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The expression of inhibitor of growth 4 is reduced in cervical cancer tissues

J. YUAN[1], Y.-L. HAN[2], T.-Y. WANG[2], S. WU[2], L. TONG[2], L.-X. LIN[2], M. LI[3], L.-L. WEI[2], Z.-H. ZHONG[2], G.-M. ZHANG[1], Y. WANG[2]

[1]Department of Obstetrics and Gynecology, The First Affiliated Hospital of Harbin Medical University, Harbin, China
[2]Department of Microbiology, Harbin Medical University, Harbin, China
[3]Institute of Iodine Deficiency Disorders, Center for Endemic Disease Control, Chinese Center for Disease Control and Prevention, Harbin Medical University, Harbin, China

Abstract. – OBJECTIVE: Inhibitor of growth 4 (ING4) is a candidate tumor suppressor which plays an important role in multiple processes including DNA repair, apoptosis, cell cycle control, tumor metastasis and angiogenesis. However, clinical data about the role of ING4 in the development and progression of cervical cancer are still limited. This study aimed to examine ING4 expression in cervical cancer and analyze its correlation with the progression of the malignancy.

PATIENTS AND METHODS: RT-PCR, Western blot and immunohistochemistry analysis were performed to determine ING4 expression in 18 clinical specimens from cervical cancer patients. The correlation of ING4 expression with the clinical-pathological features of the patients was analyzed. Moreover, the correlation between ING4 and HPV E6/E7 transcription level in SiHa cells was analyzed.

RESULTS: ING4 expression was decreased significantly at mRNA and protein levels in the tissues of cervical cancer compared with paracarcinoma tissues. Analysis of the subcellular localization of ING4 showed that ING4 expression was decreased in the nucleus of cervical cancer tissues. Ectopic expression of ING4 reduced the proliferation of SiHa cells, accompanied by decreased HPV E6/E7 transcription.

CONCLUSIONS: ING4 expression is decreased in human cervical cancer tissues. Reconstitution of ING4 expression in cervical cancer cells is correlated with decreased HPV E6/E7 transcription. These data suggest that ING4 expression has diagnostic and prognostic significance for cervical cancer.

Key Words:
Inhibitor of growth 4, Cervical cancer, HPV, E6/E7, SiHa cells.

Introduction

ING4 is a member of the inhibitor of growth (ING4) family with 249-amino acid, encoded by a gene located on chromosome 12p13-31. ING4 contains three nuclear localization signals and a highly conserved C-terminal plant homeodomain (PHD) zinc finger motif[1]. ING was initially identified as a tumor suppressor candidate by subtractive hybridization screen[2]. ING4 plays an important role in multiple cellular processes, including cell proliferation, cell cycle control, cell senescence, and apoptosis[3-6]. Furthermore, it has been documented that ING4 interacted with histone acetyl transferase (HAT) and histone deacetylase (HDAC) to regulate gene transcription[7,8]. Recent data suggest that ING4 interacted with A-U rich binding element factor (AUF1) to downregulate proto-oncogene MYC[9]. However, the molecular mechanism underlying the role of ING4 on cancer progression still need to be elucidated.

Cervical cancer is the second most frequent female malignancy worldwide, and is associated with oncogenic human papillomavirus (HPV)[10]. High-risk human papillomavirus (hr-HPV) is a major risk factor for cervical cancer and closely related to the development of high-grade cervical intraepithelial neoplasia (CIN2-3) and invasive cancer[11]. It has been confirmed that HPV viral protein E6 and E7 are predominantly responsible for pathological change of HPV-positive cervical cancers. E6/E7 are expressed in the early infection and play multifunctional roles in pathogenesis[12]. E6/E7 expression is vital for malignancy and is required to maintain a malignant transformed phenotype[13].

Corresponding Authors: Yan Wang, MD; e-mail: wangyan@hrbmu.edu.cn

In this study, we examined ING4 expression in tumor tissues and paracarcinoma tissues from patients with cervical cancer, and analyzed the correlation of ING4 expression with the clinical-pathological features of the patients. Furthermore, the effects of ING4 expression on SiHa cervical cancer cell proliferation and E6/E7 transcription level were determined. Our results provided a new insight into the role of ING4 in HPV-positive cervical cancer.

