Long non-coding RNA growth arrest-specific transcript 5 (GAS5) protects ovarian cancer cells from apoptosis

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Received March 16, 2016; Accepted July 12, 2016; Epub September 1, 2016; Published September 15, 2016

Abstract: Epithelial ovarian cancer (EOC) is a main cause of death in malignant tumor of women genital system. This study aims to investigate the underlying role of growth arrest-specific transcript 5 (GAS5) in EOC. In vivo expression of GAS5 in 60 EOC specimens was evaluated by quantitative reverse transcription QRT-PCR, which used to study the differences of GAS5 expression between EOC tissues and normal ovarian epithelium. There were no significant differences of GAS5 expression between normal ovarian epithelium and benign epithelial lesions; however, GAS5 expression was lower in EOC tissues compared with normal ovarian epithelial tissues (6.44-fold), which was closely related to lymph node metastasis (P=0.025) and tumor node metastasis stage (P=0.035). Moreover, exogenous GAS5-inhibited proliferation promoted apoptosis and decreased migration and invasion in ovarian cancer cells. Finally, through Western blot analysis, overexpression of GAS5 protein could decrease the expression of Cyclin A, Cyclin D, Cyclin E, and PCNA. Conclusions: This study revealed that GAS5 is down-regulated in EOC specimens, and GAS5 inhibits EOC cell proliferation, migration, and invasion, and promotes the cell apoptosis. GAS5 can serve as a novel therapeutic target in patients with EOC.

Keywords: Long non-coding RNA, epithelial ovarian cancer, GAS5, apoptosis, mitochondrion

Introduction

Ovarian cancer is the main cause of death in malignant tumor of women genital system, which owns a high morbidity in developing countries. Although, rapid progress in diagnosis and treatment of ovarian cancer [1], it is still the fifth major cause of death in women cancer patients. Of all ovarian carcinoma cases, Epithelial ovarian cancer (EOC) accounts for 90% of morbidity [2]. Moreover, EOC can spread to peritoneal cavity via peritoneal fluid, leading to the inefficiency of surgery and chemotherapy treatment. Thus, a better understanding of the mechanisms involved in EOC and more effective therapeutic approaches are urgently needed. It is also reported that the loss of function proteins such as, Kras, Brcal/2, Tp53, Rb, and PTEN will induce epithelial ovarian cancer [3-5].

Long non-coding RNA (lncRNA) is longer than 200 nucleotides but does not translate into proteins [7]. Nowadays, lncRNA is emerging as important regulators of tumor initiation and progression [6]. The tempting potential in most of the lncRNAs has stimulated keen interests, especially in the cancer complexity. Recently, MALAT1, lncRNA, has been reported to promote cancer metastasis [8] and resist rapid RNA decay [9-11]. CCAT1-L has been shown to play a role in MYC transcriptional regulation and promote a long-range chromatin looping [12-14]. GAS8-AS1 and LPAR4 were identifies as novel papillary thyroid carcinoma driver alteration [15]. These findings indicate that lncRNAs may act as important regulators in tumorigenesis.

This study describes the lncRNA GAS5, which is alternatively spliced and is transcribed from a locus of 1q25.1 [16]. GAS5 has a valuable biological function because it is a multiple-small-nucleolar-RNA (snRNA) host gene [17]. GAS5 competes with the glucocorticoid response elements in the genome for binding to these recep-
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tors and promotes cells apoptosis [18], which had been originally identified in leukemia cells and NIH3T3 cells [19]. It is also reported that the inhibition of mammalian target of rapamycin (mTOR) pathway via rapamycin depends on GAS5 [20]. GAS5 negatively regulates miR-21 possibly through the RNA-induced silencing complex [21]. Additionally, it had also shown that GAS5 is down-regulated in some cancer cell lines and tissues [22-25]. Some research showed GAS5 inhibited malignant pleural mesothelioma (MPM) cell growth by inhibiting hedgehog and PI3K/mTOR signal pathway in MPM [24]. GAS5 as a tumor suppressor in non-small-cell lung cancer (NSCLC) was mediated by p53-independent and p53-dependent pathways [26]. Furthermore, GAS5 could inhibit E2F1 and cyclin D1 and thus leads to decreased gastric cancer cell proliferation [25]. However, the role of GAS5 in ovarian cancer is not known up to now.

