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Long Noncoding RNA GAS5, Which Acts as a Tumor Suppressor via microRNA 21, Regulates Cisplatin Resistance Expression in Cervical Cancer

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Objectives: The aims of this study were to investigate the functions of GAS5 as a tumor suppressor in cervical cancer and explore the mechanism.

Methods: The expression of GAS5 and microRNA 21 (miR-21) was detected in primary cervical cancer tissue specimens, as well as in cervical cancer cell lines. We identified the interaction of GAS5 and miR-21 by quantitative polymerase chain reaction, Western blot, and dual-luciferase reporter assay. We also studied the functions of GAS5 in proliferation, apoptosis, migration, and invasion in cervical cancer cells in vitro and vivo. Finally, the impact of GAS5 on cisplatin resistance and its mechanism in cervical cancer cells was also identified.

Results: The expression of GAS5 and miR-21 was detected in primary cervical cancer tissue specimens, as well as in cervical cancer cell lines. GAS5, which is a tumor suppressor playing roles in inhibiting the malignancy of cervical cancer cells, including proliferation in vivo and vitro, migration, and invasion, has a low expression in cervical cancer tissue and cervical cancer cell lines, whereas miR-21 expression is high. GAS5 significantly decreased the expression of miR-21, and there is a reciprocal repression of gene expression between GAS5 and miR-21. Besides, most importantly, we found that high expression of GAS5 and low expression of miR-21 can enhance the sensitivity of SiHa/cDDP cancer cells to cisplatin. A further experiment for identifying the mechanism of cisplatin resistance by GAS5 showed that GAS5 can not only regulate phosphatase and tensin homolog through miR-21 but also influence the phosphorylation of Akt.

Conclusions: Our results indicate that GAS5 is a direct target of miR-21 and can predict the clinical staging of cervical cancer. Most importantly, GAS5 can also influence cisplatin resistance in cervical cancer via regulating the phosphorylation of Akt. All of these suggest that GAS5 may be a novel therapeutic target for treating cervical cancer.

Key Words: Cervical cancer, GAS5, miR-21, Cisplatin resistance, PI3K/Akt/mTOR pathway

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Cervical cancer (CC) is one of the most commonly diagnosed lethal malignancies among gynecological malignant tumors in China.¹ Although the human papillomavirus vaccine has been launched for years, the mortality of CC still has a 10-fold increase in developed countries than in developing countries. It has been shown that CC is one of the leading causes of cancer death in China, mostly in the rural areas.² The present treatment of CC is radical hysterectomy and pelvic lymphadenectomy, radiation with concomitant chemotherapy, or a combination thereof. However, multiple chemotherapeutic resistance in CC treatment remains as a leading cause of treatment failure and poor outcomes. Standard drug treatment of CC includes a combination regimen of paclitaxel and a platinum compound such as cisplatin.³ Damaged DNA double-strand is one of the mechanisms of cisplatin, including phosphatidylinositol 3-kinase (PI3K)/Akt activation downstream of DNA-PK.^{3,4} Overactivation of Akt can be found in cisplatin-resistant epithelial ovarian cancers.^{5,6} It was reported that inhibition of Akt could enhance platinum-induced apoptosis in cancer cell lines such as ovarian cancer cell lines.^{5,6} The phosphorylated Akt exerts its biological functions as inhibiting apoptosis and promoting cell survival via phosphorylating of its downstream targets.^{5,6} Many studies showed that Akt activation can be frequently seen in patients with relapse, and it was believed that Akt plays an essential role in cisplatin resistance acquired cells.⁴⁻⁷ Besides, PI3K/Akt pathway is considered as a well-known regulator of growth and malignancy of cancer cells. Thus, identification of new potential therapeutic targets aiming at the PI3K/Akt pathway in CC is necessary for improving the treatment and prognosis.

Recent studies have revealed that long noncoding RNAs (lncRNAs), which are RNAs with more than 200 nucleotides in length and cannot be translated into a protein, play important roles in tumor development.^{8,9} GAS5 is a putative tumor suppressor and apoptosis-promoting lncRNA that hosts multiple small nucleolar RNAs. It is localized at chromosomal 1q25 and was originally isolated from mouse NIH3T3 cells.^{10,11} As a member of 5'-terminal oligopyrimidine tract gene family, GAS5 is composed of 12 exons and can be

accumulated in the growth arrested cells.^{12,13} It has been reported that GAS5 could be accumulated in the permanent cells such as brain cells, although the expression of GAS5 was relatively lower in the labile cells such as hepatic and splenic cells.¹⁴ Studies demonstrated down-regulation of GAS5 expression in various cancer cell lines and tissues, as a tumor suppressor.¹⁵⁻¹⁹ The accumulation of GAS5 in growth-arrest cells is regulated by mTOR pathway and nonsense-mediated decay pathway.¹¹⁻¹³ In actively growing cells, the activity of mTOR pathway is relatively high, and then the translation of GAS5 will be activated, which can lead to a low cellular GAS5 lncRNA level. However, suppression of cell growth and activity of mTOR pathway can prevent the translation of GAS5 transcripts.^{11,13}

It is well known that lncRNAs play an important regulatory role in biological activities of tumor cells. However, in the regulation of tumor cellular function, microRNAs (miRNAs) are also essential.²⁰ miRNAs are approximately 22 nucleotide noncoding RNAs functioning as mRNA translation inhibitors by an imperfect base pairing with 3' untranslated region of target mRNAs.^{21,22} microRNA 21 (miR-21) is a well-studied miRNA and has been shown as an oncogene in various types of cancers.²³⁻²⁶ The growth, migration, and invasion of cells with miR-21 knockdown were suppressed in vitro and in vivo.^{27,28} Phosphatase and tensin homolog (PTEN), a well-known tumor suppressor, was validated as the first target gene of miR-21 in hepatic cancer.²⁹ It also plays an important role of the suppressor in the PI3K/Akt pathway, which is associated to chemotherapeutic drug resistance in cancer cells.^{30,31}

There are other tumor suppressors, such as PDCD4, TPM1, RECK, maspin, and TIMP3, that were shown to be the direct targets of miR-21.^{28,32,33} microRNA 21 can function as an oncogene by silencing multiple tumor suppressors. Overwhelming evidence suggests that miR-21 targets are protein coding genes. However, a large number of lncRNAs play important roles in cancer biology, raising the possibility of whether miR-21 can target lncRNAs. A recent study has identified that miR-21 can induce 5 lncRNAs over 2 folds including GAS5 in breast cancer cells.³⁴

TABLE 1. Association of GAS5 and miR-21 expression with clinicopathological features in CC

Parameters	Groups	Total	GAS5 Expression		miR-21 Expression	
			High	Low	High	Low
Histology	Squamous	30	14	16	15	15
	Adenocarcinoma	7	4	3	4	3
	Adenosquamous carcinoma	2	2	0	0	2
	Undifferentiated carcinomas	1	0	1	1	0
Differentiation	Well	2	0	2	2	0
	Moderate	12	8	4	5	7
	Moderate-poor	11	6	5	5	6
	Poor	15	4	11	10	5
FIGO stage	Ia1-Ib2	20	17	3	2	18
	IIa1-IIb	20	3	17	18	2

In this study, we found a negative correlation between GAS5 and miR-21 in CC tissue and cell lines and identified a repression feedback between GAS5 and miR-21. GAS5 can function as a tumor suppressor by regulating growth, apoptosis, invasion, and migration in CC cells by regulating PTEN and PDCD4 that are also miR-21 targets. Moreover, we demonstrated that GAS5 and miR-21 can regulate cisplatin resistance via regulating phosphorylation of Akt in CC cells.