Patients and Methods

Patients and Specimens

All paired specimens of carcinoma and paracarcinomatous tissues were collected from patients with cervical cancer who underwent surgical cervical resection from March to May 2012, at the Department of Surgery, the Third Affiliated Hospital of Harbin Medical University (Harbin, China). Informed consents were obtained from all patients. The usage and collection of human specimens in this study were permitted by Ethics Review Board of the Third Affiliated Hospital of Harbin Medical University, China. The details of specimens were list in Table I.

Cell Culture

SiHa cell line was provided by Microbiology Department, Harbin Medical University and cultured in DMEM (Invitrogen, CA, USA) supplemented with 10% fetal bovine serum (FBS). pEGFP-ING4 was previously constructed by our group.

Quantitative Reverse Transcription Polymerase Chain Reaction

Total mRNA was isolated from 18 paired frozen tissues using Trizol reagent (Invitrogen, Camarillo, CA, USA) following the manufacturer's protocol. Reverse transcription was performed using 1 μg of RNA and TaKaRa reverse transcription Kit (TaKaRa, Dalian, China) according to the procedures provided by the supplier. Real-time PCR was performed by LightCycler 2.0 (Roche, Basel, Switzerland). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as internal control. The primers were as follow: *ING4* forward 5'-TCGTGCTCGTTCCAAAGG-3' and reverse 5'-GGCAATAGGTGGGTTCGTT-3'; HPV *E6* forward 5'-GACCCAGAAAGTTACCACAG-3'and reverse 5'-CACAACGGTTTGTTGTATTG-3'; *GAPDH* forward 5'-ATCACTGCCACCCAGAAGAC-3' and reverse 5'-TTTCTAGACGGCAGGTCAGG-3'. PCR was performed with 20 μl of SYBR Premix Taq mix (TaKaRa, Dalian, China). Reaction mixtures were initially denatured at 95°C for 30 s followed by 45 cycles of 95°C for 20 s, 60°C for 20 s and 72°C for 20 s, with fluorescence acquisition in single mode. To confirm the amplification specificity, RT-qPCR products were subjected to a melting curve analysis. Data were analyzed with the LightCycler analysis software 4.05.

ING4 expression level in tissues of cervical cancer tissues (designated as T) or in the paracarcinomatous tissues (designated as N) was normalized by the corresponding GAPDH expression level (T = *ING4/GAPDH*; N = *ING4/GAPDH*). T/N ratio represented relative ING4 mRNA level in cervical cancers.

Table I. The clinical information of the specimens.

Characteristic	Information	Number
Age	< 50 years old	14
	≥ 50 years old	4
Histology	Adenomatous	5
	Squamous	13
Differentiation	High	0
	Medium	12
	Low	1

Western Blot Analysis

The tissues preserved in liquid nitrogen were ground and incubated on ice with lysis buffer (Pierce, Rockford, IL, USA) containing a protease inhibitor cocktail (Sigma, St. Louis, MO, USA) for 30 min. The lysate was centrifuged at 12,000 g for 10 min at 4°C, and the supernatant was collected. The protein concentration of the lysate was determined by Bradford assay (BIO-RAD, Hercules, CA, USA). Equal amounts of protein were subjected to 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to polyvinylidene fluoride (PVDF) membrane (Millipore, Billerica, MA, USA). PVDF membrane was blocked with 5% non-fat milk solution containing 0.1% Tween (Amresco, Solon, OH, USA) at 37°C for 1 h. The membrane was then incubated with ING4 rabbit polyclonal antibody (diluted at 1:1000; Invitrogen) at 4°C overnight. The membrane was washed with TBS-Tween buffer for 5 min 3 times and incubated with goat anti-rabbit immunoglobulin G (diluted at 1:4000, Zhongshan Goldenbridge Biotechnology, Beijing, China) at

37°C for 1 h. Membrane was stained with DAB (Boster, Wuhan, China) and visualized by Fuji-Film LAS-4000 Imaging System (Tokyo, Japan). β-actin was used as a loading control. ING4 protein level in cervical cancer tissues and the corresponding paracarcinoma tissues of the same patient was designated as T and N, respectively. T/N represented relative ING4 protein level in the specimens of human cervical cancers.

