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Correlation between long strand non-coding RNA GASS expression and prognosis of cervical cancer patients

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Abstract. – OBJECTIVE: Cervical cancer is the second popular female specific malignant tumors. Long-strand non-coding RNA (lncRNA) GAS5 (Growth Arrest Specific 5) participates in pathological processes of various malignant tumors. This study aimed at investigating the correlation between lncRNA GAS5 expression and prognosis of cervical cancer patients.

PATIENTS AND METHODS: Cancer tissues were collected from 48 cervical cancer patients. GAS5 expression in cervical cancer cells was determined by qRT-PCR and in situ hybridization (ISH). The correlation between GAS5 expression and pathological parameters of patients was analyzed. Cervical cancer cell line HeLa was used as the in vitro model, RNA interference approach was adopted to suppress lncRNA GAS5 expression. MTT assay was employed to analyze cell proliferation potency. Transwell assay was conducted to analyze the cell migration potency, and tumor cell invasion was measured.

RESULTS: qRT-PCR and ISH results showed that GAS5 expression in cervical cancer tissues was significantly inhibited compared to that in adjacent tissues ($p<0.05$). GAS5 expression was correlated with FIGO stage and metastatic tumor parameters of cervical cancer patients ($p<0.01$). RNA interference data showed that the down-regulation of GAS5 significantly enhanced cell proliferation and invasion potency ($p<0.05$).

CONCLUSIONS: GAS5 is down-regulated in cervical cancer cells, and this is probably related to patient clinical stage and tumor invasion or metastasis. The regulatory role of GAS5 on cell proliferation provides the academic basis for the future therapy of cervical cancer.

Key Words:
Cervical cancer, GAS5, LncRNA, Cell proliferation, Invasion.

Introduction

Cervical cancer represents the second most common type of cancer in women worldwide[1]. Primary cancer is prevalent in the population of 30-35 years old, and invasion cancer frequently occurred in the age group of 45-55 years, but presenting a trend of younger population[2]. In recent decades, the promotion of cervical cytology screening made possible an early diagnosis and treatment of cervical cancer, leading to the decrease of incidence or mortality of cervical cancer. However, the problem of recurrence and drug resistance still exist[3]. Therefore, the deep research of molecular mechanism, as well as its correlation with the survival or prognosis of the patient, is critical for the treatment of cervical cancer.

Long strand non-coding RNA (lncRNA) is a group of RNA molecules with longer than 200 nt[4]. Recent studies showed that lncRNA modulated gene expression level at multiple layers including epigenetic regulation, transcriptional regulation, and post-transcriptional regulation[5]. Some lncRNA can bind with transcriptional factors to modulate gene expression and DNA methylation, further mediating expression of certain genes[6]. In recent years, lncRNA is found to be aberrantly expressed in malignant tumor cells, and shows close correlation with cancer pathogenesis[7]. For example, MALAT, an evolutionary conservative lncRNA, has been found to be up-regulated in various malignant tumors including lung cancer and cervical cancer, and is correlated with cancer metastasis and recurrence. Further investigations[8,9] about cervical cancer also found abnormal expression of lncRNA, such as ANRIL and MEG3.

Current opinions proposed that human papillomavirus (HPV) infection in reproductive tract became the major reason of the cause of cervical cancer[10]. The previous study[11] showed that during the integration of HPV into host cell genomic DNA, certain abnormality in gene function may lead to cancer pathogenesis. Gibb et al[12] used SAGE (serial

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analysis of gene expressions) approach to analyze lncRNA expressional profile in human cervical cancer tissues and found abnormal expression levels of lncRNA, including GAS5 (Growth Arrest Specific5), which was thus proposed to be involved in precancerous lesion, occurrence, and progression of cervical cancer. Thus, this work aimed to investigate the relationship between level of lncRNA GAS5 and prognosis of cervical cancer patients.

Patients and Methods

Patients

A total of 48 cervical cancer patients aged between 32 and 54 years (average age = 40.1±6.5 years) were admitted from January 2014 to December 2015 and were recruited in Central Hospital of Zaozhuang Mining Group (Shandong, China). All patients were diagnosed as primary tumors, without any chemo-, radio- or immune therapy. All patients received radical cervical resection in our hospital, and were diagnosed as cervical cancer by histopathology. There were 38 squamous carcinomas, and 10 adenoma cases. Based on FIGO staging criteria, there were 7, 20, and 21 patients of stage Ib1, stage Ib2, and IIa, respectively.