MATERIALS AND METHODS

Tissue Samples

The study was performed in accordance with the Declaration of Helsinki and has been approved by the ethical committee of the Third Affiliated Hospital of Guangzhou Medical University. Informed consent was obtained from all patients. Forty CC and 40 normal cervical tissue specimens were collected from patients who underwent hysterectomy in the Third Affiliated Hospital of Guangzhou Medical University. All cases were diagnosed histologically according to International Federation of Gynecology and Obstetrics (FIGO) criteria (Table 1) and not subjected to preparative radiotherapy and/or chemotherapy. These tissue specimens were snapped frozen in liquid nitrogen and stored at -80°C .

Cell Lines and Cell Culture

Cervical cancer cell lines HeLa, SiHa, and CaSki were obtained from the American Type Culture Collection (ATCC, Manassas, VA). HeLa and SiHa were cultured in Dulbecco modified Eagle medium supplemented with 10% fetal bovine serum (FBS) and 1% streptomycin/penicillin at 37°C in a humidified incubator with 5% CO_2 , and CaSki cells were cultured in RPMI 1640 with the same condition as previously mentioned.

Establishment of Cisplatin-Resistant SiHa Cell Line

SiHa cells were cultured with Dulbecco modified Eagle medium with 10% FBS and 1% streptomycin/penicillin at 37°C with 5% CO_2 . Briefly, cell lines were serially treated with increasing doses of cisplatin with intermittent cell recovery/expansion phases to induce resistance. At the first month, SiHa cells were grown in the medium with $0.1\text{-}\mu\text{g/mL}$ cisplatin and cultured with $0.5\text{-}\mu\text{g/mL}$ cisplatin for the following 2 months. The surviving cells were further maintained in $1.0\text{-}\mu\text{g/mL}$ cisplatin for 3 months. Then, the cells were transferred to the medium with $1.5\text{-}\mu\text{g/mL}$ cisplatin for 2 months. The final surviving cells were cultured in the presence of $2\text{-}\mu\text{g/mL}$ cisplatin. Even when SiHa/cDDP cells were grown in the absence of cisplatin for 30 passages, their resistant properties were sustained.

Design and Construction of GAS5 Lentivirus Vector, GAS5 Small Interfering RNA, and miR-21 Mimic and Inhibitor

To assess the role of GAS5 in CC and the correlation between GAS5 and miR-21, we overexpressed and knocked down GAS5 expression using GAS5 lentivirus vector (constructed by GenePharma, Shanghai, China) and GAS5 small interfering RNA (siRNA) (constructed by Ribobio, Guangzhou, China). In brief, we designed and constructed 3 sets of

GAS5 siRNA. The sequences were as follows: GAS5 siRNA1 (sense strand, 5' CUUGCCUGGACCAGCUUAA dTdT 3'; antisense strand, 3' dTdT GAACGGACCUGGUCGAAUU 5'), GAS5 siRNA2 (sense strand, 5' GCUCUGGAUAGCACCUUAAU dTdT 3'; antisense strand, 3' dTdT CGAGACCUAUCGUGG AAUA 5'), and GAS5 siRNA3 (sense strand, 5' GCAGUGG CCUUUGAAGCUU dTdT 3'; antisense strand, 3' dTdT CGU CACCGGAAACUUCGAA 5'). microRNA 21 mimic and inhibitor (Ribobio, Guangzhou, China) were used for overexpressing and silencing the expression of miR-21. Briefly, CC cells were seeded in a 6-well plate. When the cell amount reached 70% to 80% confluency, cells were transfected with GAS5 lentivirus vector, GAS5 siRNA1, siRNA2, siRNA3, or miR-21 mimic or inhibitor using riboFECT CP transfection reagents (Ribobio, Guangzhou, China) according to the manufacturer's instructions. Cells were then harvested and subjected to quantitative real-time polymerase chain reaction (qRT-PCR) analysis of GAS5 or miR-21 expression. Because GAS5 siRNA1 was most effective in achieving knockdown of GAS5 expression, all subsequent experiments were performed using this vector.

Establishing GAS5 Stable Transfected Cell Lines

Cervical cancer cell lines were seeded in a 24-well plate. When cell amount reached 70% to 80% confluency, lentivirus (pGLVU6/green fluorescent protein/Puro) with GAS5 or NC was added for infection (GenePharma, Shanghai, China). Green fluorescent protein expression per well was observed 48 hours after lentivirus infection. Then, the cells were transferred to a 6-well plate. Puromycin of $1\text{ }\mu\text{g/mL}$ was added for screening of stable transfected cells. The concentration of puromycin was increased gradually until $12\text{ }\mu\text{g/mL}$.

RNA Isolation and qRT-PCR

Total cellular RNA was isolated from tissues and cell lines using TRIzol reagent (Takara Bio, Inc, Shiga, Japan) and then reversely transcribed into complementary DNA using PrimeScript RT Master Mix (Takara Bio, Inc) according to the manufacturer's instructions. Quantitative real-time PCR was performed to determine the expression of GAS5 and miR-21 using a SYBR GREEN MIX kit (Takara Bio, Inc, Shiga, Japan) according to the manufacturer's protocols. Primers sequences of GAS5—forward, GGATGCAGTGTGGCTCT GGATA, and reverse, TGTGTGCCAATGGCTTGAGTTAG (Generay, Shanghai, China)—and sequences of miR-21 were not offered (Ribobio, Guangzhou, China).

Cell Proliferation Assay

To assess the effect of GAS5 regulation in CC cells, HeLa, SiHa, and CaSki cells were seeded into 96-well plates at a density of 3×10^3 and cultured overnight. Transfected cell proliferation was measured using the CCK-8 assay (Dojindo, Kyushu, Japan) in 24-hour increments for up to 96 hours. Briefly, we added $10\text{-}\mu\text{L}$ CCK-8 solution into each well of cell culture, and the plates were further incubated for another 2 hours. The optical density was then measured by microplate reader analysis at the absorbance of 450 nm. Experiments were performed in triplicate and repeated at least 3 times independently.

Cell Migration and Invasion Assay

The Transwell chambers with 8- μm pores were obtained from Corning (Corning, NY). In the migration examination, the transfected cells were harvested and resuspended in 100- μL serum-free medium and then transferred to the upper chambers (2×10^4 cells per well). A medium of 600 μL supplemented with 10% FBS was added to the lower chamber. After incubation for 24 hours, the Transwell membrane was fixed with methanol, stained with crystal violet, and then counted under a light microscope. For the invasion assay, the Transwell membrane (filter) was precoated with 30 μL of Matrigel (1:3 mixed with PBS; BD Biosciences, Heidelberg, Germany) and incubated for 48 hours; the remaining experimental procedures were similar to the migration assay.

