Abstract. Previous studies have shown that microRNA-31 (miR-31) functions as a tumor-suppressor in various types of cancer. In the present study we found that miR-31 was significantly downregulated in gastric cancer (GC) as determined by microRNA (miRNA) array screening analysis. Although aberrant expression of miR-31 has been found in different types of cancer, its pathophysiological effect and role in tumorigenesis still remain to be elucidated. In the present study, we detected miR-31 expression in both metastatic GC cell lines and tissues that are potentially highly metastatic by real-time polymerase chain reaction (PCR). Transwell and scratch healing assays were conducted to examine whether the ectopic expression of miR-31 could modify the invasion and migration abilities of GC cells in vitro. We found that miR-31 inhibited GC metastasis in a nude mouse xenograft model of GC. Luciferase reporter assays demonstrated that miR-31 could directly bind to the 3’ untranslated region of RhoA and downregulate the expression of RhoA. Significant downregulation of miR-31 in 78 GC tissues and four GC cell lines was examined by real-time reverse transcription-PCR. Moreover, the decreased expression of miR-31 was demonstrated to be associated with lymph node metastasis, poor pT and pN stage, and invasion ability into lymphatic vessels as determined by the Mann-Whitney U test. We also found that miR-31 could inhibit cell invasion and migration abilities in vitro using the Transwell and scratch healing assays in BGC-823, SGC-7901, MGC-803 as well as AGS cells. Experiments in a nude mouse model revealed that miR-31 suppressed tumorigenicity in vivo. The luciferase activity assay and western blotting indicated that RhoA was the potential target of miR-31 in GC cells. Collectively, our results provide important evidence that the downregulation of miR-31 inhibited the invasion and migration abilities of GC cells through direct targeting of the tumor metastasis-associated gene, RhoA. These findings suggest that miR-31 may be a promising therapeutic candidate in the treatment of GC metastasis.

Introduction

Gastric cancer (GC) is the fourth most common cancer and the second leading cause of cancer-related deaths worldwide (1). Widespread metastasis is a major factor accounting for the poor prognosis and outcome of GC. Metastasis is considered a complex, multi-step process whereby cancer cells migrate from the primary location to a distant organ (2). This process initiates when primary tumor cells invade their surrounding tissues, followed by tumor cells entering into the blood system (intravasation), translocating through the vasculature, penetrating from the blood stream (extravasation) into the adjacent parenchyma tissue, forming micrometastases and finally proliferating to become secondary gross tumors (3). Many molecular regulators involved in this process have been identified, among which microRNAs (miRNAs) often play vital roles (4).

miRNAs are small non-coding RNA molecules and play important roles in regulating the expression of various genes by targeting mRNA through translational suppression or cleavage (5). Numerous studies have indicated that these short RNAs (usually 19-25 nucleotides in length) are involved in various biological processes including cell differentiation, proliferation, apoptosis, stress resistance, fat metabolism, tumorigenesis, as well as tumor metastasis (6-8). Numerous studies have revealed that miR-31 expression was specifically attenuated in metastatic breast cancer cells and could inhibit breast cancer metastasis by targeting multiple genes (9). Furthermore, Wang et al found that miR-31 may play an important role in colon cancer metastasis (10). We previously revealed that miR-31 was significantly downregulated in GC tissues through microarray analysis. However, its role in the metastasis of GC has remained largely unknown (11).

In the present study, we investigated the role of miR-31 in the metastasis of GC as well as its underlying mechanisms. We examined the expression of miR-31 in GC and studied its role in GC metastasis by both in vitro and in vivo analysis. We further examined the association of miR-31 expression with lymph node metastasis and found that low expression of miR-31 was associated with lymph node metastasis, poor...
pN stage and invasion into lymphatic vessels. Furthermore, we used bioinformatics analysis and the luciferase reporter assay to identify the potential target of miR-31. The present study, to the best of our knowledge, evaluated for the first time the role of miR-31 in the metastasis of GC using detailed data.