Immunohistochemistry

Tumor tissues and normal tissues were fixed in 40 g/L paraformaldehyde and paraffin embedded. Subsequently, 4 um-thick serial sections of the tissues were cut, washed carefully with 0.01 M phosphate buffered saline (PBS) three times (10 min each), and then blocked with 2% goat serum in 0.01 M PBS containing 0.3% Triton X-100 (PBS-X) for 1 h at room temperature. Endogenous peroxidase activity was blocked with 3% hydrogen dioxide for 5 min. Sections were then washed with PBS-Tween for 5 min three times and incubated in 10% goat serum to reduce nonspecific staining. Next the sections were incubated with ING4 rabbit polyclonal antibody (1:200, Invitrogen, Carlsband, CA, USA) overnight at 4°C and then incubated in biotin-secondary antibody at 37°C for 20 min. The immunoreactivity was detected by Two-Step IHC Detection Reagent (Zhongshan Goldenbridge Biotechnology, Beijing, China). The results were independently assessed by two pathologists.

ING4 staining was evaluated by immunoreactive score (IRS), presented in the form of the intensity of immuoreactivity and the percentage of positively stained cells. ING4 staining intensities were designated 0-3 (0, negative; 1, weak; 2, moderate; and 3, strong). The percentages of ING4-positive stained cells were scored into four categories: 1 (0-25%), 2 (26-50%), 3 (51-75%) and 4 (76-100%). In the cases where there was a discrepancy between the assessment scores made by the two pathologists, the higher one was taken as the final score. According to the IRS, the staining patterns of ING4 were defined as: negative (-; IRS 0), weak (+; IRS 1-4), moderate (++; IRS 6-8) and strong (+++; IRS 9-12).

MTT Assay

The cells in logarithmic phase were seeded into 96-well culture plates. After overnight incubation, cells were transfected with different concentration plasmid of EGFP-ING4. Cell growth was measured 12 h and 24 h after transfection. 0.5 mg/ml MTT solution (Sigma) was added into each well. About 4 h later, the medium was replaced with 120 μL dimethylsulfoxide (DMSO, Sigma) and vortexed for 10 min. Absorbance (A) was recorded at 490 nm using a microplate reader (Bio-Rad, Philadelphia, PA, USA).

Statistical Analysis

Paired Student's *t* test was applied to assess the difference of ING4 mRNA and protein expression between tumor tissues and paracarcinoma tissues. Chi-square and Fish exact test were used to assess the difference of ING4 expression in paired tissues. All tests were two tailed and $p < 0.05$ was considered statistically significant. Statistical analysis was performed using Stata statistical software (version 11.0, Stata Corporation, College Station, TX, USA).

Results

The Decreased Expression of ING4 in Cervical Cancer Tissues

The expression of ING4 in paired specimens of patients with cervical cancer was examined by RT-PCR, Western blot and immunohistochemistry analysis. Significant decrease in ING4 expression at mRNA and protein levels was found in all cancer tissues examined, compared to paracarcinoma tissues (Figure 1A, $p = 0.008$; Figure 2B, $p = 0.001$).

To further analyze the relationship between ING4 expression and the clinical characteristics of cervical cancer patients, clinical specimens were classified according to patients' age and histology. However, no significant discrepancies between the tumor tissues and paracarcinoma tissues were detected at both mRNA and protein levels of ING4 (Figure 1B and 2C).