Western Blot Analysis

After transfection, cells were harvested, and protein was extracted. Protein concentrations were determined using a BCA protein assay kit (Beyotime, Beijing, China). Equal amounts (40 μg) of protein were separated by 10% sodium dodecyl sulfate denatured polyacrylamide gel and transferred onto poly(vinylidene fluoride) membranes with a pore size of 0.45 μm (Millipore, Billerica, MA). After blocking in 5% bovine serum albumin in Tris-based saline with Tween 20 for 1 hour at room temperature, membranes were incubated with rabbit antihuman antibodies at the recommended dilution (PTEN at a dilution of 1:500 [Abcam, Cambridge, MA], PDCD4 at 1:1000 [Abcam, Cambridge, MA], Akt at 1:1000 [C67E7] [Cell Signaling Technology, MA], phospho-Akt [Ser473] at 1:1000 [Cell Signaling Technology, MA]) overnight at 4°C. After washed in Tris-based saline with Tween 20, the membranes were further incubated with a secondary antirabbit (1:10000) antibody for 2 hours. Enhanced chemiluminescence solution was added onto the membranes, and protein expression was quantified using the Laboratory Work Image Acquisition and Analysis Software (UVP, Upland, CA). Protein β -actin was used as a loading control.

Cytotoxicity Assay

Cell viability was determined by CCK-8 (Dojindo, Kyushu, Japan). Briefly, 5×10^3 cells were plated in each well of 96-well plates. Cells were cultured overnight to allow attachment and then treated with cisplatin of various concentrations (1, 2, 3, 4, 5, 6, 10, 15, and 30 $\mu\text{g}/\text{mL}$). After incubation for 48 hours, CCK-8 solution was added to each well and incubated at 37°C for 2 hours. Measurement of optical density was performed by microplate reader analysis at the absorbance of 450 nm.

Dual-Luciferase Reporter Assay

Two hundred ninety-three T-cells (1×10^4) were seeded in 500- μL medium in a 12-well plate. After 18 to 24 hours of incubation, cells were transfected for 1 to 2 hours with 1- μg reporter plasmid (PSICHECK2.0-H-GAS5-WT and PSICHECK2.0-H-GAS5-Mut) (Transgen, Beijing, China) using 250- μL Optim and 3- μL Lipofectamine 2000 according to the manufacturer's instructions. After the transfection, the medium was replaced with 500- μL medium with 20% FBS and incubated for another 6 hours. Dual-luciferase reporter assay was performed after a 48- to 72-hour transfection. Cells were

harvested by adding 200- μL passive lysis buffer at room temperature. After centrifuging, 100- μL supernatant was assayed for luciferase detection using a microplate luminometer (Beyotime, Beijing, China).

Animal Work

The study was performed in accordance with the Declaration of Helsinki and has been approved by the ethical committee of the Third Affiliated Hospital of Guangzhou Medical University. Female nude (BALB/c-nu) mice (4–5 weeks old) were purchased from Vital River (Beijing, China). SiHa cells were transfected with scrambled control of 200 μL (5×10^6 cells/mL) and GAS lentivirus of 200 μL (5×10^6 cells/mL). The cells at exponential stage were harvested and were then mixed and injected to the left side of the mice. Tumor growth was monitored by measuring the tumor size every 3 days with a digital caliper. The tumor volume was calculated using the following formula: $0.5 \times a \times b^2$, where a refers to the long diameter and b refers to the short diameter of the tumor. The mice were euthanized at the end of the experiment, and the tumor xenografts were removed and weighed. Freshly frozen tumors were used for immunohistochemistry staining.

Statistical Analysis

All data were expressed as mean (SD). Differences between the 2 groups were assessed using the Fisher exact test or Student t test, whereas difference among multiple groups was analyzed using 1-way analysis of variance followed by Bonferroni multiple comparisons test. $P < 0.05$ or $P < 0.01$ was considered statistically significant.

RESULTS

GAS5 Was Down-Regulated and miR-21 Was Up-Regulated in Primary Cervical Tumors and in CC Cell Lines

GAS5 and miR-21 expression was detected in tumor and adjacent normal tissues from 40 patients. The levels of GAS5 and miR-21 expression were grouped according to the FIGO stages of 40 patients (Figs. 1A, B). GAS5 expression in tumor tissues was significantly lower than that in normal tissues (Fig. 1C). On the contrary, expression of miR-21 is higher in tumor tissues (Fig. 1D). Furthermore, expression of GAS5 negatively correlated with the FIGO stage of patients with CC (odds ratio [OR], 0.031; $P < 0.01$); however, miR-21 correlated positively (OR, 81.000; $P < 0.01$) (Table 2).

The relative expression of GAS5 and miR21 in CC HeLa, SiHa, CaSki, and SiHa/cDDP cell lines was detected by qRT-PCR. SiHa/cDDP cell line expressed the lowest level of GAS5 and the highest level of miR-21 compared with the other 3 cell lines (Figs. 1E, F), suggesting that drug resistance in CC cells might be associated with the expression levels of GAS5 and miR-21.

GAS5 Regulates miR-21 and Its Targets

Because the previously mentioned result showed that expression of GAS5 and miR-21 had a negative correlation, qRT-PCR was performed after transfection of GAS5 and miR-21 in these CC cell lines. Interestingly, GAS5 negatively