Materials and methods

Human tissue samples. Seventy-eight pairs of human GC and non-tumor adjacent tissues were obtained from patients who underwent surgical resection at the Chinese PLA General Hospital between 2012 and 2014. All of the samples were clinically and pathologically determined to be correctly labeled and frozen in liquid nitrogen and stored at -80°C. No systemic or local treatment was given to these patients before the surgery. The histological grade of the tumor was evaluated based on the World Health Organization (WHO) criteria and patients were staged according to tumor-node-metastasis (TNM) staging of the International Union Against Cancer (UICC)/American Joint Committee on Cancer (AJCC) System (2002). The present study was approved by the Research Ethics Committee of the Chinese PLA General Hospital. Informed consent was obtained from all the patients who provided samples.

Cell culture. The human GC cell lines BGC-823, MGC-803, SGC-7901 and AGS, as well as the normal gastric epithelium cell line GES-1 were obtained from the Chinese Academy of Sciences (Beijing, China) and were all maintained in our own laboratory and cultured in RPMI-1640 medium or Dulbecco's modified Eagle's medium (DMEM) (both from Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS; Gibco, Grand Island, NY, USA) in a humidified 5% carbon dioxide incubator at 37°C.

Cell culture and RNA extraction and real-time polymerase chain reaction (RT-PCR). Total RNA was extracted from tissues or cultured cells using the mirVana miRNA Isolation kit (Ambion, Foster City, CA, USA) based on the manufacturer's instructions. The poly(A) tail was added to the RNA using the Poly(A) Tailing kit (Ambion). Complementary DNA (cDNA) was synthesized using the TaqMan Reverse Transcription kit (Applied Biosystems, Foster City, CA, USA). Real-time PCR analyses were performed using the TaqMan Micro-RNA Assay kit (Applied Biosystems). miRNAs expression was calculated relative to U6 small nuclear RNA. Changes in the expression were calculated by the ΔΔCt method (12). The relative expression ratio of miR-31 was expressed as the fold change normalized to the endogenous reference (U6snRNA), and also relative to the non-tumor controls (adjacent non-tumor tissues and the GES-1 cell line). Therefore, a relative expression ratio of <1.0 was considered to be a low expression level, and a ratio >1.0 was considered to be a high expression level. The miRNA primer was purchased from Ambion. The PCR procedure and data analysis were performed using an iCycler (Bio-Rad, Hercules, CA, USA). Each sample was assessed in triplicate. The miR-31 primer sequence was as follows: 5'-AGGCAAGATGCTGGCATAGCT-3'; and the U6-RNA primer sequence was, 5'-TGACACGCAAATTCGTAATGTTTG-3'.

RNA oligoribonucleotides and cell transfection. RNA oligoribonucleotides and their corresponding normal controls (NCs) were purchased from RiboBio Co., Ltd. (Guangzhou, China). The cell lines were cultured to 50% confluence after being transfected into 6-well plates and were then transfected with a final concentration of 100 nM of RNA mimics or 200 nM of inhibitor and their corresponding NCs using Lipofectamine 2000 (Invitrogen) based on the manufacturer's instructions. The cells were harvested for further experiments at 48 h post-transfection.

In vitro migration/invasion assay. The migratory and invasive abilities of the cell lines were detected using Transwell assay. The Transwells (8-µm pore size; Corning Costar Corp., Corning, NY, USA) were placed into new 24-well plates. For the Transwell migration assay, 2.5x10⁴ cells were plated in the top chamber lined with a non-coated membrane. For the invasion assay, chamber inserts were coated with 35 µl of Matrigel (1:4 dilution) to form the basement membrane and were incubated for 4 h at 37°C. Furthermore, 5x10³ cells were plated in the top chamber. In both assays, the cells were suspended in medium without serum in the lower chamber which was used as a chemoattractant. After incubation at 37°C with 5% CO₂ in an incubator, cotton swabs were used to wipe off the upper layer of the Matrigel. After being fixed with 95% absolute alcohol and stained with 4’-diamidino-2-phenylindole (DAPI), the number of cells capable of migrating to the lower chamber was thus calculated by inverted microscopy (Olympus Corp., Tokyo, Japan) at a magnification of x200 in over 10 random fields for each well of the plate, and therefore the mean of the number of cells in each visual field represented the invasion ability of the cells. Each experiment was conducted in triplicate.