The fundamental mechanisms of GAS5 on tumorigenesis remain largely unknown, despite GAS5 has been indicated to take part in suppression on malignant tumor. The potential mechanisms can be related to the fact that GAS5 regulates nonsense-mediated RNA decay pathway [20, 27] or down-regulates c-Myc [28]. These findings provide strong evidence that GAS5 plays an important role in guiding the cell fate toward inhibiting proliferation.

Signaling pathways in cancer cell biology are increasingly being used to investigate the mechanisms underlying tumor relapse and dormant behavior in many tumors [29, 30], some evidences suggest that the MAPK pathway plays an important role in several tumors [31]. The ERK signaling cascade is one of most extensively studied MAPK pathways, hyperactivation of which has been demonstrated to play major roles in tumor cell proliferation and metastasis. while clinical and experimental evidence is consistent with most negative regulators of the ERK pathway being tumor suppressors [32]. The potential role of ERK pathway-dependent proliferation pathway in the proliferation effect of GAS5 has not been explored.

In our study, we demonstrated a significant decrease in the expression of GAS5 in EOC tissues, which was associated with clinicopathological parameters. Moreover, the overexpression of GAS5 obviously inhibited the proliferation of ovarian cancer cell lines. Together, these results reveal an evidence for proliferation regulation between IncRNA GAS5 and ovarian cancer, indicating a potential target of diagnosis and gene therapy in the disease.

Materials and methods

Cell lines and tissue samples

The human ovarian cancer HO8910 (Bioleaf Biotech Co., Ltd, Shanghai, China) and A2780 cells (Chuanbo Biotechnology Co., Ltd, Nanjing, China) were grown in RPMI-1640 (Nyclone, Beijing, China) and DMEM medium (Nyclone, Beijing, China), respectively, supplemented with 10% fetal bovine serum (FBS) (Sijiqing, Zhejiang, China) in a humidified incubator (37°C, 5% CO₂).

Specimens of EOC tissue (n=60, without radiotherapy or chemotherapy), normal ovarian epithelial tissues (n=13) and benign ovarian epithelial lesions (n=10) were collected at the Second Affiliated Hospital of Harbin Medical University (China) between Mar 2012 and Apr 2014 (median age years 55, range 37-79). The samples of normal ovarian tissue were collected from hysterosalpingo-oophorectomy following uterine myoma, endometriosis, or adenomyosis. Written informed consent was obtained from all participants. The study was approved by the Human Ethics Committee of the Second Affiliated Hospital of Harbin Medical University (No.: 2015-yan-167).

Real-time polymerase chain reaction

Total RNA was extracted from tissues and cell lines by using Trizol reagent (Invitrogen, CA, USA), and RNA purity was identified by optical density (OD) 260 nm/OD 280 nm. The reverse transcription reactions were performed using oligo dT primers and a Reverse Transcriptase Kit (BIONEER, Shanghai, China). Real-time polymerase chain reaction (PCR) was performed with the ABI 7300 real-time PCR system (Applied Biosystems, CA, USA). For all human ovarian tissues and cell lines, real-time PCR was performed using TaqMan MGB primers and probe specially for human GAS5 (designed by GenePharma, Shanghai, China; forward primer: 3'-CTTCTGGGCTCAAGTGATCCT-5'; reverse primer: 3'-TTGTGCCATGAGACTCCATCAG-5'; probe: CCTCCCAGTGGTCTTT) and eukaryotic
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18S rRNA (GenePharma, Shanghai, China; forward primer: 5'-TTGACTCAACGGGAAACC-3'; reverse primer: 5'-CACGGGAATCGAAAGAGC-TAC-3'; probe: CCGGACACGGACAGGATTGACACGAT) served as the endogenous control. All of the real-time PCRs were performed in triplicate. The relative quantification of GAS5 expression was calculated using the 2^ΔΔCT method relative to 18s rRNA.