Altered ING4 Expression in the Nucleus and the Cytoplasm of Cervical Cancer Tissues

18 paired specimens were detected by immunohistochemistry to determine the subcellular localization of ING4. ING4 staining in paracarcinoma cervical tissues was primarily distributed in the nucleus (Figure 3A and 3C). However, less staining intensity was shown in the paired cervical cancer tissue (Figure 3B and 3D). According to the IRS, the rate of high ING4 nuclear expression in cervical tumor tissues and in paired paracarcinoma tissues was 11% (2/18) and 50%

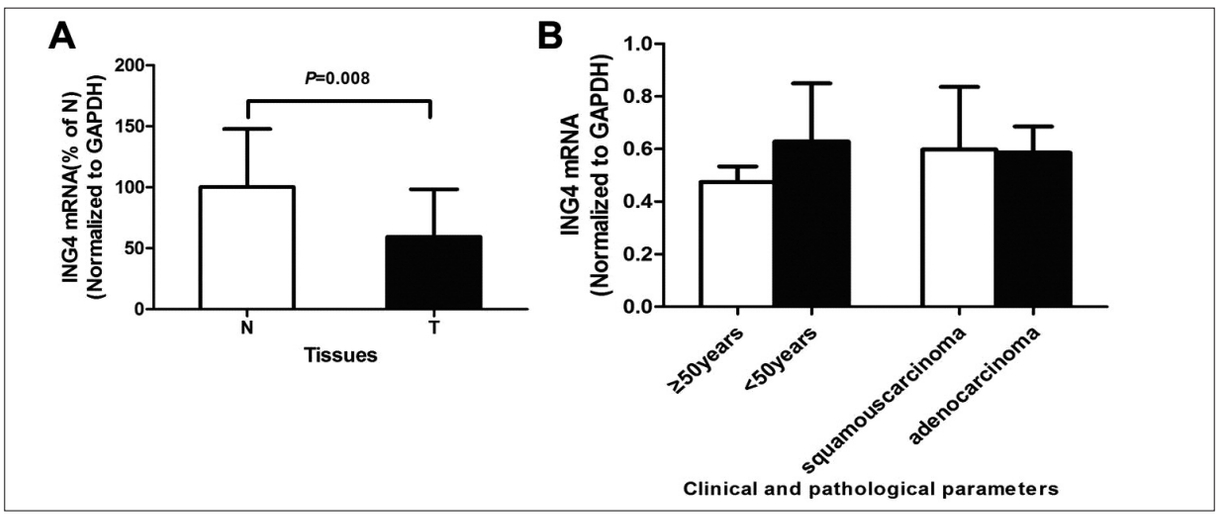

Figure 1. ING4 mRNA expression in human cervical carcinoma. ***A,*** RT-PCR analysis of ING4 mRNA level in 18 paired cervical cancer tissues. GAPDH was the internal control, N indicated paracarcinoma tissue, and T indicated tumor tissues. ***B,*** According to the age and histology type, the tissues were grouped into the subgroups (≥ 50 years old, n = 4; < 50 years old, n = 14; adenomatous, n = 5; squamous, n = 13). T/N represented relative ING4 mRNA level in tumor and paracarcinoma tissues.