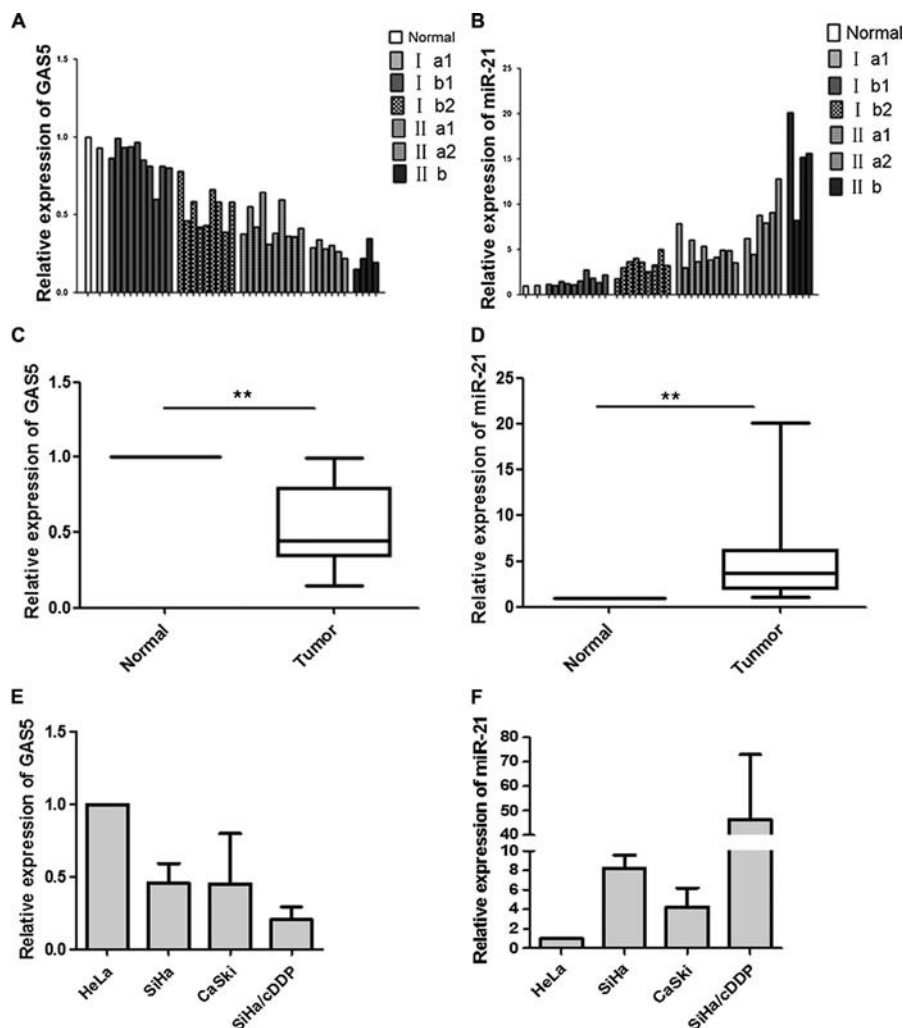


FIGURE 1. Expression of GAS5 and miR-21 in CC tissues and cell lines. Forty pairs of the tissue samples are gathered from 40 patients, including the cancer tissue and pericarcinomatous tissue. Quantitative real-time PCR was used to measure the expression of GAS5 and miR-21. The relative expression is presented as the fold change on the figure. A and B, Samples were classified by the clinical stage of CC (FIGO, 2009). C and D, Average relative expression of GAS5 and miR-21 in CC tissue compared with the normal pericarcinomatous tissue. E and F, Average relative expression of GAS5 and miR-21 in HeLa, SiHa, CaSki, and SiHa/cDDP CC cell lines. Data are means (SD) of 3 independent experiments. ** $P < 0.01$.

TABLE 2. Expression of GAS5 correlated negatively with the FIGO stage of patients with CC

FIGO Stage	GAS5 Expression		miR-21 Expression	
	High	Low	High	Low
Ia1–Ib2	17	3	2	18
IIa1–IIb	3	17	18	2
	OR, 0.031		OR, 81.000	
	$P < 0.01$		$P < 0.01$	

regulates miR-21, whereas miR-21 can also repress GAS5 expression (Figs. 2A, B). Knockdown of GAS5 increased miR-21 expression (Figs. 2C, D). Similarly, in CC cells transfected with miR-21 mimic, the GAS5 mRNA level decreased (Fig. 2E), whereas miR-21 inhibitor enhanced GAS5 expression (Fig. 2F). To further investigate whether miR-21 is a functional target of GAS5, dual-luciferase reporter assay and Western blot were performed (Figs. 2G, H). The results showed that up-regulation of GAS5 reduced the luciferase activities of the cells cotransfected with pGL3-miR-21-WT in CC cells and GAS5 regulated tumor suppressors PTEN and PDCD4 (Figs. 2H-Q). Taken together, we conclude that GAS5 not only directly regulates miR-21 in CC cells but also, as a tumor suppressor, can regulate PTEN and PDCD4 by interacting with miR-21.