Transfection

To overexpress GAS5, plasmid pCDNA-GAS5 was constructed by Shanghai GenePharma Co., Ltd of China containing the whole genome sequence of GAS5 (NCBI Reference Sequence: NR_002578.2). The plasmid carrying pCDNA-GAS5 (1 μg/μl) was transfected into ovarian cancer cell lines by using Lipofectamine 2000 (Invitrogen, CA, USA). (Lipofectamine 2000: pCDNA-GAS5=1:1.4).

Terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL)

The HO8910 and A2780 cells were transfected with pCDNA-GAS5 or empty vector, and cultured in six-well plates for 48 h. They were then fixed with 4% paraformaldehyde solution, the apoptotic cells were labeled using the in situ cell death detection kit (Beyotime, Shanghai, China), the fluorescence was detected by using a Nikon ECLIPSE TE2000-S fluorescence microscope (Nikon, Tokyo, Japan) and counted by Image Pro-Plus 6.0. (Media Cybernetics, MD, USA) in three different experiments, the red fluorescence marked cells were served as apoptotic cells.

Cell apoptotic analysis

HO8910 and A2780 cells (1~2 × 105) were treated with a pCDNA-GAS5 or an empty vector; then, these were placed in 6-well plates. After 24-h incubation, the cells were trypsinized and then fixed in 70% ethanol for 3 h at 4°C; 3 h later, the cells were incubated with propidium iodide (20 ×) and RNease-A (50 ×) for 30 min in the dark. Cells were collected and analyzed for apoptosis using a flow cytometer (BD Biosciences, NJ, USA) after propidium iodide staining. The results were analyzed by BD Accuri C6 software. The experiments were repeated at least thrice.

Transwell assay

Transwell assays were performed using a Costar chamber (Corning Costar Corp, MA, USA). The bottom chambers were filled with a culture medium containing 10% FBS. Different samples of tansfected HO8910 or A2780 cells (5 × 104) were suspended in a culture medium without FBS, and then the cells were seeded into the upper chambers. The transwell chamber containing an 8-μm pore size polycarbonate membrane filter was coated either with (for invasion) or without (for migration) matrigel. After 48 h of culture at 37°C, the upper layer of cells were removed before visualization, and the cells on the lower surface were fixed and stained with 0.5% crystal violet. The cells were counted by Image Pro-Plus 6.0. (Media Cybernetics, MD, USA) in five random fields and photographed. The experiments were performed three times.

Cell proliferation and viability

Cell proliferation and viability of HO8910 and A2780 were evaluated using 3-[4,5-dimethylthiazol-2-y1]-2,5-diphenyl-tetrazoliumromide (MTT) (Amresco, OH, USA) assay. Briefly, after 6 h transfection with pCDNA-GAS5, about 5 × 103 cells per well was seeded in a 96-well plate at 37°C. Each well was repeated six times. After further incubation with different times (24 h, 48 h, and 72 h), 20 μl MTT (0.5 mg/ml) was added to each well and further incubated for 4 h. Then the medium was removed and dimethylsulfoxide was added to dissolve the MTT formazan crystals. The cell viability and proliferation were determined by OD450 value. The experiments were performed three times.