Scratch wound-healing assays. The cells were cultured into 6-well plates at a density of 3x10⁵ cells/well. After being cultured for 24 h at 37°C in an incubator with 5% CO₂, the cells were then transfected with miR-31 mimics and NC. A straight-line scratch was made on the bottom of the cell culture plate using a sterile 200-µl yellow pipette tip 5 h post-transfection. Fresh and complete media were added, and the wound healing ability was observed for 24 h. Images were obtained every 8 h.

In vivo metastasis assay. After transfection with the miR-31 mimics or stable NC using trypsin, SGC-7901 cell lines were harvested from tissue culture flasks and were washed three times with phosphate-buffered saline. Next, 5x10⁵ cells/l were suspended in 0.2 ml of serum-free RPMI-1640 medium and were injected into the lateral tail vein of each mouse (six in each group, female BALB/c, 6-8 weeks of age). The mice were sacrificed five weeks post-injection. The number of visible tumor lesions on the lung surface was counted. The lungs were then cut into serial sections, fixed with phosphate-buffered neutral formalin, stained with hematoxylin and eosin and then examined histologically. Nude mice were manipulated and cared for according to the NIH Animal Care and Committee guidelines of the Experimental Animal Center of the Chinese PLA General Hospital.

Bioinformatics analysis. The miRNA target predictions were obtained from PicTar (http://pictar.mdc-berlin.de/),
TargetScan 7.0 (http://www.targetscan.org/vert-70/) and miRDB (http://mirdb.org/miRDB). The overlapping targets were further studied using the Expression Analysis Systematic Explorer (EASE) based on the Gene Ontology database.

Vector construction and luciferase reporter assay. Luciferase reporters were constructed based on the firefly luciferase-expressing vector pGL3-control (Promega, Madison, WI, USA). To construct the pGL3-RhoA-3’ untranslated region (3’UTR), a partial 3’UTR of the RhoA segment of human RhoA mRNA (GenBank accession no. NM_001664) containing the putative miR-31 binding sites was thus amplified and cloned into the pGL3-control vector. The following primers were used for the amplification of RhoA: forward, 5’-GGCTGCCATCCGGAAGAAA-3’; and reverse, 5’-CACAAGACAAGGCACCCAGA-3’. In addition, we constructed a luciferase reporter that had DNA segments with scrambled target sites to miR-31 as a positive control.

SGC-7901 cells were transfected using Lipofectamine 2000 in 24-well plates based on the manufacturer’s instructions, with 0.8 µg of the firefly luciferase reporter vector and 0.08 µg of the control vector containing Renilla luciferase, pRL-TK (Promega). miR-31 (40 nM) or NC was used for each well. Firefly and Renilla luciferase activities were detected using a Dual-Luciferase Reporter Assay (Promega) 24 h post-transfection using the Centro LB 960 system (Berthold Technologies, Bad Wildbad, Germany).

Western blotting. Total protein was extracted from the cultured cells using the total protein extraction kit based on the manufacturer’s instructions (KeyGen Biotech Co., Ltd., Nanjing, China). Proteins were separated by 8% SDS polyacrylamide gels and were then transferred to polyvinylidene difluoride membranes (Millipore, Billerica, MA, USA) electrothermically. The membranes were blocked with 5% non-fat milk in TBS with 0.05% Tween-20 (TBST) at 37°C for 2 h. Antibodies targeting against RhoA (1:200; Santa Cruz Biotechnology, Santa Cruz, CA, USA) and β-actin (1:4,000; Sigma-Aldrich, St. Louis, MO, USA) were used. The proteins were visualized using an enhanced chemiluminescence (ECL) kit (Pierce, Rockford, IL, USA) and MF-Chemi BIS 3.2 Pro (Micro Photonics, Allentown, PA, USA) with GelCapture software (DNR Bio-Imaging Systems, Ltd., Jerusalem, Israel). The intensity of protein fragments was quantified by FluorChem 2.01 software (Alpha Innotech, San Leandro, CA, USA).

