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...
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 for overexpressing ADAM9 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.

Cell transfection. SGC-7901 cells were transfected with pGPU6/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 (1x10⁵) 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% CO₂. 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.
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’-uagagaaauuaauuuaauu uagaauuuaauuaauuuauu uagaauuuaauuaauuuauu uagaauuuaauuaauuuauu uagaauuuaauuaauuuauu uagaauuuaauuaauuuauu uagaauuuaauuaauuuauu uagaauuuaauuaauuuauu uagaauuuaauuaauuuauu uagaauuuaauuaauuuauu uagaauuuaauuaauuuauu uagaauuuaauuaauuuauu uagaauuuaauuaauuuauu uagaauuuaauuaauuuauu uagaauuuaauuaauuuauu uagaauuuaauuaauuuauu uagaauuuaauuaauuuauu uagaauuuaauuaauuuauu uagaauuuaauuaauuuauu uagaauuuaauuaauuuauu uagaauuuaauuaauuuauu uagaauuuaauuaauuuauu uagaauuuaauuaauuuauu uagaauuuaauuaauuuauu uagaauuuaauuaauuuauu uagaauuuaauuaauuuauu uagaauuuaauuaauuuauu uagaauuuaauuaauuuauu uagaauuuaauuaauuuauu uagaauuuaauuaauuuauu uagaauuuaauuaauuuauu uagaauuuaauuaauuuauu uagaauuuaauuaauuuauu uagaauuuaauuaauuuauu uagaauuuaauuaauuuauu uagaauuuaauuaauuuauu uagaauuuaauuaauuuauu uagaauuuaauuaauuuauu uagaauuuaauuaauuuauu uagaauuuaauuaauuuauu uagaauuuaauuaauuuauu uagaauuuaauuaauuuauu uagaauuuaauuaauuuauu uagaauuuaauuaauuuauu uagaauuuaauuaauuuauu uagaauuuaauuaauuuauu uagaauuuaauuaauuuauu uagaauuuaauuaauuuauu uagaauuuaauuaauuuauu uagaauuuaauuaauuuauu uagaauuuaauuaauuuauu uagaauuuaauuaauuuauu uagaauuuaauuaauuuauu uagaauuuaauuaauuuauu uagaauuuaauuaauuuauu uagaauuuaauuaauuuauu uagaauuuaauuaauuuauu uagaauuuaauuaauuuauu uagaauuuaauuaauuuauu uagaauuuaauuaauuuauu uagaauuuaauuaauuuauu uagaauuuaauuaauuuauu uagaauuuaauuaauuuauu uagaauuuaauuaauuuauu uagaauuuaauuaauuuauu uagaauuuaauuaauuuauu uagaauuuaauuaauuuauu uagaauuuaauuaauuuauu uagaauuuaauuaauuuauu uagaauuuaauuaauuuauu uagaauuuaauuaauuuauu uagaauuuaauuaauuuauu uagaauuuaauuaauuuauu uagaauuuaauuaauuuauu uagaauuuaauuaauuuauu uagaauuuaauuaauuuauu uagaauuuaauuaauuuauu uagaauuuaauuaauuuauu uagaauuuaauuaauuuauu uagaauuuaauuaauuuauu uagaauuuaauuaauuuauu uagaauuuaauuaauuuauu uagaauuuaauuaauuuauu uagaauuuaauuaauuuauu uagaauuuaauuaauuuauu uagaauuuaauuaauuuauu uagaauuuaauuaauuuauu uagaauuuaauuaauuuauu uagaauuuaauuaauuuauu uagaauuuaauuaauuuauu uagaauuuaauuaauuuauu uagaauuuaauuaauuuauu uagaauuuaauuaauuuauu uagaauuuaauuaauuuauu uagaauuuaauuaauuuauu uagaauuuaauuaauuuauu uagaauuuaauuaauuuauu uagaauuuaauuaauuuauu uagaauuuaauuaauuuauu uagaauuuaauuaauuuauu uagaauuuaauuaauuuauu uagaauuuaauuaauuuauu uagaauuuaauuaauuuauu uagaauuuaauuaauuuauu uagaauuuaauuaauuuauu uagaauuuaauuaauuuauu uagaauuuaauuaauuuauu uagaauuuaauuaauuuauu uagaauuuaauuaauuuauu uagaauuuaauuaauuuauu uagaauuuaauuaauuuauu uagaauuuaauuaauuuauu uagaauuuaauuaauuuauu uagaauuuaauuaauuuauu uagaauuuaauuaauuuauu uagaauuuaauuaauuuauu uagaauuuaauuaauuuauu uagaauuuaauuaauuuauu uagaauuuaauuaauuuauu uagaauuuaauuaauuuauu uagaauuuaauuaauuuauu uagaauuuaauuaauuuauu uagaauuuaauuaauuuauu uagaauuuaauuaauuuauu uagaauuuaauuaauuuauu uagaauuuaauuaauuuauu uagaauuuaauuaauuuauu uagaauuuaauuaauuuauu uagaauuuaauuaauuuauu uagaauuuaauuaauuuauu uagaauuuaauuaauuuauu uagaauuuaauuaauuuauu uagaauuuaauuaauuuauu uagaauuuaauuaauuuauu uagaauuuaauuaauuuauu uagaauuuaauuaauuuauu uagaauuuaauuaauuuauu uagaauuuaauuaauuuauu uagaauuuaauuaauuuauu uagaauuuaauuaauuuauu uagaauuuaauuaauuuauu uagaauuuaauuaauuuauu uagaauuuaauuaauuuauu uagaauuuaauuaauuuauu uagaauuuaauuaauuuauu uagaauuuaauuaauuuauu uagaauuuaauuaauuuauu uagaauuuaauuaauuuauu uagaauuuaauuaauuuauu uagaauuuaauuaauuuauu uagaauuuaauuaauuuauu uagaauuuaauuaauuuauu uagaauuuaauuaauuuauu uagaauuuaauuaauuuauu uagaauuuaauuaauuuauu uagaauuuaauuaauuuauu uagaauuuaauuaauuuauu uagaauuuaauuaauuuauu uagaauuuaauuaauuuauu uagaauuuaauuaauuuauu uagaauuuaauuaauuuauu uagaauuuaauuaauuuauu uagaauuuaauuaauuuauu uagaauuuaauuaauuuauu uagaauuuaauuaauuuauu uagaauuuaauuaauuuauu uagaauuuaauuaauuuauu uagaauuuaauuaauuuauu uagaauuuaauuaauuuauu uagaauuuaauuaauuuauu uagaauuuaauuaauuuauu uagaauuuaauuaauuuauu uagaauuuaauuaauuuauu uagaauuuaauuaauuuauu uagaauuuaauuaauuuauu uagaauuuaauuaauuuauu uagaauuuaauuaauuuauu uagaauuuaauuaauuuauu uagaauuuaauuaauuuauu uagaauuuaauuaauuuauu uagaauuuaauuaauuuauu uagaauuuaauuaauuuauu uagaauuuaauuaauuuauu uagaauuuaauuaauuuauu uagaauuuaauuaauuuauu uagaauuuaauuaauuuauu uagaauuuaauuaauuuauu uagaauuuaauuaauuuauu uagaauuuaauuaauuuauu uagaauuuaauuaauuuauu uagaauuuaauuaauuuauu uagaauuuaauuaauuuauu uagaauuuaauuaauuuauu uagaauuuaauuaauuuauu uagaauuuaauuaauuuauu uagaauuuaauuaauuuauu uagaauuuaauuaauuuauu uagaauuuaauuaauuuauu uagaauuuaauuaauuuauu uagaauuuaauuaauuuauu uagaauuuaauuaauuuauu uagaauuuaauuaauuuauu uagaauuua au...
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, 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 0.016.

