculated using a paired t-test and Fisher's exact test. P-values <0.05 were considered to indicate a statistically significant result.

Results

ADAM9 is overexpressed in GC tumor specimens. Seventy-six paired specimens from GC tumors and adjacent non-cancerous tissues were examined using IHC. According to the expression intensity of ADAM9, these cases were separated into two groups: an ADAM9 low expression and an ADAM9 high expression group. Among the tumor specimens, 72.3% (55/76) of the cases presented high levels of ADAM9 expression, and only 27.6% (21/76) of the cases expressed a relatively lower level of ADAM9. On the contrary, in non-cancerous tissues, high expression of ADAM9 was detected in a small portion of the cases (13.2%, 10/76) and 86.8% (66/76) of the specimens presented a low level of ADAM9 expression. This proves that ADAM9 is expressed frequently higher in GC tissues when compared with adjacent non-cancerous tissues (Fig. 1).

ADAM9 expression is upregulated and miR-126 is downregulated in GC cell lines. Analysis of the transcription levels of miR-126 in the 4 GC cell lines (SGC-7901, MKN-28, MKN-45 and SUN-16) using qRT-PCR revealed that the expression levels of miR-126 were significantly downregulated in the GC cell lines compared with the GES-1 cells (P<0.05) (Fig. 2A). In contrast, the mRNA levels of ADAM9 were upregulated in the 4 different GC cell lines compared with the GES-1 cells (P<0.05) (Fig. 2B). Similarly, western blot analysis demonstrated that the protein levels of ADAM9 were significantly higher in the GC cell lines than that in the GES-1 cells (Fig. 2C). These results were consistent with the observations obtained in the IHC analysis of the tumor tissues.

High expression of ADAM9 is correlated with GC clinicopathological features. The correlation between the expression of ADAM9 and the clinicopathological features of the 76 GC cases was analyzed. According to Table I, there was

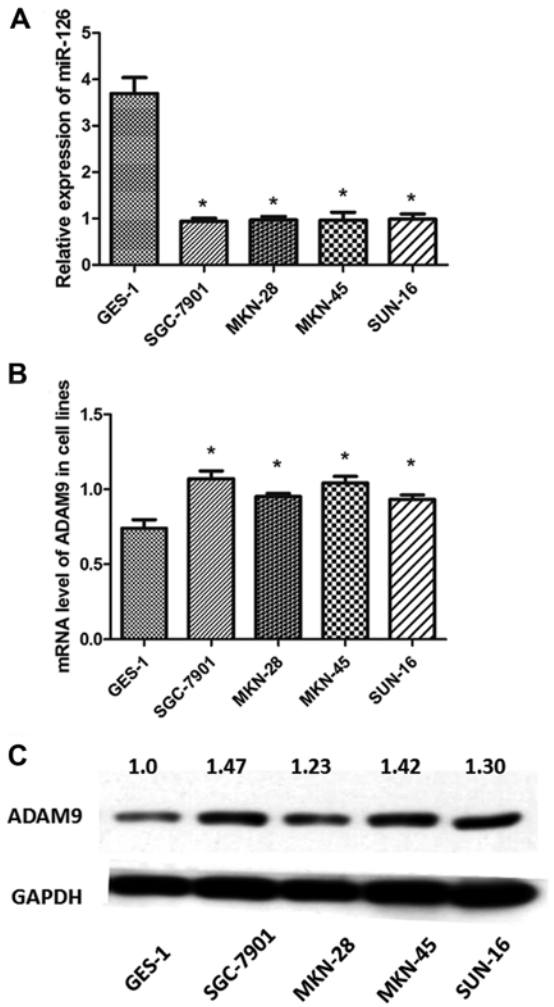

Figure 2. Expression of miR-126 and a disintegrin and metalloproteinase domain 9 (ADAM9) in gastric cancer (GC) and GES-1 cells. (A) Analysis of the transcription levels of miR-126 in cell lines using qRT-PCR. The expression of miR-126 in the 4 GC cell lines (SGC-7901, MKN-28, MKN-45 and SUN-16) was significantly downregulated compared with the GES-1 cells (*P<0.05). (B) The transcription levels of ADAM9 in the cell lines were detected using qRT-PCR. The mRNA level of ADAM9 was significantly higher in the GC cells than that in the GES-1 cells (*P<0.05). (C) The detection of the protein expression of ADAM9 in the cell lines by western blot analysis. The ADAM9 protein was significantly upregulated in the GC cells when compared with the GES-1 cells. The numbers above the blot indicate normalized protein amounts relative to the negative control, as determined by densitometry.