Statistical analysis. Data are presented as the means ± standard deviation (SD) based on at least three separate experiments. Statistical analysis was performed using Student’s t-test, a non-parametric test (Mann-Whitney U test between two groups and Kruskal-Wallis test for three or more groups). The correlations between the expression of miR-31 and RhoA protein were calculated by Chi-squared test and Spearman’s rank correlation. Differences were considered to be statistically significant for a P-value <0.05. Statistical analysis was performed using SPSS 16 (SPSS, Inc., Chicago, IL, USA).

Results

miR-31 expression and its correlation with the clinicopathological characteristics of GC. miR-31 was detected in all 78 pairs of GC tissues, and their matched non-tumor adjacent tissues using quantitative reverse transcription real-time PCR (qRT-PCR) analysis. Among the 78 patients with GC, 53 (67.95%) cases showed a >50% decrease in the miR-31 expression level relative to their matched adjacent non-tumor tissues (Table I). We then studied the correlation between miR-31 expression and the clinicopathological characteristics of GC. The Mann-Whitney U test revealed that lower expression levels of miR-31 were associated with higher lymph node metastasis, poorer pT and pN stage (Table I).

To further validate the role of miR-31 in GC cell metastasis, we examined the mRNA expression levels of miR-31 in four human GC cell lines: BGC-823, SGC-7901, MGC-803 and AGS. As shown in Fig. 2, the expression of miR-31 was lower in the BGC-823 and SGC-7901 cells, which had a relatively high metastatic potential, while miR-31 was highly expressed in the MGC-803 and AGS cells with a relatively low metastatic potential. These results revealed that the expression of miR-31 was negatively correlated with GC metastasis and may play an important role in GC metastasis.

miR-31 inhibits the invasion and migration of GC cells in vitro. The significant low expression of the miR-31 in GC cell lines with high metastatic potential prompted us to further
explore the potential biological significance of miR-31 in GC metastasis. We investigated the effects of miR-31 expression on the metastatic abilities of GC cells with different metastatic potential in vitro. An inhibitor and an NC oligonucleotide (MGC-803-inhibitor and MGC-803-NC, respectively) were thus, introduced into MGC-803 cells to perform metastasis assays in vitro. In addition, miR-31 mimics and an NC oligonucleotide (BGC-823-miR-31-mimics and BGC-823-NC) were constructed and introduced into BGC-823 cells. The results revealed that the depletion of miR-31 significantly enhanced the invasion and migration abilities of MGC-803 cells as determined by Transwell assay (Fig. 3A). Conversely, the increased expression of miR-31 significantly inhibited the invasion and migration abilities of BGC-823 cells (Fig. 3B).

Wound scratch assay results revealed that the cell migration distances in the BGC-823-miR-31-mimics and BGC-823-NC cells were 537±22 and 210±14 μm, respectively at 24 h after the wound scratch. There was a significant difference in the cell migration distance between the BGC-823-miR-31-mimics and the BGC-823-NC. In addition, the results indicated that the miR-31-mimics significantly inhibited the migration ability of the BGC-823 cells while the miR-131 inhibitor promoted the migration ability of the SGC-7901 cells.

miR-31 suppresses GC metastasis in a nude mouse xenograft model. To further confirm the aforementioned findings, we performed an in vivo study using a nude mouse xenograft model. SGC-7901 cells transfected with the miR-31 mimics or NC were injected into the lateral tail vein of nude mice, and the mice were sacrificed five weeks after inoculation. The number of lung metastatic lesions were markedly decreased in the nude mice injected with the miR-31 mimic-transfected cells compared with the negative control-injected ones (Fig. 5). These findings provide strong evidence that miR-31 also inhibited tumor metastasis in vivo.

RhoA may be a functional target of miR-31 in the process of GC metastasis. To investigate how the low expression of miR-31 contributes to the enhanced metastatic ability of GC, we explored potential regulatory targets of miR-31 using the combination of prediction tools, including PicTar, TargetScan and miRBase target. Although hundreds of different targets could be predicted, the genes involved in the migration or invasion process may be the real relevant targets related to the biological functions of miR-31. We next performed functional classification of the predicted targets using the Database for Annotation, Visualization and Integrated Discovery (DAVID) program (http://david.abcc.ncifcrf.gov/). Among these genes, RhoA attracted our attention and may possibly contribute to the metastasis of GC.