Moreover, 11 patients receiving total hysterectomy under similar conditions were recruited as the control group, the age of whom ranged between 34 and 53 years old (average age = 42.1±5.5 years). All control people had normal cytology indicators confirming no malignant transformation in cervical epithelium, without inflammation or HPV infection. Tissue samples were collected during the surgery. Partial tissues were rinsed in pre-cold sterile saline and were kept in liquid nitrogen. Other tissues were fixed in formaldehyde and were embedded in paraffin. This study was approved by the Medical Ethical Committee of Zaozhuang Mining Group (Shandong, China), and all subjects signed informed consents.

HPV Assay

Samples stored in liquid nitrogen were cut into pieces. Fresh tissues were extracted for DNA using extraction kit (Qiagen, Hilden, Germany). DNA samples were then tested for the positive rate of high-risk HPV strain (HPV16, 18, 31, 35, 39, 45, 51, 52, 56, 58, 59, 66, and 68) using HPV nucleic acid test kit (Qiagen, Hilden, Germany).

In Situ Hybridization Assay

Based on Lnc-RNA GAS5 sequence (Genebank access ID: NR_002578), *in situ* hybridization (ISH) probes for Lnc-RNA GAS5 was designed. The probe was modified by anti-sense nucleic acid approach. ISH probe sequence was: 5'-TTAATCTTCTTGTGCCATGAGACTCCA-3', in which underlined nucleic acids were locked. All paraffin-embedded tissue samples were tested by ISH using LNA-GAS5 probe under the assistance of ISH kit (Boster Biotech, Shanghai, China). In brief, paraffin-based tissues slices were de-waxed, re-hydrated, and blocked for hydrogen peroxidase activity. After digestion in proteinase K, slices were rinsed in 0.5 M PBS (pH 7.4) and were rinsed in distilled water. Pre-hybridization buffer was added for 3-4 h treatment under 63°C-65°C incubation. Hybridization buffer containing probes was then added for 12-16 h incubation at 65°C. After hybridization, SSC buffer was used for rinsing, and blocking buffer was added for room temperature incubation. Anti-DIG antibody working solution was added for 60 min incubation at 37°C. PBS and alkaline phosphatase buffer were added for rinsing. Development buffer was then added for dark incubation, which was quenched by phosphate-buffered saline (PBS). 4% paraformaldehyde was added for fixation after coverslip was rinsed with PBS and mounted. Blue-violet granules in cytoplasm or on membrane were identified as positive cells. The sample was identified as positive for lnc-RNA GAS5 if more than 40% of total cells were positive. Positive rats of all samples were calculated.

qRT-PCR Assay

Based on Lnc-RNA GAS5 sequence (Genebank access ID: NR_002578), primers were designed as Table I. One-step total RNA extraction

Table I. Primer sequence used in RT-PCR.

Primer	Sequence
β-tublin	F5'-TGTCCCGATGGCGAGTGTTT-3' R5'-CCTGTTGGCCATAGTACTGC-3'
GAS5	F5'-AGCAAGCCTAACTCAAGCCATT-3' R5'-TCAAGCCGACTCTCCATACCT-3'

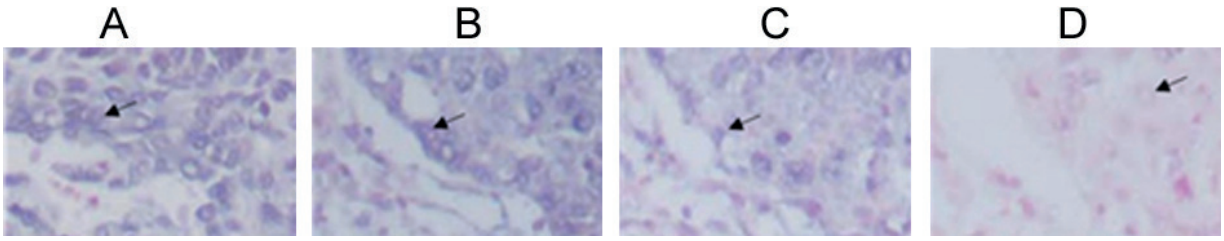

Figure 1. ISH staining results (×400). **A,** Normal cervical epithelial tissues; **B,** Stage Ib1; **C,** Stage Ib2; **D,** Stage IIa.