Table I. Correlation between ADAM9 expression and clinicopathological features of the 76 GC cases.

	ADAM9 expression		
Clinicopathological parameters	Low (n=21)	High (n=55)	P-value[a]
Age (years)			0.592
≤60	8	17	
>60	13	38	
Gender			0.196
Male	12	21	
Female	9	34	
Tumor diameter (cm)			0.023
≤5	15	19	
>5	8	36	
Location			0.778
Distal third	16	39	
Middle or proximal third	5	16	
Histological classification			0.792
Poorly-differentiated adenocarcinoma	7	22	
Middle/well-differentiated adenocarcinoma	4	6	
Signet ring cell carcinoma	3	6	
Mucinous adenocarcinoma	1	3	
Local invasion			0.016
T1,T2	13	16	
T3,T4	8	39	
Lymph node metastasis			0.030
No	12	15	
Yes	9	40	
TNM stage			0.046
I,II	10	12	
III,IV	11	43	

ADAM9 expression level associated with clinicopathological features in 76 GC patients, including age, gender, tumor size, tumor location, histological classification, local invasion, lymph node metastasis and TNM stage. Statistical significance was assessed by Fisher's exact test. ADAM9, a disintegrin and metalloproteinase domain 9; GC, gastric cancer; TNM, tumor-node-metastasis.

no significant correlation between ADAM9 expression and patient age, gender or tumor location. However, a significant trend towards a larger tumor size (P<0.05), deeper local invasion (P<0.05), more frequent lymph node metastasis (P<0.05) and more advanced tumor-node-metastasis (TNM) stage (P<0.05) in cases with higher expression levels indicates a correlation between ADAM9 overexpression and certain GC clinicopathological features.

Knockdown of ADAM9 suppresses cell proliferation and arrests the cell cycle in SGC-7901 cells. SGC-7901 cells, which expressed the highest level of ADAM9 among the 4 GC cell lines, were selected and transfected with pGU6/Neo vectors to knock down the expression of ADAM9. We verified the transfection effect through qRT-PCR and western blot analysis (Fig. 3).

We conducted flow cytometric analysis and found that, the cell cycle of SGC-7901 cells was significantly arrested at the G0/G1 phase when ADAM9 was knocked down (Fig. 4A and B). The percentage of the SGC-7901 cells in the G0/G1 phase was increased from 50.89 to 64.78% (P<0.01). The S phase was decreased from 30.63 to 18.05%, and the G2/M phase was decreased from 28.16 to 17.16% (Fig. 4B). Meanwhile, as the CCK-8 assay demonstrated, we observed a significant decrease in cell proliferation in the ADAM9-knockdown SGC-7901 cells as the P-value was <0.01 for day 1 and the P-value was

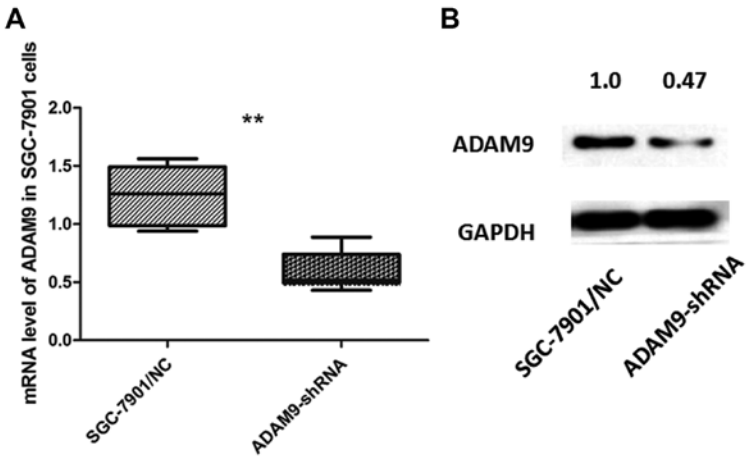

Figure 3. Knockdown of a disintegrin and metalloproteinase domain 9 (ADAM9) in SGC-7901 cells transfected with pGU6/Neo/sh-ADAM9 vector. PGU6/Neo vectors were used to transfect SGC-7901 cells to knockdown ADAM9 expression. (A) qRT-PCR analysis results indicated a significant decrease in the ADAM9 mRNA level in SGC-7901 cells after transfection (**P<0.01). (B) Western blot analysis confirmed a significant suppression of ADAM9 expression after transfection. The numbers above the blot indicate normalized protein amounts relative to the negative control, as determined by densitometry.

