GAS5 Inhibits Gastric Cancer Cell Proliferation Partly by Modulating CDK6

Xiaqiang Guo a, Kaiyuan Deng a, Hao Wang a, Jiazeng Xia a, b, Ting Shan a, Zheng Liang a, Lubing Yao a, Shimao Jin c

a Department of General Surgery, Nanjing Medical University Affiliated Wuxi Second Hospital, Wuxi, China; b Department of Translational Medicine Center, Nanjing Medical University Affiliated Wuxi Second Hospital, Wuxi, China; c Department of Gastroenterology, Nanjing Medical University Affiliated Wuxi Second Hospital, Wuxi, China

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Introduction
Gastric cancer (GC) is one of the most common malignant tumors of the alimentary system, especially in East Asia. In 2008, worldwide, approximately 989,600 cases of GC were diagnosed and 738,000 patients with GC died [1]. With the improvement of clinical treatment, the 5-year survival of patients with early GC has significantly increased, but the prognosis of patients with advanced GC is still poor. Hence, early diagnosis and treatment are effective ways to improve the prognosis of GC patients, and it is of vital clinical value to seek novel biomarkers for early diagnosis or therapeutic targets.

Introduction: As it is not clear whether growth arrest-specific 5 (GAS5) inhibits gastric cancer (GC) cell proliferation by regulating cell cycle, we analyzed the effect of GAS5 on cell cycle regulation of GC cells and explored the underlying mechanism. Methods: We measured GAS5 levels in GC tissues and corresponding normal tissues, and analyzed the role of GAS5 in regulation of cell proliferation and cell cycle in GC cells using CCK-8 assay and flow cytometry. We also measured the expression of P21 and CDK6 proteins after transfection of AGS and MGC-803 cells with pLJM-GAS5 and GAS5 siRNA, respectively, by western blotting. Results: GAS5 expression was significantly lower in GC tissues relative to normal tissues, and its lower expression was correlated with larger tumor size and a more advanced clinical stage of GC. GAS5 induced growth arrest of GC cells through inhibition of G1–S phase translation. The action of GAS5 may be mediated by upregulation of P21 and suppression of CDK6. Conclusion: These data enhance our understanding of the important role that GAS5 plays in the molecular etiology of GC and suggest a potential of GAS5 as a new therapeutic target for GC treatment.

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in a humidified environment containing 5% CO2 at 37 °C. 

The lncRNA GAS5 is encoded by the gas5 gene, which was originally isolated from mouse NIH 3T3 cells and comprises 12 exons that lack protein-coding capacity and has numerous snoRNAs in its introns [10, 11]. Recently, evidence has been found that a decrease in GAS5 expression is associated with tumor formation in several types of cancer, and that an increase in GAS5 expression can suppress tumors probably via inhibition of cell proliferation and induction of apoptosis [12–15]. Moreover, decreased expression of GAS5 is found in GC tissues as well as cell lines, and can promote cell proliferation and induce apoptosis by regulation of P21 and E2F1 protein [16]. However, it is not yet clear whether GAS5 inhibits GC cell proliferation by regulation of the cell cycle. Hence, in this study, we analyzed the effect of GAS5 on the cell cycle regulation of GC cells and explored the underlying mechanism.

Materials and Methods

Clinical Samples Collection

Paired GC tissues and adjacent normal gastric mucosa were obtained from 40 patients who underwent radical resection for GC between October 2012 and July 2013 at Nanjing Medical University Affiliated Wuxi Second Hospital, Wuxi, China. Informed consent was obtained from all patients before operation. After resection, all samples were immediately frozen in liquid nitrogen and stored at 80°C until RNA extraction. None of the patients had received other anti-tumor therapy before surgery, and the Ethics Committee of Nanjing Medical University approved the study protocol.