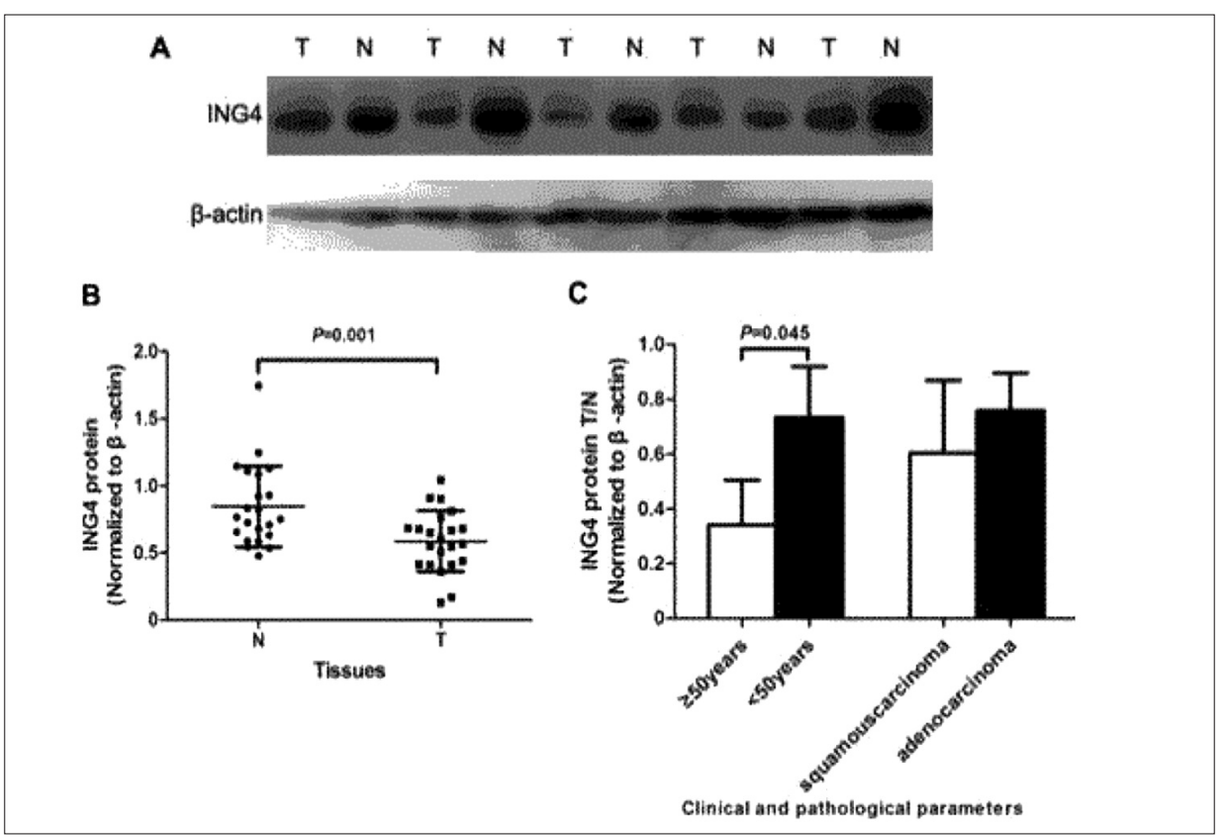

Figure 2. ING4 protein expression in human cervical carcinoma. ***A,*** Western blot analysis of ING4 protein level in 5 paired cervical cancer tissues. β-actin was the loading control, N indicated paracarcinoma tissue, and T indicated tumor tissues. ***B,*** The average expression level of ING4 in the 18 paired cervical cancer tissues and paracarcinoma tissues. N indicated paracarcinoma tissue, and T indicated tumor tissues. ***C,*** According to the age and histology type, the tissues were grouped into the subgroups (≥ 50 years old, n = 4; < 5 0 years old, n = 14; adenomatous, n = 5; squamous, n = 13). T/N represented relative ING4 protein level in tumor and paracarcinoma tissues.