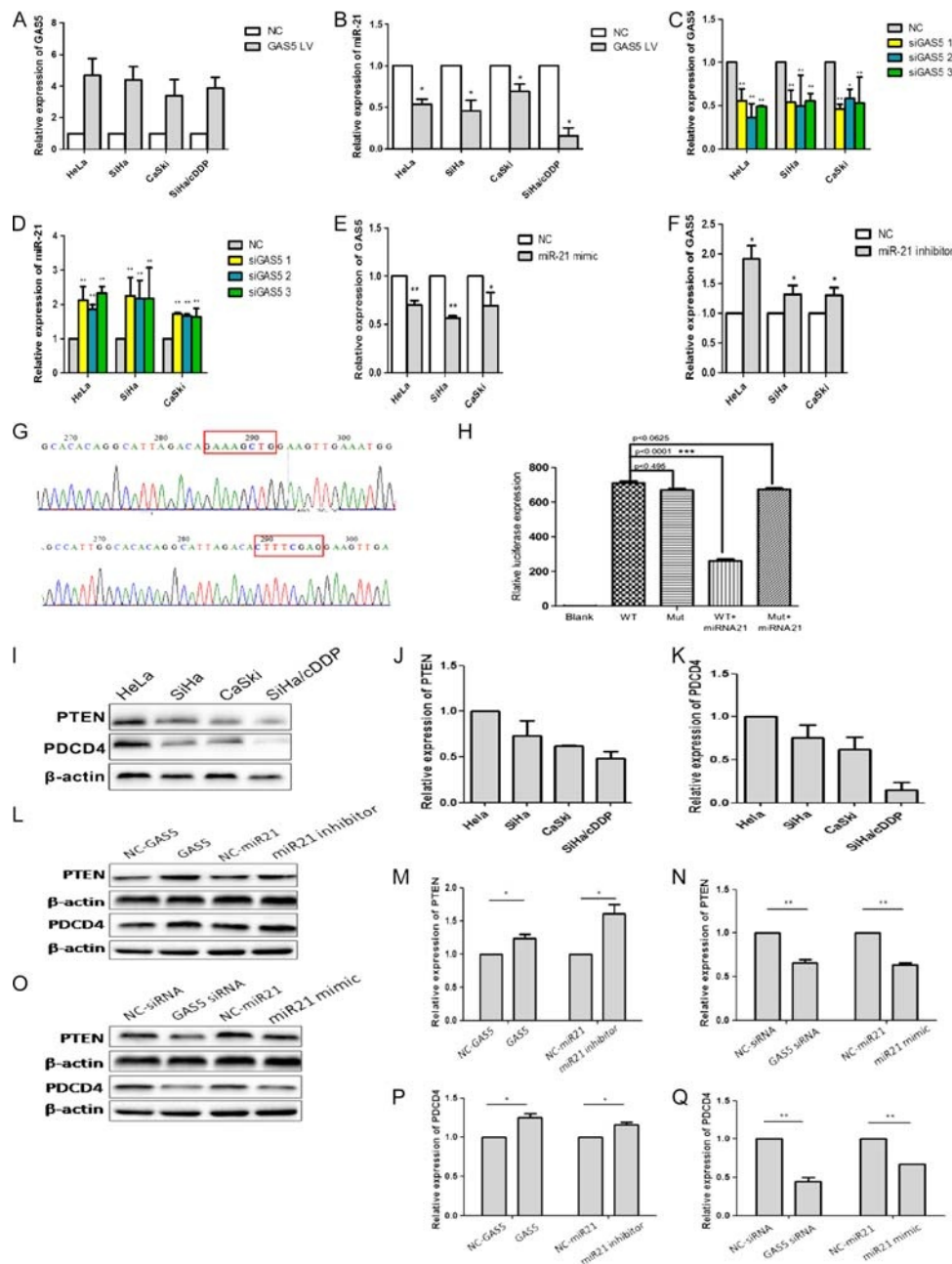


FIGURE 2. Identification of GAS5 as a target of miR-21 and effect of regulation to miR-21 target proteins PTEN and PDCD4 by GAS5. A, Expression of GAS5 after GAS5 lentivirus transfection. Four cervical cell lines were transfected with either GAS5 or blank lentivirus (Ctrl). The cells were harvested after screening the stable GAS5 transfected cell lines, and total RNA was isolated, followed by detecting the expression of GAS5 using qRT-PCR. B, Detection of miR-21 in the stable GAS5 transfected cell lines using qRT-PCR; experimental procedure was the same as in A. C and D, Three CC cell lines HeLa, SiHa, and CaSki were transfected with con-siRNA or GAS5 siRNA1, GAS5 siRNA2, or GAS5 siRNA3 for 24 hours; the expression of GAS5 or miR-21 was determined by qRT-PCR. E and F, Identification of the GAS5 expression in the 3 CC cell lines by transfecting miR-21 mimic and miR-21 inhibitor by qRT-PCR. G and H, Dual-luciferase reporter assay of GAS5 and miR-21 and the sequence of mutation site. WT, luciferase vector of GAS5; Mut, GAS5 mutant vector; WT+ miR-21, luciferase expression with vector of GAS5 after transfecting miR-21 mimic; Mut + miR-21, luciferase expression with mutant vector of GAS5. I, Identification of the basic level of PTEN and PDCD4 in 4 CC cells HeLa, SiHa, CaSki, and SiHa/cDDP. Cellular protein was isolated from the cells and used for Western blot. J and K, Relative protein levels (mean [SD], n = 3) of PDCD4 and PTEN were determined. L-Q, Effects of GAS5 and miR-21 on expression of the tumor suppressors PDCD4 and PTEN. Error bars represent SD, n = 3. **P* < 0.05 or ***P* < 0.01.

Overexpression of GAS5 Promotes Apoptosis and Inhibits Migration and Invasion of CC Cells

To investigate the biological role of GAS5 in CC cells, we first determined the effect of GAS5 on CC cell growth. CCK-8 was

performed after transfection of lentivirus expressing GAS5 or GAS5 siRNA. Proliferation of different CC cell lines decreased significantly in groups overexpressing GAS5, whereas knockdown of GAS5 can promote cell proliferation (Figs. 3A-C). We further assessed the effects of GAS5 on cell migration and invasion, a key determinant of malignant

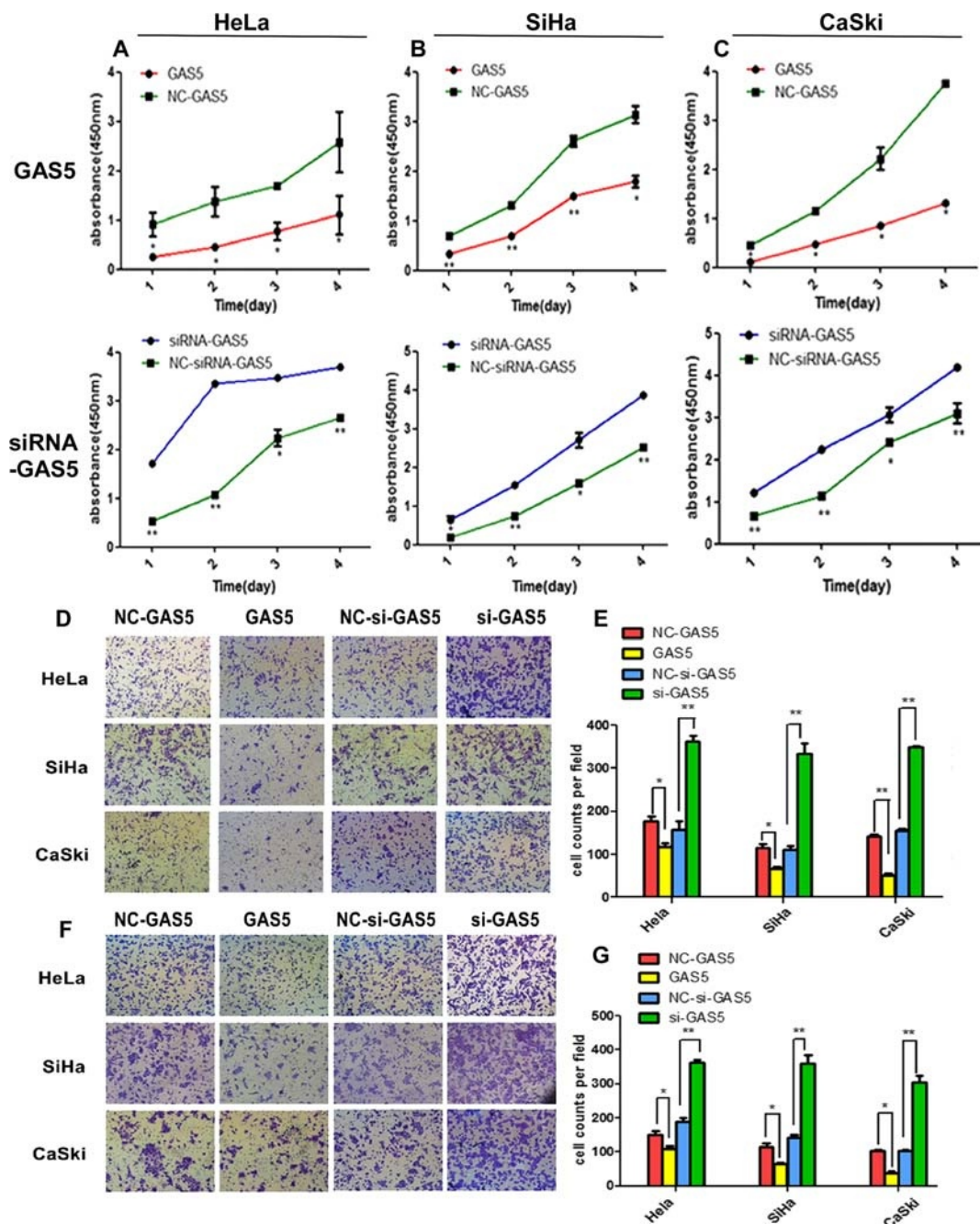


FIGURE 3. Effect of GAS5 on proliferation inhibition, migration, and invasion in CC cells HeLa, SiHa, and CaSki. A-C, Cell proliferation CCK-8 assay. Three cell lines were firstly grown and transiently transfected with GAS5 lentivirus and GAS5 siRNA and then subjected to CCK-8 assay. **P* < 0.05 versus control groups. Then, 3 cell lines were grown and transiently transfected with GAS5 lentivirus and GAS5 siRNA and then subjected to (D) migration and (F) invasion assay. E and G, Quantitative data of D and F, respectively. Error bars represent SD, n = 3. **P* < 0.05; ***P* < 0.01.

progression and metastasis. As shown in Figures 3D to G, overexpression of GAS5 significantly suppressed the cell migration and invasion of CC cells HeLa, SiHa, and CaSki. On the contrary, knockdown of GAS5 by siRNA increased the cell migration and invasion of all the CC cells. These results suggested a functional role of GAS5 in mediating cell migration and invasion in CC and down-regulation of GAS5 may contribute to tumor metastasis in CC.

