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Correlation between methylation of the p16 promoter and cervical cancer incidence

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Abstract. – OBJECTIVE: To study the methylation of the promoter of the p16 gene in cervical cancer patients and explore the correlation between methylation and the incidence of cervical cancer.

PATIENTS AND METHODS: We recruited 78 patients with cervical cancer and 48 healthy individuals. The methylation-specific PCR was used to detect the methylation status in the promoter of the p16 gene. The mRNA expression of p16 was studied by quantitative fluorescence PCR. The protein expression of p16 was monitored by Enzyme-linked immunosorbent assay (ELISA) and Western blot. Immunohistochemistry was applied to detect the expression and distribution of p16 in cervical tissues.

RESULTS: The methylation sequencing results showed that samples from cervical cancer patients had a methylation rate of 78.52% in the p16 gene promoter region compared with a much lower rate of 9.8% in the control group (9.8%). Quantitative fluorescence PCR indicated that the p16 mRNA expression was significantly reduced in cervical cancer patients compared with controls. ELISA and Western blot results showed that expression of the p16 protein in cancer tissue was 0.81 ± 0.12 μg/l, whereas in the healthy controls it was 3.21 ± 0.24 μg/l. Immunohistochemical results showed that the p16 protein was mainly present in the cytoplasm. The rate of p16 positive cells in the healthy cervical tissue 83.29% was higher than in cervical cancer 10.18%.

CONCLUSIONS: The methylation of the p16 gene promoter could significantly reduce p16 expression, losing its tumor suppressor activity and promoting the development of cervical cancer.

Key Words:
p16, Methylation, Cervical cancer, Correlation, Treatment.

Introduction

Statistics show that cervical cancer is a common disease of the female reproductive system with high incidence and mortality rate[1]. As of 2015, the incidence of cervical cancer showed a yearly rising trend in the world. Understanding the pathogenesis of cervical cancer is critical for improving women's health[2]. Recent studies showed that p16 is a multiple tumor suppressor gene[3] that prevents the proliferation of cancer cells[4]. One study demonstrated that the p16 protein competed with cyclin D1 to inhibit its interactions with cyclin-dependent kinase[5], which inhibited the phosphorylation of substrates such as retinoblastoma, and inhibited cell proliferation. Recent studies showed that the expression p16 was undetectable in many tumor and cancer lesions[6]. Sequencing analysis found that the *p16* promoter region was methylated in breast cancer and other diseases, which significantly inhibited p16 expression[7]. In the lesions from patients with pancreatic cancer, the methylation of the 5'CpG island in the *p16* promoter region was nearly 20 times of that in healthy pancreatic tissues. Additionally, p16 protein expression in pancreas cancer was significantly lower than in healthy pancreatic tissues[8]. These results suggested a significant cor-

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relation between *p16* promoter methylation and the incidence of pancreatic cancer. In the present study, we first investigated the correlation between *p16* promoter methylation and the incidence of cervical cancer to determine the diagnostic value of p16 and its potential therapeutic use in late stages of cervical cancer.

Patients and Methods

Patients

We recruited 78 patients with a diagnosis of cervical cancer (observation group) treated in our hospital from January 2014 to January 2016. Their average age was 45.6 ± 8.5 years old. 48 healthy individuals were selected as the control group, with an average age of 47.2 ± 7.3 years old. Patients and their families signed the informed consent. Ethics Committee Approval of our hospital was obtained before the research samples were collected.

Tissue Genomic DNA Extraction

In this study, every subject was treated as a different sample. Genomic DNA was extracted using the Animal cell genome extraction kit (Axygen, Tewksbury, MA, USA) as follows: 1) Cell lysis buffer A was added to 0.2 g of tissue followed by vigorous vortex for about 2 min. 2) Lysis buffer B was added, and the mixture was slightly shaken for 30 sec. 3) Lysis buffer C was added and, after a vigorous vortex, the samples were centrifuged at 10,000 x g for 10 min. 4) The supernatant was transferred to a collection tube and centrifuged at 10,000 x g for 5 min. 5) The columns were washed with 75% ethanol twice, and centrifuged each time for 1 min at 12,000 rpm. 6) Elute DNA with 50 µl eluent buffer and store at 4°C[9].