To confirm that RhoA was the direct target of miR-31, we constructed the luciferase reporter pGL3-RhoA-3’UTR. The scrambled target site pGL3-RhoA-MUT was also constructed as a negative control. All reporters were transfected into SGC-7901 cells. The luciferase activity of the pGL3-RhoA-3’UTR was significantly suppressed in the SGC-7901-miR-31-mimic cells compared with that in SGC-7901-NC cells when normalized to the control vector pRL-TK, containing Renilla luciferase. (Fig. 6). However, no significant difference in the relative luciferase activity of the pGL3-RhoA-MUT reporters was observed in the SGC-7901-miR-31-mimics compared with that in the SGC-7901-NC. These results revealed that RhoA proteins were negatively and directly regulated by miR-31 in GC cells, and that RhoA may serve as a target of miR-31.

Table I. Expression levels of miR-31 according to the clinicopathological features of patients with gastric cancer.

<table>
<thead>
<tr>
<th>Feature</th>
<th>N</th>
<th>miR-31a</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>≤50</td>
<td>30</td>
<td>0.41 (0.08-0.79)</td>
<td>0.373</td>
</tr>
<tr>
<td>&gt;50</td>
<td>48</td>
<td>0.45 (0.14-1.20)</td>
<td></td>
</tr>
<tr>
<td>Sex</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>51</td>
<td>0.43 (0.10-0.89)</td>
<td>0.500</td>
</tr>
<tr>
<td>Female</td>
<td>27</td>
<td>0.39 (0.09-1.21)</td>
<td></td>
</tr>
<tr>
<td>Tumor size (cm)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>≤3.5</td>
<td>35</td>
<td>0.44 (0.12-1.25)</td>
<td>0.621</td>
</tr>
<tr>
<td>&gt;3.5</td>
<td>43</td>
<td>0.42 (0.15-1.19)</td>
<td></td>
</tr>
<tr>
<td>Histological grade</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Well</td>
<td>25</td>
<td>0.41 (0.15-0.98)</td>
<td>0.500</td>
</tr>
<tr>
<td>Moderate and poor</td>
<td>53</td>
<td>0.44 (0.06-1.34)</td>
<td></td>
</tr>
<tr>
<td>pT stage</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T1+T2</td>
<td>36</td>
<td>0.53 (0.18-1.07)</td>
<td>0.01*</td>
</tr>
<tr>
<td>T3+T4</td>
<td>42</td>
<td>0.37 (0.05-0.96)</td>
<td></td>
</tr>
<tr>
<td>pN stage</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N0</td>
<td>24</td>
<td>0.73 (0.19-1.38)</td>
<td>0.03*</td>
</tr>
<tr>
<td>N1</td>
<td>20</td>
<td>0.36 (0.11-0.94)</td>
<td></td>
</tr>
<tr>
<td>N2</td>
<td>19</td>
<td>0.29 (0.05-0.66)</td>
<td></td>
</tr>
<tr>
<td>N3</td>
<td>15</td>
<td>0.24 (0.03-0.71)</td>
<td></td>
</tr>
<tr>
<td>Invasion into lymphatic vessels</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>33</td>
<td>0.63 (0.19-1.34)</td>
<td>0.000*</td>
</tr>
<tr>
<td>Positive</td>
<td>45</td>
<td>0.25 (0.07-0.67)</td>
<td></td>
</tr>
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</table>

*Median relative expression of miR-31, with means ± SD. The mean fold changes in miR-31 expression were calculated using the 2^(-ΔΔCt) method. *Statistically significant at P<0.05.
To further confirm these findings, we examined RhoA protein levels in both miR-31-transfected and anti-miR-31-transfected cells, as well as in their corresponding NC and parental SGC-7901 cells, using western blotting and real-time qRT-PCR analysis. We observed a significant decrease in the level of the endogenous RhoA proteins in SGC-7901-miR-31-mimic cells than that in the SGC-7901-NC cells when normalized to the endogenous reference β-actin protein. Overexpression of RhoA proteins was also observed in the anti-miR-31-transfected SGC-7901 cells compared with that in the anti-NC-transfected and parental SGC-7901 cells (Fig. 7). These results demonstrated that miR-31 may target and regulate RhoA in GC.
RhoA and RhoA-MUT with miR-31 mimics or normal controls (NCs) in the SGC-7901 cells. A greater decrease in the level of the endogenous RhoA proteins was demonstrated in the miR-31-transfected SGC-7901 cells compared with the NC-transfected parental SGC-7901 cells as determined by western blotting. A greater significant difference between groups (P<0.05). GC, gastric cancer.