Overexpression of GAS5 Inhibits Tumorigenicity

To further demonstrate the tumor-suppressive role of GAS5, we injected SiHa cells infected with lentivirus GAS5 into female nude mice. Tumor grew much slower in GAS5-transfected SiHa cells than the control group, and the tumor also weighs significantly less than that of the control group

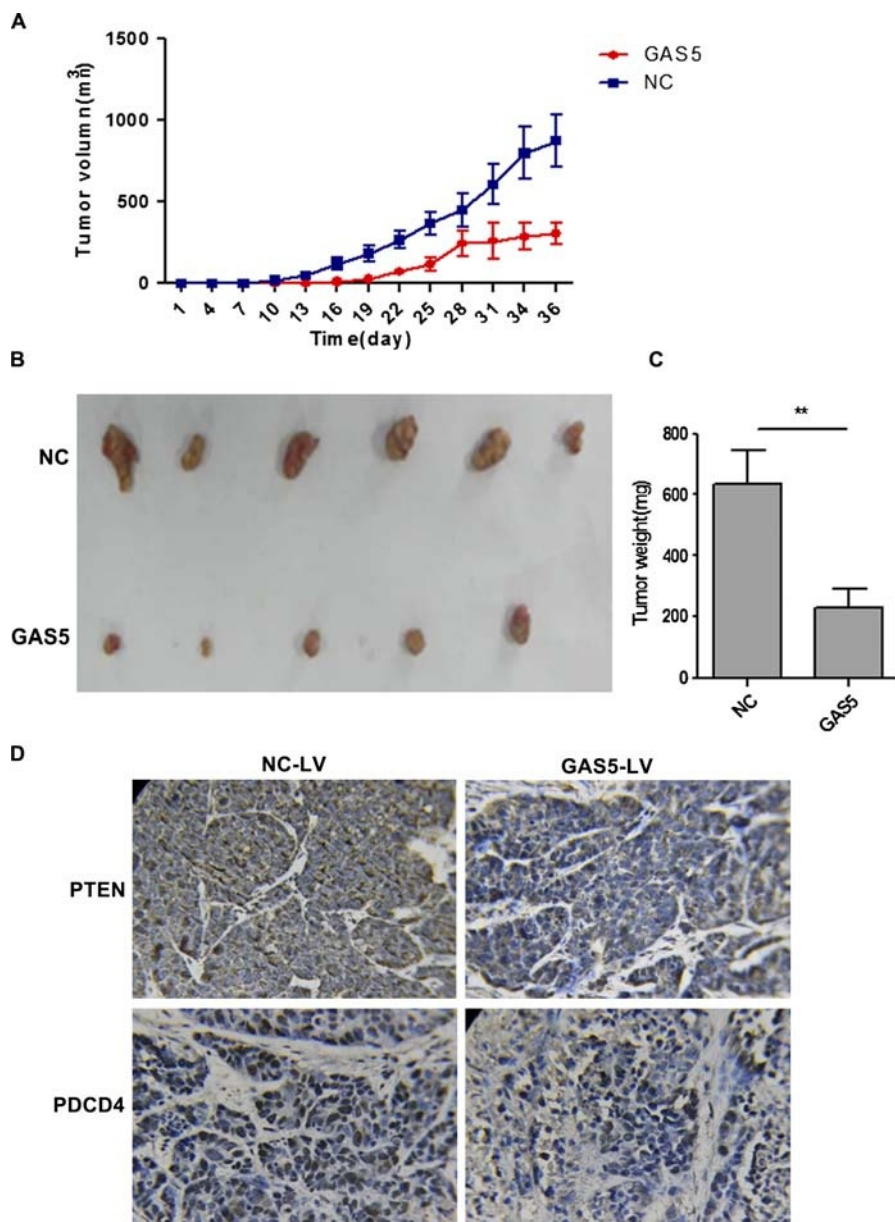


FIGURE 4. Suppression of tumor growth by GAS5 in the xenograft mouse model. SiHa cells were transfected with GAS5 lentivirus and NC-lentivirus and then injected into the left subaxillary, as described in the text. A, Tumor growth was measured every 3 days of injection. B and C, Tumors were then harvested on day 36 and weighed. D, Immunohistochemical evaluation of PTEN and PDCD4 in the tumor tissue derived from the GAS5 group and the NC group of the xenograft mice, respectively.

(Figs. 4A-C). Expression of PTEN and PDCD4 was also examined in tumor tissues by immunohistochemistry. A significant increase of PTEN and PDCD4 was detected in the lentivirus GAS5 tumors compared with control tumors (Fig. 4D).

CC Cell Survival in Cisplatin Is Correlated to GAS5 and miR-21 Levels

Tumor staging and response to chemotherapeutic drugs are 2 major factors that impact the survival of patients with