Colon formation assay

About 800 pCDNA-GAS5- or empty vector-transfected HO8910 and A2780 cells/wells were placed onto a six-well plate and maintained in a medium containing 10% FBS, replacing the medium every 3 days. After 14 days, the colonies were fixed with methanol and stained with 0.5% crystal violet (Amresco, Shanghai, China). The visible colonies were manually counted. The experiments were performed three times.
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Western blot analysis

The pCDNA-GAS5- or empty vector-transfected HO8910 and A2780 cells were lysed using a lysis buffer (Beyotime, Shanghai, China) that contained the phenylmethanesulfonyl fluoride. The protein concentrations were determined by bicinchonininc acid protein assay. The samples (50 µg) were electrophoresed on a 10% sodium dodecyl-sulfate-polyacrylamide gel electrophoresis and transferred onto nitrocellulose membranes (BioTrance, MI, USA). Then these were incubated with specific antibodies. Specific bands were detected using an Enhanced Chemiluminescent (Beyotime, Shanghai, China) chromogenic substrate. The protein expression was analyzed using densitometry (Quantity One Software; Bio-Rad, CA, USA). β-actin (ZSGB-BIO, Beijing, China) was used as a control. Additionally, anti-Cyclin A and anti-Cyclin D antibodies were purchased from Santa Cruz Biotechnology (CA, USA). Anti-p38, anti-p-p38, anti-JNK, anti-p-JNK, anti-ERK, anti-p-ERK antibodies were purchased from Beyotime Institute of Biotechnology (Shanghai, China).

Statistical analysis

All statistical analysis was performed with SPSS 17.0 (Chicago, IL, USA). The data were presented as means ± standard error of mean from at least three independent experiments. Statistical analysis was performed using chi-

Table 1. Correlation between GAS5 expression and clinicopathological parameters of EOC

<table>
<thead>
<tr>
<th>Clinicopathological parameters</th>
<th>No. of cases</th>
<th>Relative GAS5 expression</th>
<th>P-value&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td></td>
<td>Low</td>
<td>High</td>
</tr>
<tr>
<td>≤50</td>
<td>22</td>
<td>13</td>
<td>9</td>
</tr>
<tr>
<td>&gt;50</td>
<td>38</td>
<td>18</td>
<td>20</td>
</tr>
<tr>
<td>Differentiation</td>
<td></td>
<td>Low</td>
<td>High</td>
</tr>
<tr>
<td>Well, moderate</td>
<td>26</td>
<td>14</td>
<td>12</td>
</tr>
<tr>
<td>Poor</td>
<td>34</td>
<td>18</td>
<td>16</td>
</tr>
<tr>
<td>Location</td>
<td></td>
<td>Low</td>
<td>High</td>
</tr>
<tr>
<td>Unilateral</td>
<td>25</td>
<td>13</td>
<td>12</td>
</tr>
<tr>
<td>Bilateral</td>
<td>35</td>
<td>18</td>
<td>17</td>
</tr>
<tr>
<td>Lymph node metastasis</td>
<td></td>
<td>Low</td>
<td>High</td>
</tr>
<tr>
<td>Positive</td>
<td>40</td>
<td>28</td>
<td>12</td>
</tr>
<tr>
<td>Negative</td>
<td>20</td>
<td>8</td>
<td>12</td>
</tr>
<tr>
<td>FIGO stage</td>
<td></td>
<td>Low</td>
<td>High</td>
</tr>
<tr>
<td>I</td>
<td>15</td>
<td>5</td>
<td>10</td>
</tr>
<tr>
<td>II/III/IV</td>
<td>45</td>
<td>29</td>
<td>16</td>
</tr>
</tbody>
</table>

<sup>a</sup>Chi-squared test; <sup>b</sup>P<0.05. GAS5, growth arrest-specific transcript 5; EOC, epithelial ovarian cancer.