The microRNA-31 (miR-31) gene is located at 9p21.3. Numerous studies have shown that miR-31 has different expression patterns in different types of cancer, for example, it was downregulated in urothelial carcinoma of the bladder (13), breast (9) and serous ovarian cancer (14), while it was upregulated in colorectal cancer (CRC) (15,16), head and neck squamous cell carcinoma (HNSCC) (17), hepatocellular carcinoma (18) and lung cancer (19). Although numerous studies have investigated the different expression levels of miR-31 in different types of cancer, the function of miR-31 still remained unclear.

In the present study, we demonstrated that miR-31 expression was markedly downregulated in both GC tissues and cell lines, suggesting that the low expression of miR-31 may be associated with GC development. Furthermore, we demonstrated that the low expression of miR-31 was significantly associated with a higher rate of lymph node metastasis, poorer pT and pN stage, and invasion into lymphatic vessels. Lymph node metastasis, pT and pN stage were independent prognostic factors for the overall survival rates of GC patients, and lymphatic vessel invasion was identified as an independent prognostic factor predicting lymph node metastasis. GC patients with lymph node metastasis, poor pT and pN stage and invasion into lymphatic vessels tended to have a lower survival rate (20,21). This suggested that the expression of miR-31 may act as an independent prognostic factor for the overall survival rates of GC patients.

Recent studies revealed that miR-31 expression was specifically attenuated in metastatic breast cancer cell lines, and miR-31 could inhibit breast cancer metastasis by targeting multiple genes (9). In the present study, we found that overexpression of miR-31 suppressed GC cell invasion and metastatic abilities. These results were consistent with a previous study, which revealed that blockade of miR-31 expression significantly decreased the invasion and migration abilities of the HNSCC cell line (17). The development of GC metastasis is characterized by multiple genetic alterations. Previous studies have investigated the effects of specific miRNAs on the pattern of GC metastasis. Zheng et al found that miRNA-145 inhibited the metastasis and angiogenesis abilities of GC cells by targeting the 3'UTR of Ets1 (22). The expression of let-7f was decreased in gastric tumors compared with that in normal gastric tissue and inhibited tumor metastasis by targeting MYH9 (23). Tie et al found that decreased miR-218 expression was associated with advanced clinical stage lymph node metastasis and poor prognosis in GC patients, and that the overexpression of miR-218 in metastatic cells inhibited migration, invasion, and metastasis formation as shown both by in vitro and in vivo experiments (24). Although numerous studies have investigated the role of miRNA in GC metastasis, the underlying mechanisms remain unclear. Notably, to the best of our knowledge, few studies have investigated whether GC metastasis is regulated by miRNA-31.

To examine the molecular mechanism by which miR-31 functioned as a metastasis suppressor in GC, we used the luciferase reporter assay and western blotting to confirm that RhoA was a potential target of miR-31 in GC cells. RhoA, with a molecular mass of 21 kDa, is the most widely studied member of the Rho GTPase family, and belongs to the Ras superfamily of small G proteins. The Rho GTPase family consists of at least 11 members sharing ≥50% sequence identity, with one of the most well known members being RhoA. RhoA acts as a molecular switch in cells, regulating signal transduction from cell surface receptors to intracellular target molecules, and is involved in various biological process, including cell morphology (25), motility (26),...
cytokinesis (27,28), smooth muscle contraction (29,30) and tumor progression (31,32).

In conclusion, the present study demonstrated that miR-31 could suppress the metastasis of GC by directly binding to the 3'UTR of RhoA. Although there is still a lot to learn concerning the role of miR-31 in GC tumorigenesis, miR-31 may serve as a promising potential target for GC treatment.

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