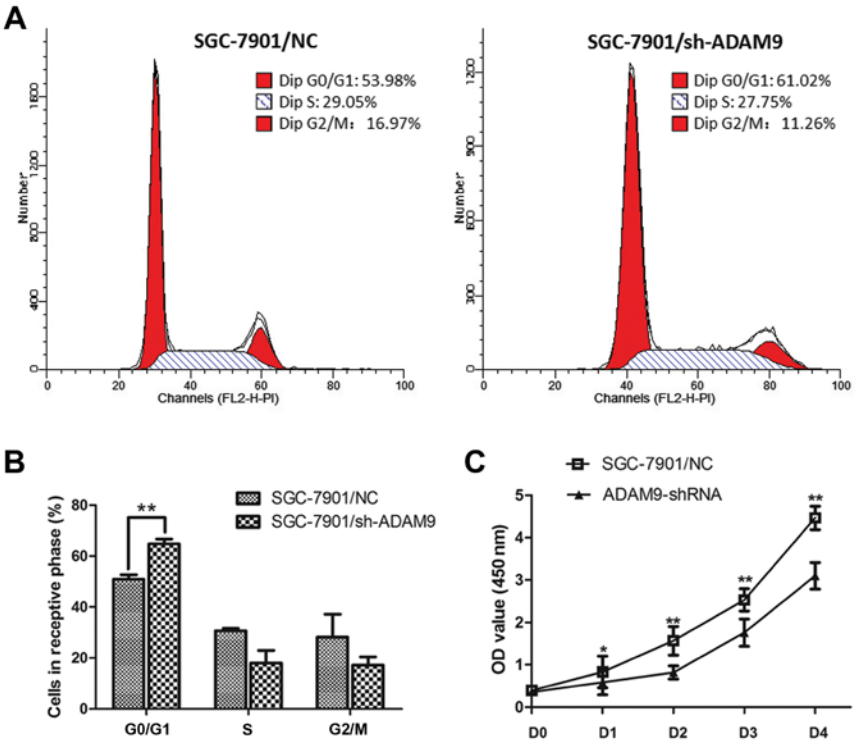

Figure 4. Effects on the cell cycle and cell proliferation following downregulation of the expression of a disintegrin and metalloproteinase domain 9 (ADAM9) in SGC-7901 cells. Flow cytometry was used to analyze the effect of ADAM9 on the cell cycle and cell proliferation. (A and B) Representative histograms describing the cell cycle profiles of SGC-7901 cells stably transfected with pGU6//Neo/sh-ADAM9 and the negative control cells. The cell cycle was significantly arrested in the G0/G1 phase following downregulation of ADAM9 in SGC-7901 cells (**P<0.01). (C) A CCK-8 assay was applied to profile the effect of ADAM9 on cell proliferation. The ability of cell proliferation in the SGC-7901 cells was significantly decreased by suppressing the expression of ADAM9 (**P<0.01, *P<0.05).

<0.05 for days 2-4 (Fig. 4C). These results indicate that knockdown of ADAM9 in SGC-7901 cells significantly impacts tumor cell growth.

ADAM9 is a direct target post-transcriptionally regulated by miR-126 in GC cells. We used microcosm, bioinformatics analysis employing an online prediction software, to predict ADAM9 as a potential target of miR-126 (Fig. 5A). Demonstration of the direct interaction between ADAM9 mRNA and miR-126 was carried out using a dual-luciferase reporter assay. Luciferase reporter vectors containing a 206-bp 3'UTR sequence of ADAM9 (WT-UTR) and the corresponding control luciferase vectors containing a mutated miR-126 target site (MUT-UTR) were constructed. As shown in Fig. 5B, overexpression of miR-126 in the SGC-7901 cells (SGC-7901/miR-126) significantly decreased the luciferase signal of ADAM9/pMIR/WT, compared with the negative control (SGC-7901/NigmiR). In addition, this suppressive