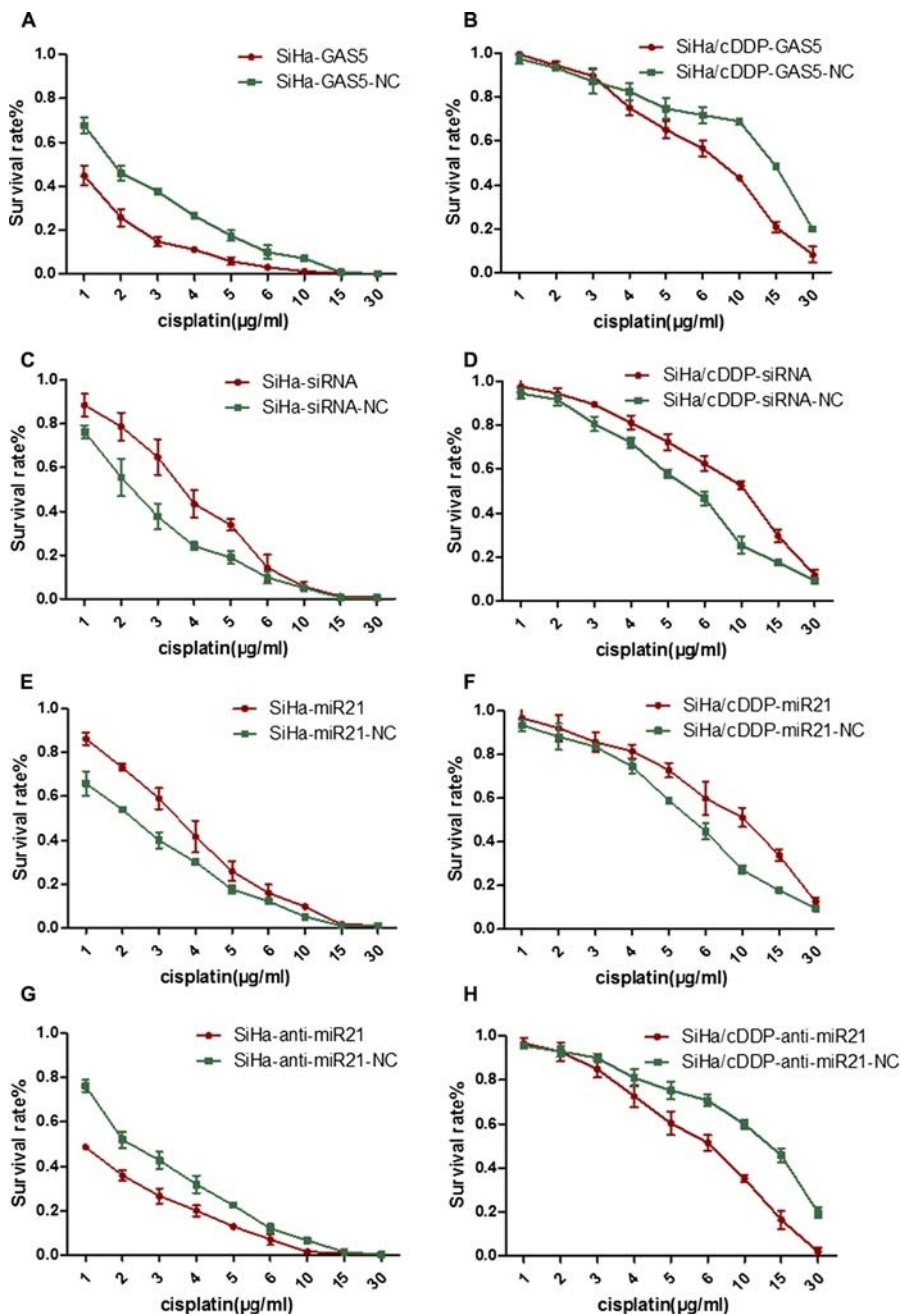


FIGURE 5. The effects of GAS5 and miR-21 on cisplatin-induced cytotoxicity in SiHa and SiHa/cDDP cells. Cells with stable GAS5 expression and cells (SiHa and SiHa/cDDP cells) were cultured for 24 hours after transfecting GAS5 siRNA, miR-21 mimic, and miR-21 inhibitor. Cells were treated with different concentrations of cisplatin (1, 2, 3, 4, 5, 6, 10, 15, and 30 µg/mL). A, C, E, and G, Effects of GAS5 and miR-21 on cytotoxicity of cisplatin in normal SiHa cells. B, D, F, and H, Effects of GAS5 and miR-21 on cytotoxicity of cisplatin in SiHa/cDDP cells. The cytotoxicity was then determined 24 hours after cisplatin treatment using the CCK-8 assay. Each data point represents the survival rate of the cells.

CC. To examine the effects of GAS5 expression on chemotherapeutic drug resistance, the survival of CC cells transfected with lentivirus GAS5, GAS5 siRNAs, miR21 mimic, and miR-21 inhibitor was compared after cisplatin treatment in different concentrations (1, 2, 3, 4, 5, 6, 10, 15, and 30 $\mu\text{g}/\text{mL}$) (Fig. 5). The results showed that the survival of SiHa cells decreased significantly by overexpressing GAS5 or transfecting miR-21 inhibitor, whereas transfecting GAS5 siRNA or miR-21 mimic enhanced SiHa cell survival (Figs. 5A, C, E, and G), suggesting that GAS5 and miR-21 can regulate the response of CC cells to cisplatin treatment.

GAS5 and miR-21 Regulate Cisplatin Resistance in SiHa/cDDP Cells

SiHa/cDDP cells are cisplatin-resistant CC cells, established by incubating the cells with stepwise increasing concentrations of cisplatin. SiHa/cDDP cells (IC_{50} , 12.268 $\mu\text{g}/\text{mL}$) were 5.7-fold more resistant to cisplatin than the parental SiHa cells (IC_{50} , 2.14 $\mu\text{g}/\text{mL}$). Overexpression of GAS5 or knockdown of miR-21 decreased the survival of SiHa/cDDP cells treated by cisplatin, whereas GAS5 knockdown and miR-21 mimic enhanced SiHa/cDDP cell survival when treated by cisplatin (Figs. 5B, D, F, and H).

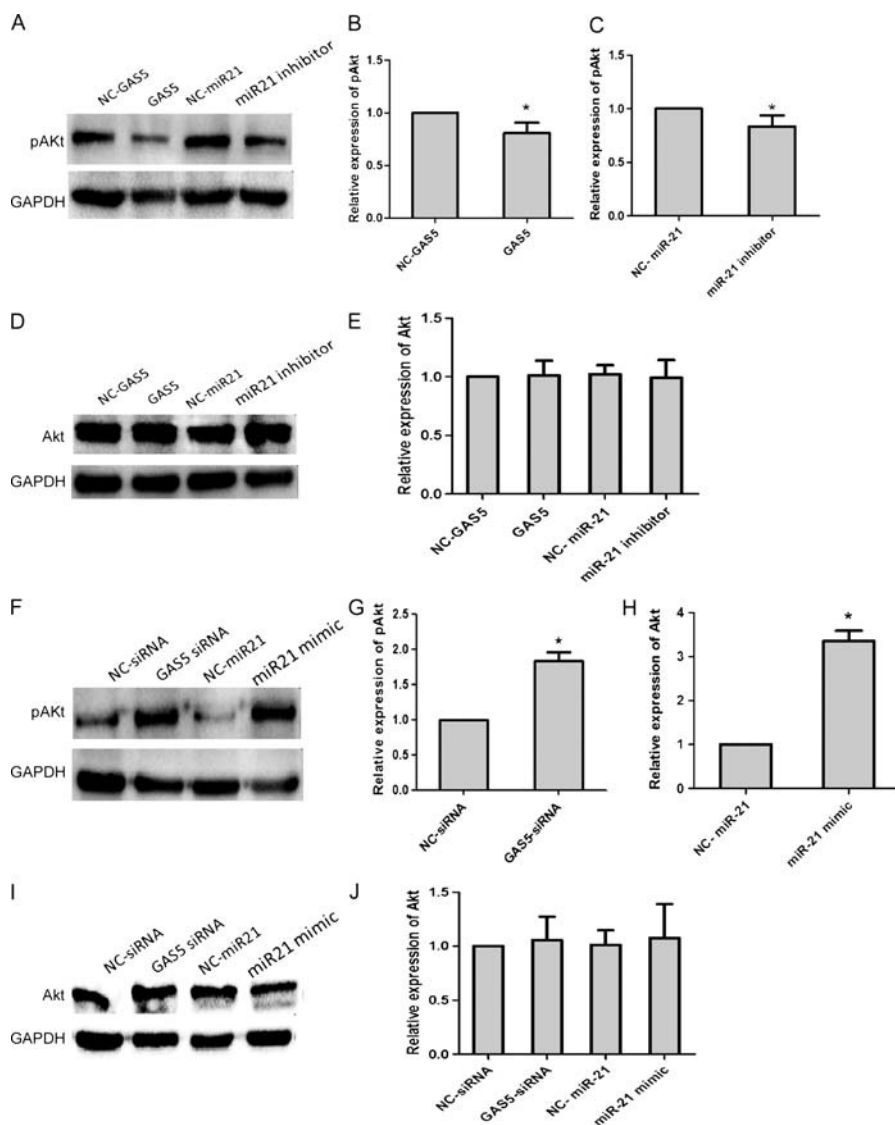


FIGURE 6. The effects of GAS5 and miR-21 on regulating the phosphorylation of Akt in SiHa/cDDP cells. SiHa/cDDP cells with stable GAS5 expression and SiHa/cDDP cells were cultured for 24 hours after transfecting GAS5 siRNA, miR-21 mimic, and miR-21 inhibitor. Cellular protein was isolated from the transfected cells and used for Western blot. A, D, F, and I, Relative protein levels (mean [SD], $n = 3$) of pAkt and Akt were determined. B and C, Quantitative data of A. E, Quantitative data of D. G and H, Quantitative data of F. J, Quantitative data of I. Error bars represent SD, $n = 3$. * $P < 0.05$ or ** $P < 0.01$.