Cell Culture

Three GC cell lines (AGS, BGC-823 and MGC-803) were obtained from the National Platform of Experimental Cell Resources for Sci-tech (China). MGC-803 was maintained in DMEM medium (Gibco, USA) with 10% fetal bovine serum (Hyclone), whereas BGC-823 and AGS were maintained in RPMI-1640 medium with 10% fetal bovine serum (Gibco), Cat. no.15596–018) according to the manufacturer's protocol. To eliminate genomic DNA (gDNA) contamination, we synthesized cDNA using a vitrogen, Cat. no.15596–018) according to the manufacturer's protocol. To determine the expression quantities of GAS5 in GC and adjacent normal tissue samples, 31 (77.5%) cases exhibited decreased expression of GAS5 and its control siRNA were purchased from GenePharma (Suzhou, China). The sequences of siRNAs were: GAS5 siRNA sense 5'-CUUCGCGCCAGCUAAUAUU-3', anti-sense 5'-UUAACCGUGCCAGCAGAAUU-3' [17]; and control siRNA sense 5'-UUCUCGAAACGUGUACUG-3', antisense 5'-ACGUACCAGCUUGCAGATT-3'. CDK6 siRNAs were also purchased from GenePharma.

Expression of GAS5 Significantly Decreased in GC Tissues

To construct the pLJM-GAS5 plasmid, human GAS5 cDNA from AGS cells was amplified by RT-PCR using the forward primer: aataataccggtTTTCGAGGTAGAGTCGCA and reverse primer: aataatgtgtTGAGACAAAATT-TATATAATTG, and then subcloned into a pLJM vector. siRNA against the human GAS5 and its control siRNA were purchased from GenePharma (Suzhou, China). The sequences of siRNAs were: GAS5 siRNA sense 5'-CUUCGCGCCAGCUAAUAUU-3', anti-sense 5'-UUAACCGUGCCAGCAGAAUU-3' [17]; and control siRNA sense 5'-UUCUCGAAACGUGUACUG-3', antisense 5'-ACGUACCAGCUUGCAGATT-3'. CDK6 siRNAs were also purchased from GenePharma.

Plasmid Construction and Cell Transfection

To construct the pLJM-GAS5 plasmid, human GAS5 cDNA from AGS cells was amplified by RT-PCR using the forward primer: aataataccggtTTTCGAG- GATAGAGTCGCA and reverse primer: aataatgtgtTGAGACAAAATT-TATATAATTG, and then subcloned into a pLJM vector. siRNA against the human GAS5 and its control siRNA were purchased from GenePharma (Suzhou, China). The sequences of siRNAs were: GAS5 siRNA sense 5'-CUUCGCGCCAGCUAAUAUU-3', anti-sense 5'-UUAACCGUGCCAGCAGAAUU-3' [17]; and control siRNA sense 5'-UUCUCGAAACGUGUACUG-3', antisense 5'-ACGUACCAGCUUGCAGATT-3'. CDK6 siRNAs were also purchased from GenePharma.

Statistical Analyses

An independent-sample t-test or a 1-sample t-test was performed to analyze the differences between groups. All data represent as means ± standard deviation (SD) from 3 independent experiments. All statistical analyses were performed using SPSS 20.0 software (SPSS, USA). A significant difference was assumed when the p value was less than 0.05.

Results

Expression of GAS5 Significantly Decreased in GC Tissues

To verify whether aberrant expression of GAS5 is correlated with GC, we determined GAS5 expression levels in 40 GC tissues and corresponding normal mucosal tissues using qRT-PCR. Among the 40 paired tissues, 31 (77.5%) cases exhibited decreased expression of GAS5 in GC tissues relative to normal mucosal tissues (fig. 1a). Furthermore, the expression of GAS5 was significantly lower in GC tissues than that in adjacent normal tissues (p < 0.001).
We then explored the correlation between GAS5 expression levels in GC tissues and clinicopathological factors in GC patients, and found that lower GAS5 expression was associated with larger tumor sizes and more advanced stages (fig. 1c, 1d).