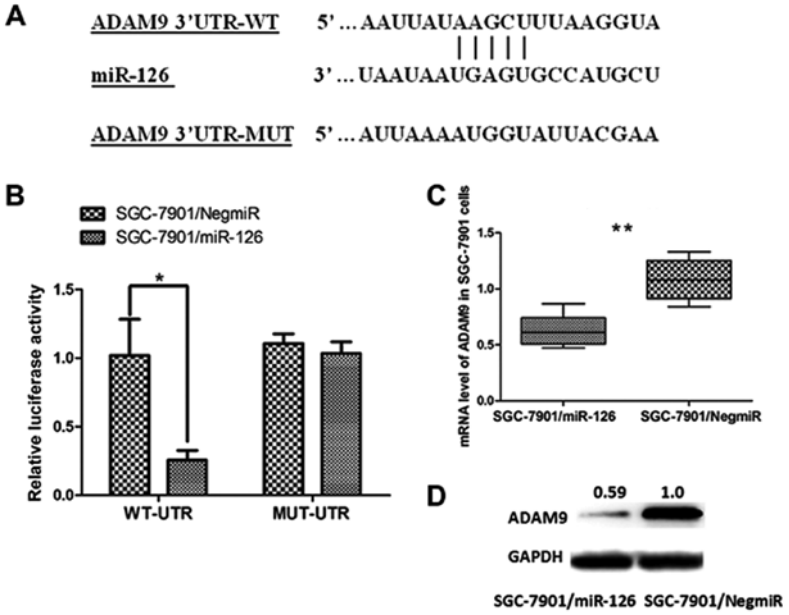

Figure 5. Modulation of a disintegrin and metalloproteinase domain 9 (ADAM9) in SGC-7901 cells as regulated by miR-126. (A) The predicted miR-126 binding site in the wild-type ADAM9 mRNA 3'UTR (3'UTR-WT), and in the mutant construct (3'UTR-MUT). (B) The direct interaction between miR-126 and ADAM9 was detected by Dual-luciferase reporter assay. Overexpression of miR-126 in SGC-7901 cells (SGC-7901/miR-126) significantly decreased the luciferase signal of ADAM9/pMIR/WT compared with the negative control (SGC-7901/NigmiR). In addition, mutation of the putative miR-126-binding site abolished this suppressive effect (*P<0.05). (C) qRT-PCR was undertaken to detect the effect of miR-126 on ADAM9 mRNA levels in SGC-7901 cells. The mRNA expression of ADAM9 was significantly decreased by overexpressing miR-126 in SGC-7901 cells (**P<0.01). (D) Western blot analysis was carried out to detect the effect of miR-126 on the ADAM9 protein expression in SGC-7901 cells. The ADAM9 protein expression was significantly suppressed by overexpression of miR-126 in SGC-7901 cells.