GAS5 and miR-21 Regulate Cisplatin Resistance in SiHa/cDDP Cells via the PI3K/Akt Pathway

Because GAS5 and miR-21 were found to regulate the cisplatin resistance in SiHa and SiHa/cDDP cells, a further experiment for identifying the mechanism of drug resistance was performed. Western blot for detecting the Akt and pAkt protein level in SiHa/cDDP cells showed that SiHa/cDDP cells with GAS5 overexpression (SiHa/cDDP-GAS5) and SiHa/cDDP cells with miR-21 knockdown (SiHa/cDDP anti-miR-21) had a down-regulated level of pAkt; however, SiHa/cDDP cells with GAS5 knockdown (SiHa/cDDP-GAS5-siRNA) and SiHa cells with miR-21 overexpression (SiHa/cDDP-miR-21) had an up-regulated level of pAkt (Figs. 6A-J). All of these groups showed no difference in expression of Akt.

DISCUSSION

The morbidity of CC decreased because the human papillomavirus vaccine is widespread in the western countries. However, CC is still prevailing in China, necessitating CC prevention and diagnosis at the early stages. In addition, the chemoresistance of CC remains a major challenge. Therefore, it is essential to understand the molecular mechanisms of CC development and identify new therapeutic drugs for this deadly disease.³⁵ Long noncoding RNAs have been shown to play important roles in the development and progression of cancer.³⁶ They may provide mechanistic insight on CC diagnosis and treatment.

Previous studies demonstrated down-regulation of GAS5 in numerous tumor types, including renal cell carcinoma, hepatocellular carcinoma, and breast cancer.^{15,17,37} However, there are few reports regarding GAS5 expression and function in CC. In this study, we found that GAS5 was down-regulated in primary tumor tissues and CC cell lines and CC progression was associated with GAS5 expression. Because low GAS5 expression in CC tissues was associated with a poor prognosis, GAS5 could be a prognostic indicator for CC. As expected on being an oncogene, miR-21 was overexpressed in primary cervical tumor tissues and CC cell lines. Of the 4 CC cell lines (HeLa, SiHa, CaSki, and SiHa/cDDP), the highly malignant SiHa/cDDP cells expressed the lowest level of GAS5 and the highest level of miR-21, whereas the weakly malignant HeLa cells expressed the highest level of GAS5 and the lowest level of miR-21. Taken together, these data suggest that GAS5 may function as a tumor suppressor lncRNA and miR-21 may function as an onco-miR in CC.

Emerging evidence suggests that lncRNAs may participate in the “competitive endogenous RNAs” regulatory network and act as endogenous miRNA sponges to bind to miRNAs and regulate their function.^{38,39} We found that knockdown of GAS5 increases miR-21 expression in CC cell lines, whereas ectopic expression of GAS5 represses miR-21 expression. We further demonstrated reciprocal repression of GAS5 and miR-21 in several CC cell lines. To assess whether GAS5 can serve as a miR-21 sponge, we performed dual-luciferase reporter assay to ascertain whether miR-21 is the direct target of GAS5. The results showed that up-regulation

of GAS5 reduced the luciferase activities of the cells co-transfected with pGL3-miR-21-WT in CC cells. Thus, GAS5 may directly target miR-21.

microRNA 21 has been characterized for its role in cancer as an oncogene. It can target a large number of protein-coding genes that are important in tumor growth and metastasis, such as PDCD4, PTEN, and Spry1.^{40–42} This study shows that PDCD4 and PTEN are down-regulated in CC tissues and that GAS5 regulates miR-21 expression and affects expression of PDCD4 and PTEN in SiHa cells. Thus, we infer that GAS5 may regulate PDCD4 and PTEN via miR-21. The results demonstrated that miR-21 partially eliminated GAS5-induced change of PDCD4 and PTEN in CC cells.

To highlight the impact of altered expression and function of GAS5, we show the biological role of GAS5 in CC cells by applying gain-of-function and loss-of-function approaches. Ectopic expression of GAS5 inhibited the CC cell growth in vitro, whereas down-regulation of GAS5 could promote CC cell proliferation. Our results also indicated that GAS5 inhibits migration and invasion, which is similar to the previous research that GAS5 is associated with migration and invasion of cancer cells.¹⁶

Cisplatin resistance is a major obstacle for successful treatment of CC. Our study showed that SiHa/cDDP cells had a significantly lower expression of GAS5 and protein PTEN than normal SiHa cells. We found that GAS5 could also have an impact on cisplatin resistance in SiHa and SiHa/cDDP cells, supported by reports that GAS5 could regulate response to chemotherapeutic drugs, such as 5-fluorouracil, imatinib, and docetaxel, in breast cancer cell lines.¹⁵ The underlying mechanism may be the modulations of chemotherapeutic agents on cellular apoptosis through the PI3K/Akt pathway.⁴³ A recent study demonstrated that PI3K/Akt is activated in cisplatin-resistant ovarian cancer OVCAR-3/cDDP cells, and level of Akt and its kinase activity was higher than the normal OVCAR-3 cells.⁴⁴ Akt is the survival signal that is an important regulator of cell proliferation, growth, survival, and metabolism. It had been implicated in the development of drug resistance and the progression of many different cancer cells.^{5,6} This indicated that PI3K/Akt was also an important part of cisplatin resistance in tumor cells. mTOR is not only a pathway that can regulate the expression of GAS5 but also a highly conserved protein kinase as an effector in the PI3K/Akt pathway. Moreover, PTEN, a tumor suppressor gene, functionally antagonizes PI3K activity by converting PI(3,4,5)P3 back to PI(4,5)P2.⁴⁵ Then, a further experiment for proving the antidrug ability of GAS5 was performed with SiHa/cDDP cells, and the results showed that GAS5 and miR-21 not only can regulate the level of protein PTEN but also may regulate the level of phosphorylation of Akt via PTEN. Thus, we suppose that GAS5 can regulate the cellular growth and drug resistance through the PTEN/PI3K/Akt/mTOR pathway. The low level of GAS5 can down-regulate PTEN by interacting with miR-21 because PTEN is one of the genes in the PI3K/Akt/mTOR pathway that can be regulated by GAS5 negatively. In the end, the low expression of PTEN activates the PI3K/Akt pathway, thus forming a circulation.

In summary, we have demonstrated that GAS5 is markedly down-regulated in human CC tissues and cell lines.

Overexpression of GAS5 inhibits cell growth, invasion, and migration. microRNA 21 is identified as the direct target gene of GAS5. GAS5 may impact on the cisplatin resistance in cervical cells through miR-21 by regulating the level of PTEN and then influencing the phosphorylation of Akt. Therefore, our data highlight the significance of the miRNA-lncRNA interaction in CC tumorigenesis in which GAS5 suppressed CC malignancy mainly by inhibiting miR-21, suggesting that GAS5 may serve as a novel biomarker and a therapeutic target in CC therapy.

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