Figure 1. Relative IncRNA GAS5 expression in EOC tissues and the clinical significance. Relative expression of IncRNA GAS5 was analyzed by real-time PCR in ovarian normal epithelial tissues (N, n=13), ovarian benign epithelial lesions (B, n=10), and epithelial ovarian cancers (C, n=60). IncRNA GAS5 expression was normalized to 18s rRNA expression. The data are presented as a fold-change in the tumor tissues relative to the normal tissues. B. The correlation between GAS5 and lymph node metastasis. Expression of IncRNA GAS5 was significantly lower in patients with lymph node metastasis than those without lymph node metastasis. C. The correlation between GAS5 expression and clinical stage. Expression of IncRNA GAS5 was significantly lower in patients with an advanced clinical stage than those with an early stage. All values are denoted as means ± SEM. from at least three separate experiments. *P<0.05, **P<0.01. *P<0.05 represents negative lymph node metastasis compared with positive lymph node metastasis, **P<0.01 represents normal ovarian epithelium compared with EOC tissues and FIGO stage of I compared with II/III/IV.
square test, Student t test, or one-way analysis of variance followed by a post hoc test, where appropriate. Correlation were estimated by Pearson’s correlation analysis. Differences were considered to be significant at P<0.05.

Results

GAS5 is down-regulated in EOC tissues

To investigate the expression of IncRNA GAS5 in normal and EOC patients, real-time PCR was used to examine the GAS5 expression in normal ovarian epithelial tissues, benign ovarian epithelial lesions, and EOCs. The expression of GAS5 in normal ovarian epithelial tissues (N) and benign epithelial lesions (B), showed no statistical significance (Figure 1A). While, there appears to be a significant reduction of GAS5 expression in EOCs (C) compared with normal ovarian epithelial tissues (6.44-fold) (Figure 1A). Then, the clinicopathological parameters, such as age, differentiation, location, lymph node metastasis, and FIGO stage, were analyzed to assess the expression of GAS5 and clinical significance of EOC. As shown in Figure 1B, 1C, and Table 1, samples with lymph node metastasis and advanced FIGO stage owned the lower expression of GAS5. These data indicate that the decreased expression of GAS5 is related to EOC.

Effect of GAS5 on apoptosis in ovarian cancer cell lines

Apoptosis-resistant phenotype is the major feature of cancer cells [33]. To study the role of GAS5 in apoptosis, A2780 and HO8910 cells transfected with pCDNA-GAS5 or empty vector were monitored using flow cytometry. As shown in Figure 2A-C, ectogenic GAS5 obviously promoted the apoptosis of the cells. Similarly, TUNEL staining showed that the number of apoptotic cells was more in the GAS5-over-
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expressed cells than in the controls (Figure 2D-F). The results mentioned above suggest that GAS5 displays a critical function in pro-apoptosis of ovarian cancer cells.

**Effect of GAS5 on migration and invasion in ovarian cancer cell lines**

Most ovarian cancer patients die of tumor metastasis, and there are a few research reports about the relationship between IncRNAs and neoplastic metastasis [34, 35]. To study the effect of GAS5 in vitro on migration and invasion of ovarian cancer cell lines, a Costar chamber without (for migration) or with (for invasion) matrigel was used. The treatment of A2780 and HO8910 cells is the same as before. Compared with the ones treated with empty vector, the ability of migration and invasion had been weakened in GAS5-overexpressed A2780 (Figure 3A-C) and HO8910 (Figure 3D-F) cells. Conclusively, GAS5 can inhibit migration and invasion in ovarian cancer cells.