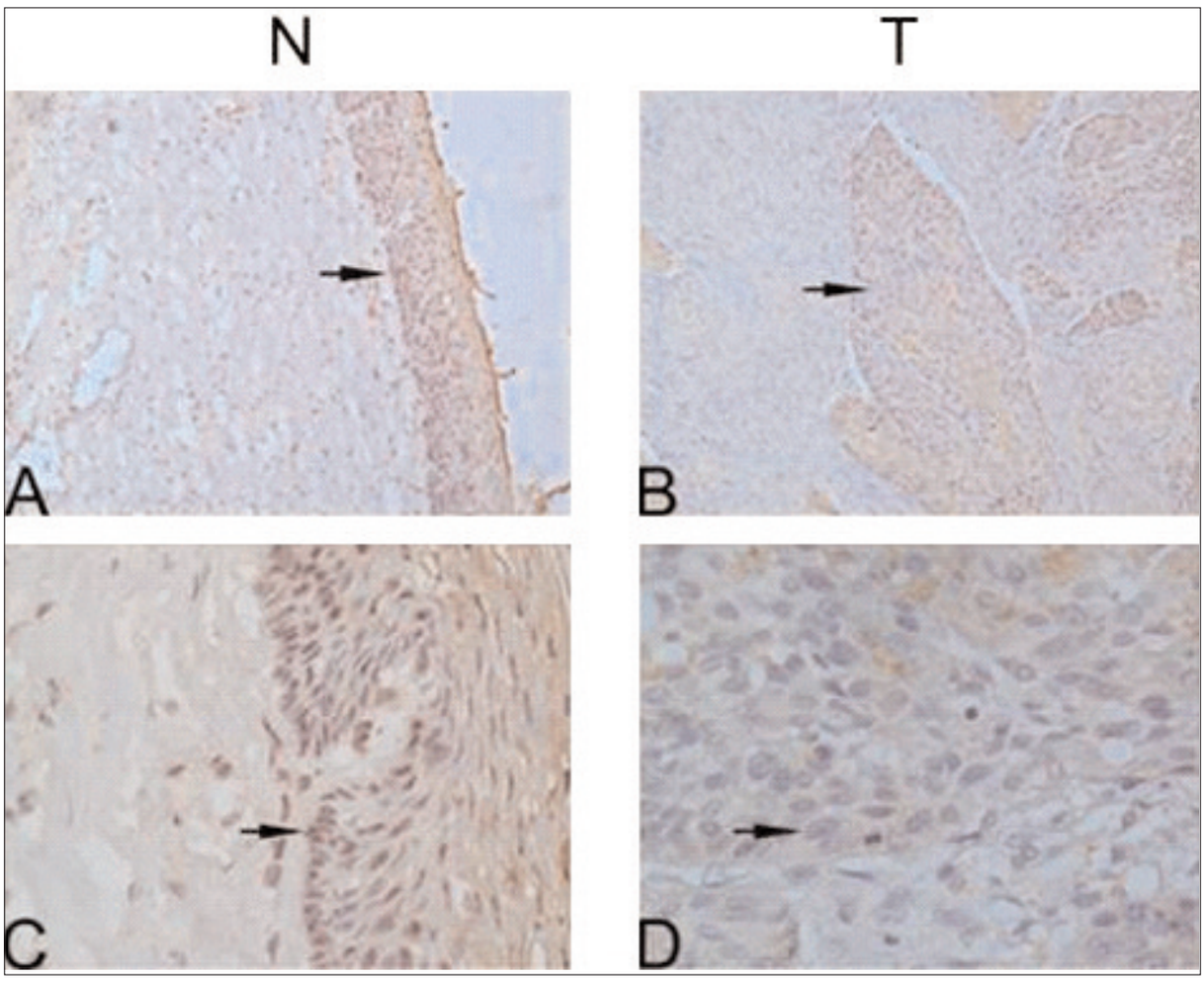

Figure 3. Immunohistochemistry analysis of ING4 expression in human cervical carcinoma. ***A,*** and ***C,*** Tissues from paracarcinoma tissues; ***B,*** and ***D,*** Tissues from cervical cancer tissues. ING4 was stained in claybank, ***A,*** and ***B,*** 100 × magnification; ***C,*** and ***D,*** 400 × magnification.

(9/18), respectively, while the rate of high ING4 cytoplasmic expression in cervical tumor tissues and in paired paracarcinoma tissues was 22% (4/18) and 33% (6/18), respectively (Table II). Taken together, these data showed that compared to paracarcinoma tissues, ING4 nuclear expression was decreased significantly ($p = 0.027$), while there was no significant discrepancy in ING4 cytoplasmic expression between tumor tissues and paracarcinoma tissues ($p = 0.711$) (Table II). Furthermore, we found no significant correlation between ING4 subcellular localization and the clinical-pathological features of cervical tumor tissues (Data not shown).

Table II. Subcellular localization of ING4 protein in human cervical carcinoma.