Genome Processing

The extracted genomic DNA was quantified by nucleic acid micro spectrophotometer (ABI, Foster City, CA, USA). We used the Methylation kit from Kangwei Biotech (China). Methylation reagent A was added to 1 µg genome and incubated at 30°C for 30 min. Methylation reagent B was added and incubated at 70°C for 15 min. Reagent C was added and incubated at room temperature for 20 min. The above mixture was added to a collection tube and centrifuged at 12,000 rpm for 10 min. Washed with the elution buffer once at 12,000 rpm. The collected liquid was stored at 4°C[10].

PCR Amplification

After the above treatment, TaKaRa synthetic primers (Table I) were added to amplify the promoter region of the *p16* gene. The PCR products were sent to BGITech for sequencing.

Quantitative Fluorescence PCR

RNA Extraction: The total RNA was extracted (TaKaRa, Dalian, China) and the A260/ 280 readings were determined by a microspectrophotometer (ABI, Foster City, CA, USA)[10]. Quantitative fluorescence PCR: 500 ng of RNA was used as the template to synthesize cDNA. Quantitative fluorescence PCR was conducted with the Fluorescence quantitative PCR kit (TaKaRa, Dalian, China) on a Fluorescence quantitative PCR instrument (ABI, Foster City, CA, USA) following the manufacturer's instructions (TaKaRa, Dalian, China). The total reaction volume was 10 µl. Sequences of primers are shown in Table II.

Enzyme-linked Immunosorbent Assay (ELISA)

The total protein samples extracted from both groups with a kit from Axygen (Tewksbury, MA, USA) were quantified by Coomassie blue staining. 2.5 µg of protein from each sample was used for the ELISA experiment. 100 µl samples were added to the 96-well plate, and then 35 µl test solution were added following the manufacturer's instructions, and the absorbance was measured at 455 nm[11].

Western Blot

The Axygen cell protein extraction kit was used to extract the total proteins by following the manufacturer's instructions. 0.5 mg of tissue was precisely measured, and proteins were extracted from them. The Coomassie blue staining was used to quantify the proteins. 20 µl treated protein samples (15 µl total protein + 5 µl loading buffer) were separated by SDS-PAGE electrophoresis (Beijing Liuyi, China) and transferred to a membrane. After the membrane had been blocked for 2 h, diluted (1:1000) anti-p16 primary antibody (Alpha Suzhou, China) was added and incubated at room temperature for 2 h. HRP-labeled monoclonal secondary antibody (Tiangen Biotech, Beijing, China) was then added and incubated for 2 h at room temperature. 5 washes were done with the washing solution for 10 min each. The chromogenic solution was used for the color development and documented on a Gel imager (Bio-Rad, Hercules, CA, USA)[12].

Table I. Sequences of primers to detect methylation.

P16 Methylation-F	GTCGATCGATCGATCGATCGATGC
P16 Methylation-R	CGTAGCTAGCGCATCGATCGATCG

Immunohistochemistry

The streptavidin-peroxidase (S-P) method was used for antibody incubation and staining of the healthy cervix tissues and cervical cancer lesions. Immunohistochemical evaluations were as follows: membrane staining < 10% or no staining was considered a negative result. Membrane staining > 10% or sole membrane staining were regarded as positive results[13].

Table II. Sequences for quantitative fluorescence PCR primers.

Primer Name	Primer sequence
P16-F	CGTAGCTAGGCTAGCTAGCTACGTC
P16-R	CGTAGCTAGCGGTCAGCTAGCATCG
GAPDH-F	CGTCGTCGGGACAGCTAGCTGAG
GAPDH-R	CGTAGTCGACAGCTGATCGCAGCTG

Statistical Analysis

SPSS 19.0 software (IBM, Armonk, NY, USA) was used to do the analyses. Percentage (%) was used to express the enumeration data, and the chi-square test was used for data analysis. $p < 0.05$ was considered statistically significant.