kit TRIZOL (Invitrogen, Waltham, MA, USA) was used to extract total RNA. qRT-PCR was performed using total RNA from control group as the control. RT-PCR kit (TianGen, Beijing, China) was used for RT-PCR assay. Reverse transcription was firstly performed at 37°C for 1 h. cDNA produced from reverse transcription was used as the template in RT-PCR under the following conditions: 95°C for 5 min, followed by 40 cycles each containing 95°C for 1 min, followed by 56°C for 30 s and 72°C for 1 min. PCR products were analyzed in 1% agarose gel electrophoresis. Relative expression of GAS5 was calculated after analysis with gel imaging system.

Design of RNA Interference Sequence

RNA interference sequence was designed based on Lnc-RNA GAS5 sequence. 29 bp length sequence with 50% GC contents was identified and was compared with other gene mRNA sequence from human genome database to exclude homology with other genes. GAS5-RNAi sequence was chosen, and randomly re-assigned sequences without homology with other genes were used as negative control scramble-RNAi. Sequences were shown in Table II and were synthesized by Sangon (Shanghai, China).

Transfection of Cervical Cancer Cells

Cervical cancer cell line HeLa was purchased from Cell Bank, Chinese Academy of Science (Beijing, China). Cells were resuscitated and cultured until log-growth phase. Cells were then digested with trypsin, counted and diluted in fresh medium to inoculate into 96-well plate. After 24 h incubation, transfection was performed using liposome INTERFERin™ transfection kit (Polyplus transfection, New York, NY, USA) following the manual instruction. qRT-PCR was used to measure relative expression of Lnc-RNA GAS5 in those cells with successful transfection.

MTT Assay for Cell Proliferation

Hela cells with successful transfection were cultured until log-growth phase and were digested with trypsin. After centrifugation, the supernatant was discarded and cells were re-suspended in culture medium. 6 μL Trypan blue solution was mixed with 6 μL cell suspension. 10 μL mixture was added into cell enumeration slide with coverslip, for counting under the microscope. Cells were re-suspended into 24-well plate and incubated for 12 h at 37°C with CO_2 incubator. After mixture, the supernatant was added with DMSO. A570 nm value was measured in Multiskan MK3 microplate reader (Thermo Fisher, Waltham, MA, USA) after complete resolving.

Transwell Assay for Cell Invasion and Migration

Following manual instruction of Transwell kit, 60 μL 5 mg/mL matrix gel (BD, San Jose, CA, USA) was added into the upper chamber of Transwell, which was air-dried at 4°C for bottom chamber. Cells were cultured until log-growth phase and were digested with trypsin, and were washed in serum-free medium for re-suspension. Cell density was adjusted to 1×10^6/mL, and 200 μL cell suspension was added into the upper chamber

Table II. RNA interference sequence.

Primer	Sequence
GAS5-RNAi	GTGTGGCTCTGGATAGCACCTTA
	GTCACACCGAGACCTATCGTGGA
Scramble-RNAi	GACCAGCTGTCTAGGACTGACTT
	CACTGGTCGACAGATCCTGACTG

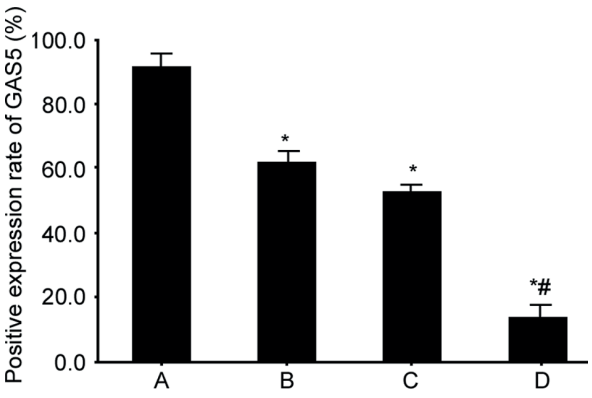

Figure 2. Positive expression rate of Lnc-RNA GAS5. ***A,*** Normal cervical epithelium; ***B,*** Stage Ib; ***C,*** Stage Ib2; ***D,*** Stage IIa. *, $p<0.05$ compared to A; #, $p<0.05$ compared to C.

of Transwell, whilst 600 μL culture medium containing 10% fetal bovine serum (FBS) was added into the lower chamber, which was incubated at 37°C with CO_2 incubator for 24 h. Residual matrix gel and cells were washed out by the cotton. Cells in the lower chamber were stained by crystal violet for 30 min, and were washed with 10% acetic acid. OD_{750} was measured by a microplate reader in triplicates.