**Effect of GAS5 on proliferation in ovarian cancer cell lines**

With the aim of manipulating the GAS5 expression in ovarian cancer cells, the pCDNA-GAS5 or an empty vector was transfected into A2780 and HO8910 cells. After 24 h of transfection, the level of GAS5 was well up-regulated in A2780 (74-fold) and HO8910 (63-fold) cells, respectively (Figure 4A). To ascertain the role of GAS5 in the proliferation of EOC, the overexpression of A2780 and HO8910 cells of GAS5 were analyzed. MTT assay was used to assess the biological role of GAS5 in proliferation. Compared with the cells transfected with empty vector, the ones transfected with pCDNA-GAS5 demonstrated significantly decreased viability.
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(Figure 4B and 4C). Besides, it was found that the overexpression of GAS5 greatly weakened the ability of colony forming using the colony formation assay (Figure 4D-F). It is well known that Cyclin A, Cyclin D, Cyclin E and PCNA are the symbols of proliferation, which were used to study the role in GAS5-inhibited proliferation of ovarian cancer cells. As shown in Figures 5A, 4B-E, there was a decrease in the expression of Cyclin A, Cyclin D, Cyclin E and PCNA in A2780 and H08910 cells that were downexpressed by the gene of GAS5. The results demonstrated that GAS5 could inhibit the proliferation of ovarian cancer cells.

Discussion

Several important observations were demonstrated in this study. First, compared with epithelial tissues of normal ovary and benign epithelial ovarian lesions, the depressed expression of GAS5 was detected in EOC. Meanwhile, GAS5 expression appeared to be significantly correlated to lymph node metastasis and FIGO stage of EOC. Second, in vitro, the capability of apoptosis was strengthened, the ability of invasion and migration were weakened, and the ability of proliferation was decreased by GAS5 overexpression.

GAS5 was found down-regulated in EOC tissues than in the normal ovarian epithelium, and lower GAS5 correlated with more transferred lymph nodes and advanced FIGO stage. Consistently, earlier studies showed that GAS5 was down-regulated in NSCLC compared with the adjacent normal lung tissues; more importantly, lower GAS5 expression correlated with larger tumor size and advanced clinical stage [26]. Additionally, findings showed that GAS5 expression was markedly depressed in gastric cancer tissues, and the lower GAS5 mainly appeared in the larger tumor size-an advanced pathologic stage. Moreover, patients with lower GAS5 expression had poorer disease-free survival and overall survival [25]. More recently, studies showed that the GAS5 expression was decreased in cervical cancer tissues than in
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the adjacent normal tissues, and depressed GAS5 expression was correlated with the advanced FIGO stage, deeper invasion, and more lymph node metastasis. Patients with lower GAS5 expression had shown poorer overall survival [36]. Taken together, GAS5 may serve as a tumor suppressor in human tumor.

Cell proliferation is a complex process that involves a variety of regulatory mechanisms and is closely related to tumorigenesis. The findings have indicated that GAS5 inhibits proliferation through significantly different intron or exon composition of these GAS5 transcripts [23]. Meanwhile, the current study found that GAS5 induced proliferation related proteins, Cyclin A, Cyclin D, Cyclin E and PCNA, (released more from the ovarian cancer cells) were expressed decreasingly by GAS5, indicating that the proliferation pathway is required for the effect of GAS5 in human ovarian cancer cells. Taken together, these findings prove that IncRNA GAS5 acts as a tumor suppressor in human EOC.

However, there are some points that still need to be resolved: (1) more patients samples should be collected to verify the credibility of GAS5 repression function in EOC, (2) the interacting molecular mechanisms and signal pathways on how GAS5 changes the mitochondrion-independent apoptosis should be clarified, and (3) the mechanisms of decreased capabilities of migration and invasion by GAS5 in ovarian cancer cells should be explicated.

In conclusion, the findings of this study have shown that GAS5 overexpression can inhibit proliferation, promote apoptosis, and reduce migration and invasion in ovarian cancer cells. In addition, GAS5 expression is lower in EOC tissues than in the normal ovarian epithelium, and the lower expression of GAS5 is correlated to more lymph node metastasis and advanced FIGO stage of EOC, indicating that GAS5 may play a role as a suppressor of tumorigenesis for EOC patients. These findings have major implications for devising a strategy to ovarian cancer treatment target.

Acknowledgements

This work was supported by Heilongjiang province science and technology research Foundation (GA14C101-06).
Disclosure of conflict of interest

None.

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