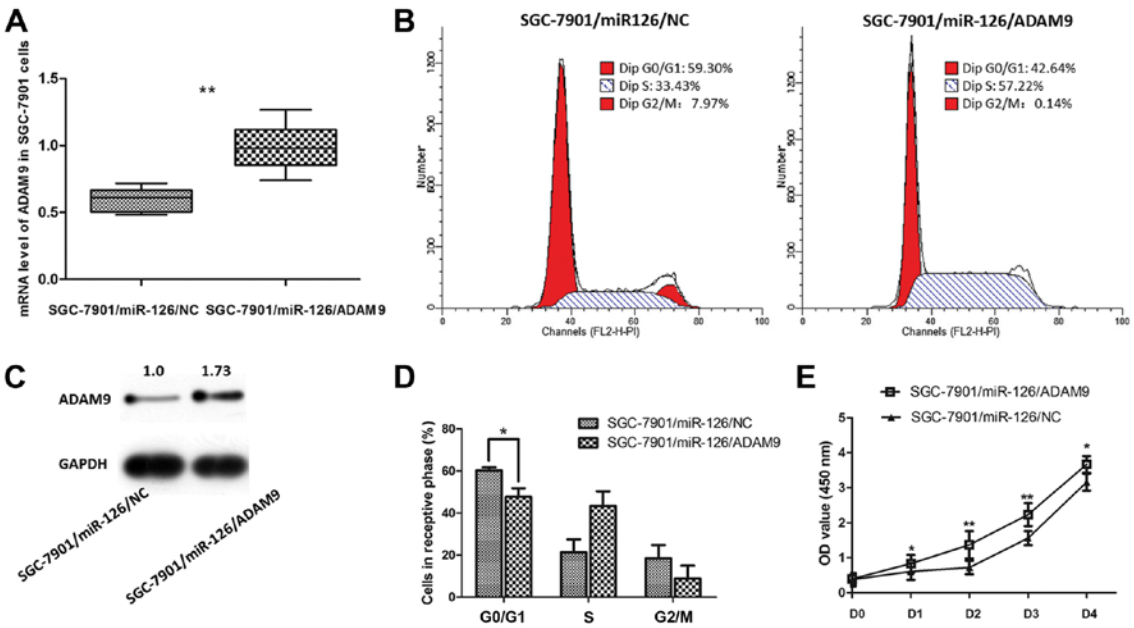

Figure 6. A disintegrin and metalloproteinase domain 9 (ADAM9) reverses the biological phenomena induced by miR-126. (A) A qRT-PCR assay was carried out to confirm the effect of transfection. A significant increase of ADAM9 mRNA expression in SGC-7901 cells overexpressing miR-126 was observed after transfection (**P<0.01). (B) Representative histograms describing the cell cycle profiles of SGC-7901 cells overexpressing ADAM9 and miR-126 and the negative control cells. (C) The results from the western blot analysis show a significant increase in ADAM9 protein expression after transfection. The numbers above the blot indicate normalized protein amounts relative to the negative control, as determined by densitometry. (D) The proportion of cells in various phases of the cell cycle. Overexpression of ADAM9 rescued the cell cycle arrest at the G0/G1 phase induced by miR-126 in GC cells. The results are the means of three independent experiments ± SD. (*P<0.05). (E) A CCK-8 assay was performed every 24 h for 4 days. Overexpression of ADAM9 reversed the inhibitory effect of miR-126 in GC cells The results were the means of three independent experiments ± SD (**P<0.01, *P<0.05).

effect induced by miR-126 was significantly abolished in the SGC-7901 cells with the putative binding site of mutated miR-126 (Fig. 5B). Moreover, both the mRNA level and the protein expression of ADAM9 were significantly decreased in

SGC-7901/miR-126 cells (Fig. 5C and D). Collectively, these results revealed that ADAM9 is a direct target of miR-126 in GC and was post-transcriptionally downregulated by miR-126.

Introduction of ADAM9 in SGC-7901 cells reverses the phenotype of growth arrest induced by overexpression of miR-126. The aforementioned results indicate that ADAM9 is one of the direct targets suppressed by miR-126 in GC. Based on this, we assumed that introducing ADAM9 in the miR-126-overexpressing SGC-7901 cells would at least relatively reverse the phenotypes caused by overexpression of miR-126. Recombinant adenovirus Ad5/F35 was applied in the present study to upregulate the expression of ADAM9 in the SGC-7901 cells. As shown in Fig. 6, the inhibitory effect on SGC-7901 cell proliferation by miR-126 was significantly reversed when ADAM9 expression was increased. Meanwhile, the cell cycle arrest effect induced by miR-126 was also reverses after ADAM9 introduction. Thus, ADAM9 is a molecule that promotes GC cell growth, which may be targeted by miR-126 as a part of its post-transcriptional mechanism for suppressing GC.

Discussion

The initiation and development of human tumors are under the control of a multitude of factors. With respect to gastric cancer (GC), a large number of molecules and their relative mechanisms, which are involved in GC tumorigenesis have been discovered. A variety of molecules participate in the process of GC with different types of mechanisms such as signaling pathways and post-transcriptional regulation, which are accompanied by huge networking between the molecules discovered or those which need to be further studied. For example, nucleophosmin (NPM)/B23 was found to be aberrantly overexpressed and regulated in GC, functioning as an indicator of GC associated with advanced TNM stage, poor prognosis and recurrence (26). Plant homeodomain finger protein 10 (PHF10) was ascertained as a promoter of GC enhancing the ability of cell proliferation (27). In addition, concerning non-coding RNAs, for example, miR-223 targets EPB41L3 in GC and promotes tumor cell invasion and migration (28). miR-107 downregulates CDK6 mRNA, and induces inhibition of GC cell invasion (29).