Statistical Analysis

SPSS19.0 statistical software was used to analyze all data, in which measurement data were presented as mean±standard deviation (SD). The comparison was performed by x^2-test. One-way analysis of variance (ANOVA) with LSD method as post-hoc test was used for multiple comparisons. $p<0.05$ was considered as statistical significance.

Results

ISH Staining Results

The differential expressions of Lnc-RNA GAS5 between normal cervical tissues and cervical cancer tissues were compared by using ISH approach. Results (Figure 1) showed that hybridization probe can bind with Lnc-RNA GAS5 inside cells. With the increase of Lnc-RNA GAS5 level, staining intensity was elevated. Figure 1 showed that dark ISH staining in cervical cancer tissues was observed compared with that in normal cervical hyperplasia tissues. Calculation of positive expression rates among different samples was shown in Figure 2; positive expression rate increased as clinical stage of cervical cancer was advanced.

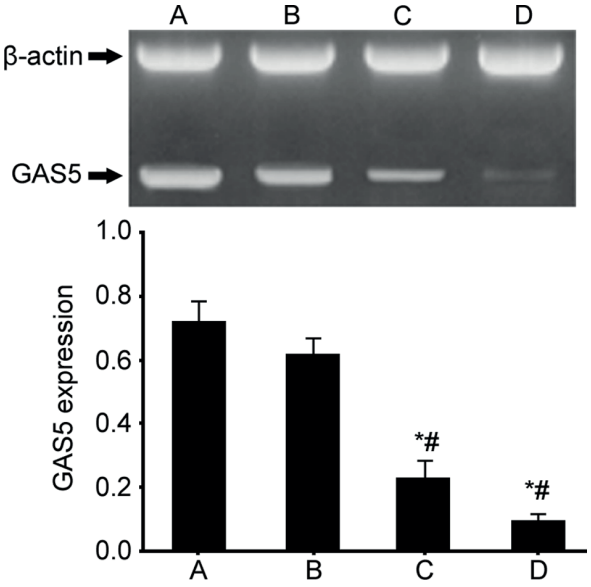

Figure 3. GAS5 expression in cervical cancer tissues. ***A,*** Normal cervical epithelium; ***B,*** Stage Ib; ***C,*** Stage Ib2; ***D,*** Stage IIa. *, $p<0.05$ compared to A; #, $p<0.05$ compared to B.

qRT-PCR

qRT-PCR was employed to confirm the result of ISH. qRT-PCR products were analyzed by agarose gel electrophoresis. As shown in Figure 3, fluorescent intensity was used to measure relative expression level of GAS5. By qRT-PCR, we found relatively higher GAS5 expression in normal cervical tissues, whilst relatively lower GAS5 expression was shown in cervical cancer tissues. Moreover, GAS5 expression was gradually decreased as the clinical stage was advanced ($p<0.05$), which was consistent with ISH results.

Correlation Between GAS5 Expression and Clinical Parameters of Patients

According to the qRT-PCR results, all cervical cancer patients were divided into GAS5 high-expres-

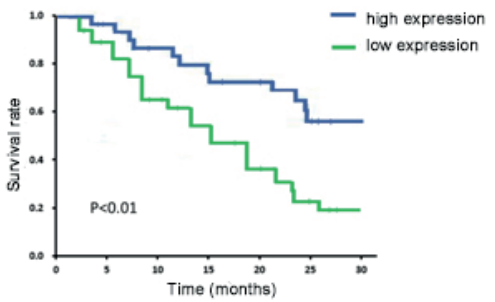

Figure 4. Relationship between the expression of GAS5 and the survival rate of patients.