**Manipulation of GAS5 Expression in GC Cells**

We next determined the expression of GAS5 in GC cell lines by qRT-PCR, and showed that the expression of GAS5 was lower in GC cell lines (AGS, BGC-823) compared with that in the human gastric epithelial mucosa cell line GES-1 (fig. 1e). To manipulate the expression of GAS5 in GC cells, a pLJM-GAS5 plasmid was transfected into AGS cells. After transfection for 48 h, cells were harvested and the level of GAS5 was analyzed by qRT-PCR. As shown in fig. 2a, GAS5 expression was increased approximately 70-fold in AGS cells relative to control cells. In addition, we downregulated the expression of GAS5 by transfection of MGC-803 cells with siRNA, and qRT-PCR analysis showed that GAS5 expression dropped to 30% (fig. 2b).

**Effect of GAS5 on GC Cell Proliferation In Vitro**

Because of the ectopic expression of GAS5 in GC tissues and cells we investigated the biological role of GAS5 in tumorigenesis. To increase the expression of GAS5, pLJM-GAS5 was transfected into AGS cells, and overexpression of GAS5 significantly inhibited AGS cell proliferation (fig. 2c). In contrast, GAS5 siRNA was used to suppress the GAS5 expression in MGC-803 cells and led to the promotion of cell proliferation (fig. 2d).

**Effect of GAS5 on Cell Cycle in GC Cell Lines**

Next, we explored the potential mechanisms by which GAS5 could affect GC cell proliferation. Using a cell cycle assay, we found that knockdown of GAS5 in MGC-803 cell line led to a decrease in the percentage of cells in G0/G1 phase and an increase in the percentage of cells in S and G2/M phase (fig. 2e). By contrast, the proportion of cells in the G0/G1 phase significantly increased in AGS cells transfected with pLJM-GAS5 compared with those transfected with empty plasmid. Meanwhile, compared with the control group, pLJM-GAS5 group displayed fewer cells in S phase (fig. 2f).

**GAS5 Modulated the Expression of P21 and CDK6 in GC**

Further study was performed to explore the underlying mechanism involved in GC cell cycle arrest induced by GAS5 overexpression. As shown in fig. 3a and 3b, GAS5 overexpression significantly increased the protein levels of P21 and inhibited the protein levels of CDK6 in AGS cells. However, suppression of GAS5 significantly suppressed P21 expression and enhanced the CDK6 expression in MGC-803 cells (fig. 3c, 3d). We also investigated whether GAS5 inhibited GC cell proliferation by regulating CDK6. The results
CDK6. More recently, Shi et al. [17] have demonstrated the silencing of GAS5 could regulate cell proliferation and cell cycle by modulation of its introns [11]. However, Kino et al. [20] reported that GAS5 was capable of competing with DNA glucocorticoid response elements for binding to the DNA-binding domain of the glucocorticoid receptor, resulting in the inhibition of the induction of several response genes mediated by glucocorticoid. Therefore, mature GAS5 can also exert many cellular functions by other mechanisms. More recently, GAS5 has been shown to be differentially expressed in normal and tumor tissues, and to function as a tumor suppressor through induction of growth arrest and apoptosis. Lu et al. [13] found that the expression of GAS5 in pancreatic cancer samples was significantly lower than that in normal tissues. Moreover, gain-of-function and loss-of-function analysis indicated that GAS5 could significantly inhibit the proliferation of AGS cells 

GAS5 Inhibits GC Cell Proliferation Partly by Regulation of CDK6 Protein

In conclusion, GAS5 can inhibit GC cell growth by modulation of cell cycle, which may be mediated by regulation of P21 and CDK6 protein. These results enhance our understanding of the important role of GAS5 in the molecular etiology of GC and suggest that GAS5 may potentially present a new therapeutic target for GC treatment.

Online Supplemental Material

Fig. 4. GAS5 regulates GC cell cycle by regulating CDK6. (a, b) Western blotting was performed to determine the CDK6 protein expression after transfection of MGC-803 with GAS5 siRNA or co-transfection of MGC-803 with GAS5 siRNA and CDK6 siRNA. (c) When GAS5 siRNA and CDK6 siRNA were co-transfected into MGC-803 cells, the proliferation induced by suppression of GAS5 is diminished. *p < 0.05, **p < 0.01.

To access fig. 4, please refer to www.karger.com/?DOI=433499.
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Disclosure Statement

The authors declare no conflicts of interest.

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