Table 1. Correlation between ADAM9 expression and clinicopathological features of the 76 GC cases.

<table>
<thead>
<tr>
<th>Clinicopathological parameters</th>
<th>ADAM9 expression</th>
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<tbody>
<tr>
<td></td>
<td>Low (n=21)</td>
</tr>
<tr>
<td>Age (years)</td>
<td></td>
</tr>
<tr>
<td>≤60</td>
<td>8</td>
</tr>
<tr>
<td>&gt;60</td>
<td>13</td>
</tr>
<tr>
<td>Gender</td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>12</td>
</tr>
<tr>
<td>Female</td>
<td>9</td>
</tr>
<tr>
<td>Tumor diameter (cm)</td>
<td></td>
</tr>
<tr>
<td>≤5</td>
<td>15</td>
</tr>
<tr>
<td>&gt;5</td>
<td>8</td>
</tr>
<tr>
<td>Location</td>
<td></td>
</tr>
<tr>
<td>Distal third</td>
<td>16</td>
</tr>
<tr>
<td>Middle or proximal third</td>
<td>5</td>
</tr>
<tr>
<td>Histological classification</td>
<td></td>
</tr>
<tr>
<td>Poorly-differentiated adenocarcinoma</td>
<td>7</td>
</tr>
<tr>
<td>Middle/well-differentiated adenocarcinoma</td>
<td>4</td>
</tr>
<tr>
<td>Signet ring cell carcinoma</td>
<td>3</td>
</tr>
<tr>
<td>Mucinous adenocarcinoma</td>
<td>1</td>
</tr>
<tr>
<td>Local invasion</td>
<td></td>
</tr>
<tr>
<td>T1,T2</td>
<td>13</td>
</tr>
<tr>
<td>T3,T4</td>
<td>8</td>
</tr>
<tr>
<td>Lymph node metastasis</td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>12</td>
</tr>
<tr>
<td>Yes</td>
<td>9</td>
</tr>
<tr>
<td>TNM stage</td>
<td></td>
</tr>
<tr>
<td>I,II</td>
<td>10</td>
</tr>
<tr>
<td>III,IV</td>
<td>11</td>
</tr>
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</table>

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.
<0.05 for days 2-4 (Fig. 4C). These results indicate that knock-down 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
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 cells. The ADAM9 protein expression was significantly suppressed by overexpression of miR-126 in SGC-7901 cells.
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 reversed 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 protein 10 (PHF10) was ascertained as a promoter 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 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.

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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