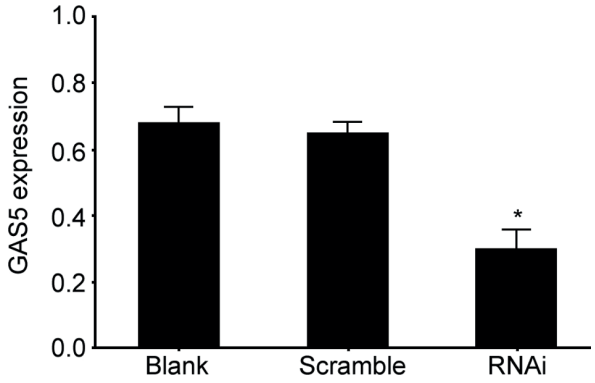

Figure 5. GAS5 relative expression in cells after transfection. *, $p<0.05$ compared to control group.

sion (relative expression>0.3) and low-expression (relative expression<0.3). Follow-up was performed after 10-30 months, and pathology parameters were collected (Table III). We found that GAS5 expression was not significantly correlated with patient age, tumor size, and tissue pathology type ($p>0.05$), but it showed close correlation with FIGO stage, or distal lymph node metastasis ($p<0.01$). The relationship between the survival rate and the expression of GAS5 in patients was analyzed. The results (Figure 4) showed that the survival rate of patients with high expression of GAS5 was significantly higher than that of patients with low level of GAS5 ($p<0.01$).

RNAi Results

Artificially synthesized oligonucleotide sequence GAS5-RNAi or Scramble-RNAi was transfected into human cervical cell line Hela. Using non-transfected cells as the empty control, qRT-PCR was performed (Figure 5). Our data showed that GAS5 expression was decreased by 58.8±6.2% in those cells transfected with GAS5-RNAi, indicating successful inhibition of GAS5.

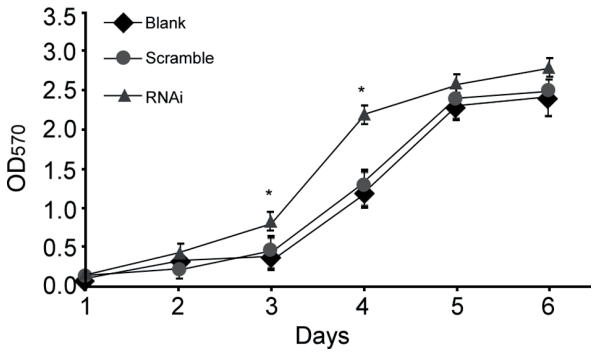

Figure 6. MTT assay for cell proliferation.

MTT Assay for Cell Proliferation

After successful transfection, MTT approach was used to measure proliferation potency of Hela cells. Using un-transfected cells as the empty control, cells transfected with scramble-RNAi oligonucleotide sequence as the negative control, cells were counted at different time points. Figure 6 showed that the amount of HeLa cells with transfection of GAS5-RNAi was significantly increased at 3d and 4d compared to control group ($p<0.05$), indicating elevating proliferation potency compared to control group.

Transwell Assay for Cell Invasion Potency

Transwell assay was used to measure invasion potency of Hela cells at 14 h after successful transfection. Figure 7A showed the invasion potency in HeLa cells with RNAi treatment was enhanced ($p<0.05$). Invasion potency of all cells was compared in Figure 7B, which showed that the invasion potency of HeLa cells with GAS5-RNAi transfection increased by 2.2 fold compared to that of un-transfected HeLa cells.

Discussion

Recent reports[9,13] showed that LncRNA played important roles in modulating transcriptional

Table III. Correlation between GAS5 expression and clinical parameters of cervical cancer.

		GAS5 expression level		
Parameters	**N**	**High**	**Low**	***p*-value**
Age (year)				
≥50	25	14	11	>0.05
<50	23	13	10	
Tumor size (cm)				
≥4	26	16	10	>0.05
<4	22	11	11	
FIGO stage				
Stage Ib	27	21	6	<0.01
Stage IIa	21	6	15	
Histo-pathology				
Adenoma	10	6	4	>0.05
Squamous carcinoma	38	21	11	
Lymph node metastasis				
Yes	22	6	16	<0.01
No	26	21	5	
Distal metastasis				
Yes	17	3	14	<0.01
No	31	24	7	