		Nuclear ING4					Cytoplasmic ING4				
		Low		High			Low		High		
Tissue	n	–	+	++	+++	*p*	–	+	++	+++	*p*
Tumor	18	6 (33%)	10 (56%)	2 (11%)	0 (0%)	0.027	3 (17%)	11 (61%)	4 (22%)	0 (0%)	0.711
Normal	18	2 (11%)	7 (39%)	9 (50%)	0 (0%)		4 (28%)	8 (44%)	6 (28%)	0 (0%)	

Ectopic Expression of ING4 Inhibits the Proliferation of SiHa Cells and Transcription of HPV16 E6/E7

MTT assay showed that compared with the control group, the proliferation of SiHa cells was obviously decreased at 24 h, 48 h, and 72 h after transfection with pEGFP-ING4 plasmid (Figure 4A).

To identify the mechanism by which ING4 inhibits cell proliferation, we examined the transcription of HPV E6//E7. RT-PCR analysis showed that HPV E6/E7 mRNA levels were both decreased in SiHa cells transfected with pEGFP-ING4 plasmid, compared to cells transfected with empty pEGFP plasmid (Figure 4B and C).

Discussion

Previous studies suggest that ING4 could be a potential tumor suppressor which could suppress the proliferation and migration of cancer cells as well as inhibit angiogenesis[1-4]. However, up to now the role of ING4 in cervical cancer remains unclear. In the present work, we demonstrated that the expression of ING4 was significantly lower in tumor tissues of cervical cancer patients than in the paracarcinoma tissues. Notably, we found that ING4 inhibited transcription of HPV E6/E7 mRNA levels in cervical cancer cells and inhibited cell proliferation. These findings indicate that ING4 may play a critical role in suppressing the progression of cervical cancer.