ADAM9 is a member of the ADAM family anchored to the membrane, and is related to various human tumors as we previously mentioned. In pancreatic ductal adenocarcinoma, ADAM9 is upregulated at the mRNA level and over 70% of pancreatic carcinomas present high protein levels (30). In prostate cancer, ADAM9 appears to be regulated, and inhibition of ADAM9 *in vivo* significantly suppressed tumor growth (10). ADAM9 was also found to modulate tumor-stromal cell interaction and sequentially promote cell motility in human hepatocellular carcinoma and lung cancer (31,32). Moreover, several reports have demonstrated high expression of ADAM9 in GC (33,34). However, the role of ADAM9 in GC and its relative upstream regulatory mechanisms remain unclear.

In the present study, we validated the expression of ADAM9 in 76 GC tumor tissues and cell lines. ADAM9 exhibited an obvious high expression level in GC tissues. By analyzing the clinicopathological features of the 76 patients, we found that high levels of ADAM9 showed a significant correlation with larger tumor size, deeper local invasion, more frequent lymph node metastasis and more advanced tumor stages. Thus, ADAM9 is an independent factor correlated with poor GC outcomes and prognosis.

Simultaneously, qRT-PCR and western blot analysis showed that expression of ADAM9 was significantly higher at both the mRNA and protein levels in GC cells than levels in GES-1 cells. Among the 4 GC cell lines, SGC-7901 cells presented the highest level of ADAM9. Considering what we observed from the specimens and cells, we believe that ADAM9 is a potential functional molecule in GC progression. To verify the function of ADAM9 in GC cells, we selected SGC-7901 cells and knocked down the ADAM9 expression by stable transfection. An *in vitro* cellular functional experiment was carried out. As we had hypothesized, when ADAM9 was knocked down in the SGC-7901 cells, the cell proliferation ability was markedly suppressed. Additionally, flow cytometric analysis demonstrated an obvious arrest of the cell cycle in GC cells at the G0/G1 phase, indicating that inhibition of ADAM9 effectively suppressed the growth of GC cells.

Furthermore, we speculated the mechanism by which ADAM9 promotes the cell growth of GC. Through the use of online bioinformatics tools, we found that a potential binding site for miR-126 exists in the 3'UTR of ADAM9 mRNA. miR-126 is an important non-coding RNA, which has been confirmed as a suppressor of GC growth. In our recent study we found that miR-126 exerts its tumor-suppressive function in various types of cancer by targeting different mRNAs, and in GC, CRKL, LAT-1, VEGF-A and CADM-1, are all targets of miR-126. We further speculated as to whether ADAM9 is a functional target of miR-126 in GC. We then conducted a dual-luciferase reporter assay to verify the direct interaction between ADAM9 and miR-126. A combination of miR with a specific 3'UTR of the target mRNA could cause an impact on luciferase gene expression. As the results showed, overexpression of miR-126 significantly decreased the luciferase signal intensity. On the contrary, mutated 3'UTR of ADAM9 mRNA failed to bind with miR-126 and presented no significant change in luciferase signal intensity. Thus, there is a direct correlation between miR-126 and the 3'UTR of ADAM9 mRNA.

To further understand whether miR-126 suppresses GC through ADAM9, we ectopically expressed ADAM9 in SGC-7901 cells overexpressing miR-126. As expected, by introducing ADAM9, the cell proliferation suppression induced by miR-126 was significantly reversed. In addition, the percentage of the cells arrested in the G0/G1 phase was notably decreased when ADAM9 was overexpressed. All the aforementioned results suggest that ADAM9 is one of the direct targets regulated by miR-126 in GC cells and by which miR-126 conducts its potential tumor suppressive function in GC.

In conclusion, according to the findings in the present study, we conclude that ADAM9 is one of the direct targets post-transcriptionally modulated by miR-126, which helps us to understand the tumor-suppressive mechanism of miR-126. High levels of ADAM9 in GC are correlated with a poor prognosis and aberrant overexpression of ADAM9 leads to the promotion of GC cell growth. ADAM9 should be considered as a potential target for GC prevention, diagnosis and therapeutic treatment.

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