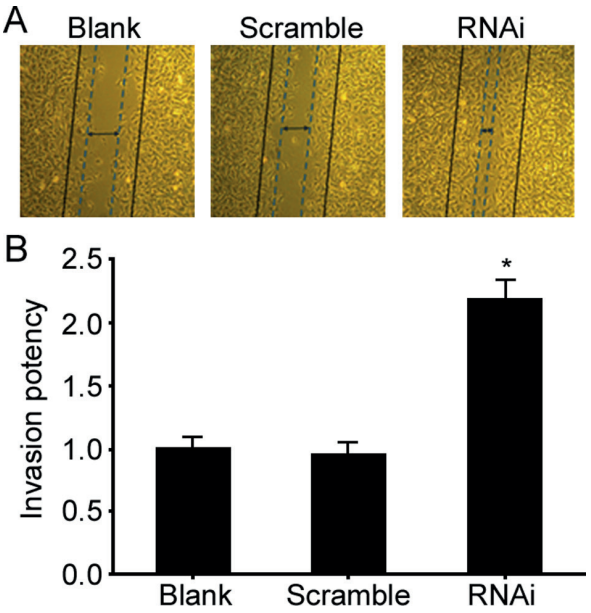

Figure 7. Transwell assay for measuring cell invasion/migration. **A,** Transwell measurement results (×200); **B,** Comparison of cell invasion potency. *, $p<0.05$ compared to control group.

factor expression, interaction or modification. Expression of LncRNA is critical for maintaining normal homeostasis of cell internal environment, whilst its abnormal expression is closely correlated with occurrence and progression of malignant tumors[5,14]. We investigated the level of GAS5 in human cervical cancer tissues, and found the correlation between GAS5 expression and FIGO stage or metastasis of cancer patients. Also, we analyzed the effect of GAS5 expression on cell proliferation and invasion by cell transfection and RNAi approach.

Via ISH and qRT-PCR, we found significantly lower GAS5 expression in cervical cancer cells. Meanwhile, we also observed the correlation between GAS5 expression and lymph node/distal metastasis of patients. Recent studies[14-17] revealed that GAS5 was significantly down-regulated in various human malignant tumors including liver cancer, gastric carcinoma, lung cancer, and cervical cancer. In those patients with GAS5 low-expression, the pathology grade of tumors was elevated and accompanied with high risk of malignancy[18]. Chang et al[19] found lower GAS5 expression was associated with higher risk of venous cancer embolism in liver cancer patient, consistent with our results.

This study demonstrated proliferation and invasion potency of cervical cancer cells were significantly enhanced, along with down-regulation of GAS5, indicating that the decrease of GAS5 might potentiate proliferation and invasion potency of cancer cells, and eventually increase malignancy of tumors and risk of invasion or metastasis. Similar phenomena were also observed in other malignant tumors. Sun et al[14] found that the increase of GAS could inhibit cell proliferation of gastric cancer cell line, whilst the inhibition of GAS5 expression by RNAi facilitated cell proliferation. Liu et al[20] performed a knock-out assay and found that the overexpression of GAS5 in prostate cancer could inhibit cancer cell proliferation, consistent with this study.

During cell regulation of cervical cancer, Jiang et al[21] proposed that lncRNA might regulate HPV carcinogenesis process via certain unknown mechanisms, including modulating gene or protein expression. The regulatory mechanism of GAS5 on cancer cells is still unclear yet. Some scholars believed that the inhibition of GAS5 could enhance the level of cellular transcriptional factor E2F1, inhibit tumor suppressor gene p21, and eventually facilitate cell proliferation[22]. Other evidence indicated that GAS5 negatively regulated expression of cell cycle-dependent kinase 6 (CDK6), and promoted cell proliferation[23]. However, the molecular mechanism of GAS5 in cervical cancer still requires more experimental evidence.

The study for lncRNA in cancer cells has become one of the major focuses in tumor molecular biology, as lncRNA might become the novel target for novel cancer drugs. The major weakness of this investigation includes insufficient research subjects, which requires further investigation of molecular mechanism regulating GAS5. Future studies will determine the effect of GAS5 expression on tumor-related genes and cycle regulatory genes, in order to provide evidence for clinical diagnosis and treatment of cervical cancer in future.

Conclusions

The reducing level of GAS5 in cervical cancer cells facilitates cell proliferation and improves cell invasion/migration potency, which is related to patient clinical stage and tumor invasion or metastasis.

Conflict of Interest

The Authors declare that they have no conflict of interest.

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