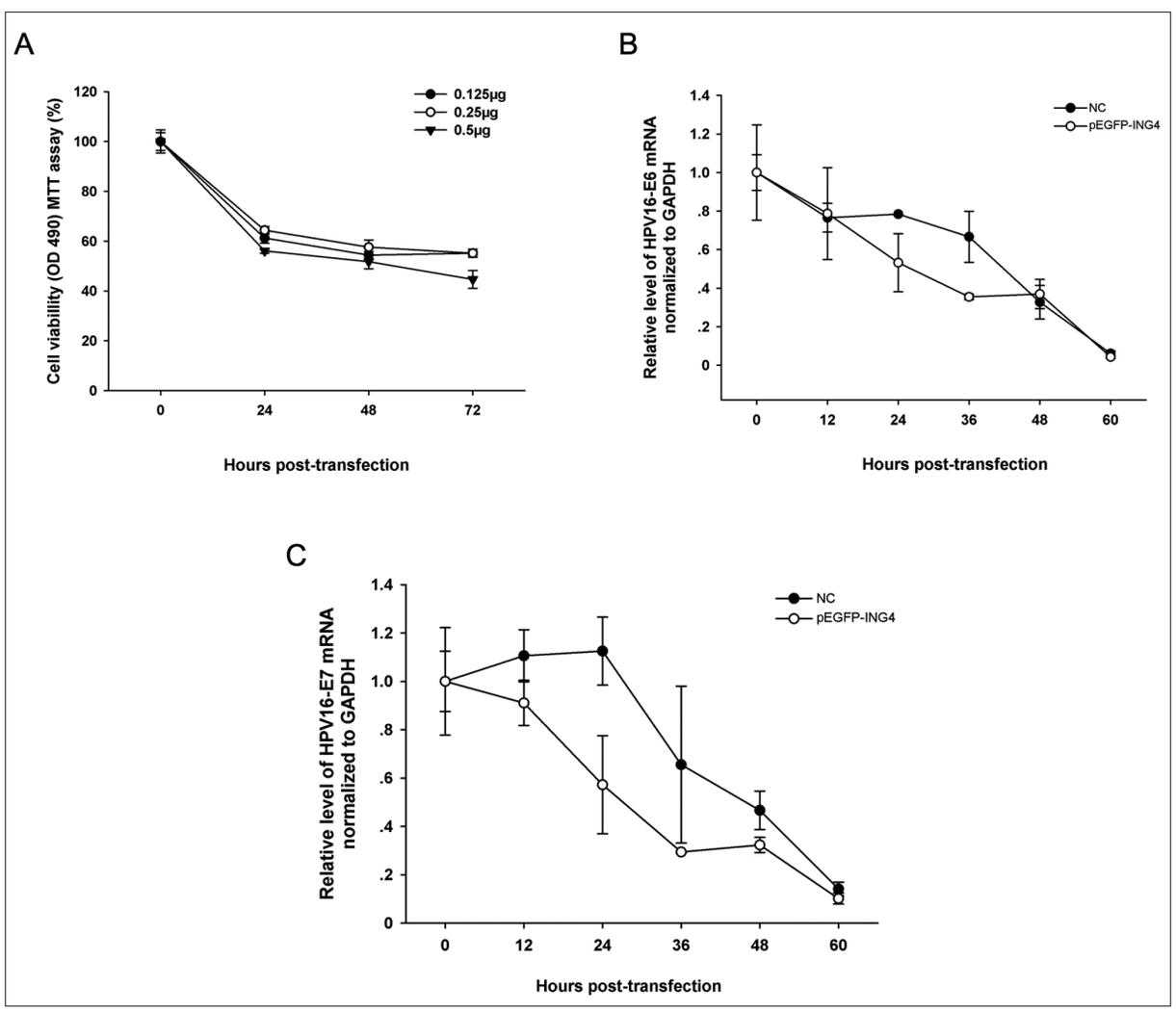

Figure 4. ING4 inhibits the proliferation of SiHa cells and transcription of HPV16 E6/E7. ***A,*** MTT assay of the proliferation of SiHa cells at 0 h, 24 h, 48 h, and 72 h after transfection with pEGFP-ING4 (0, 0.125, 0.25, and 0.5 ug). ***B,*** RT-PCR analysis of HPV16 E6 mRNA transcription level (n=3). ***C,*** RT-PCR analysis of HPV16 E7 mRNA transcription level (n=3).

We also examined the subcellular distribution of ING4 in cervical cancer tissues. While we observed high nuclear expression of ING4 in normal tissues, decreased ING4 expression in the nucleus in cervical cancer tissues was obvious. These data imply that reduced amount of ING4 in the nucleus may promote the development of cervical cancer. There are four ING4 variants (ING_v1, v2, v3, v4) due to alternative splicing of the pre-mRNA of ING4[14]. ING_v1 is primarily located in the nucleus while other variants are primarily distributed in the cytoplasm because of the partial loss of the nuclear localization signal by RNA splicing. These ING4 variants are expressed ubiquitously in various tissues but are kept at a low level except that in brain and testis[14]. Although the expression of a specific ING4 variant was not measured in this study, our data may reflect the overall decrease of ING4 expression.

Experimental data have shown that the mechanism underlying the functions of ING4 involves its interaction with p53, hypoxia-inducible factor alpha and NF-kB signaling pathway[3,4,15]. In addition, ING4_v1 was reported to interact with cytoplasmic proteins such as Liprin 1 and G3BP2, which are involved in Ras and NF-kB signaling[14]. Our results suggest that reduced expression of ING4 in the nucleus may disrupt its ability to regulate other nuclear transcription factors, resulting in increased transcription of HPV viral proteins E6/E7. Consequently, HPV driven cell proliferation is enhanced, which may favor the development of cervical cancer.

Conclusions

We demonstrated that ING4 expression was decreased in human cervical cancer tissues and decreased nuclear expression of ING4 may contribute to the progression of cervical cancer by promoting HPV viral proteins E6/E7 transcription. Our data suggest that ING4 is a potential biomarker for the diagnosis and prognosis of cervical cancer.

Acknowledgements

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Conflict of Interest

The Authors declare that there are no conflicts of interest.

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