Results

Methylation of the p16 Gene Promoter

We extracted genomic DNA from the cervix of cervical cancer and healthy control patients and treated the samples with the methylation kit. The products were then used as templates for the PCR amplification and electrophoresis of the *p16* promoter (Figure 1). Sequencing of the PCR products (BGITech) revealed that the methylation rate of the *p16* promoter was 78.52% in patients with cervical cancer, much higher than the 9.8% in healthy controls (Table III). This is a remarkable difference in the methylation of the *p16* promoter between cervical cancer and controls, suggesting a relevant role in pathogenesis.

p16 mRNA Expression in Cervix

Total RNA was extracted from cervix tissue in patients with cervical cancer and healthy controls. We used quantitative fluorescence PCR to detect the amount of *p16* mRNA. The expression of *p16* in patients with cervical cancer was significantly lower than in the healthy controls (Figure 2). The results indicated that methylation of the promoter region inhibited the expression of *p16*.

p16 Protein Expression in Cervix

Total proteins were extracted from cervical tissues from the observation and control groups. 3 μg of the total proteins were used to quantify

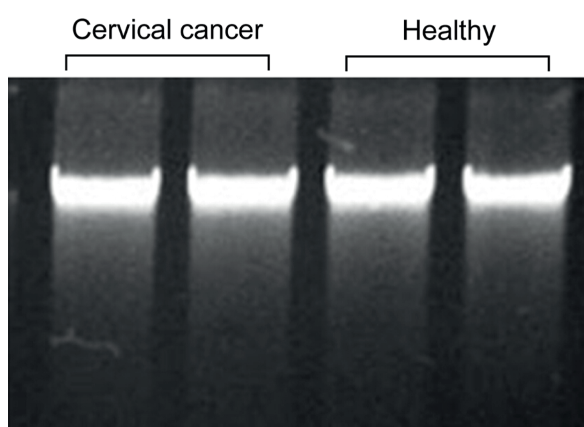

Figure 1. Electrophoresis of the PCR products of the p16 gene promoter.

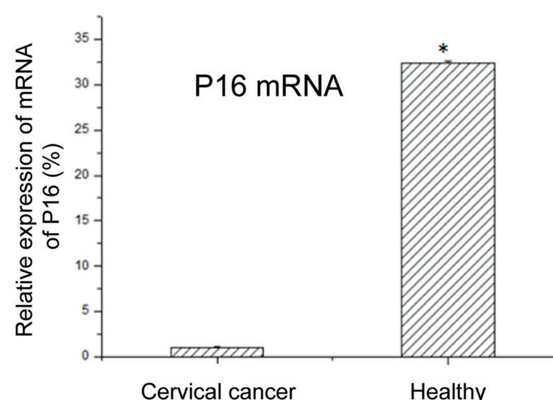

Figure 2. Detection of the p16 mRNA expression levels in different groups. *indicates a significant between-group difference.

Table III. p16 gene promoter methylation.

Group	n	Methylation of p16			
		Methylation	Non-Methylation	Methylation rate (%)	*p*-value
Observation	78	61	17	78.52	0.000
Control					
48	5	43	9.8		

Table IV. Distribution of p16-positive cells.

Group	Cells (n)	p16-positive cells	p16-positive rate (%)	p16-negative cells	p16-negative rate (%)
Control	400	333	83.29	67	16.71
Observation	400	41	10.18	359	89.92

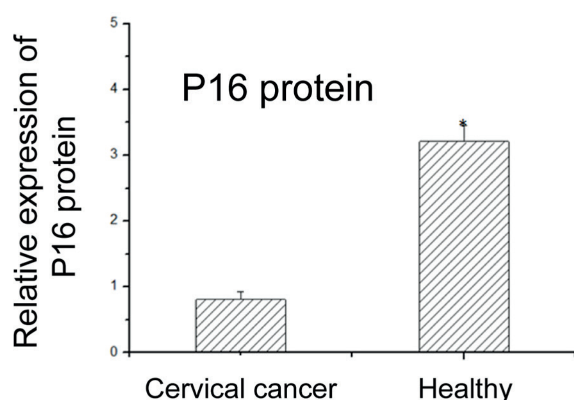

Figure 3. Quantification of the p16 protein in cervical samples from both groups. *indicates a significant between-group difference.

the amounts of p16 by ELISA. The level of p16 protein in cervical cancer lesions was 0.81 ± 0.12 μg/l, which was significantly lower than in the 3.21 ± 0.24 μg/l in healthy controls (Figure 3). This result looking at p16 protein levels is consistent with the observation of *p16* expression at the mRNA level.

Expression of p16 Protein Detected by Western Blotting

To confirm the above results by an independent technique, we next detected p16 by western blot. The expression levels of p16 were significantly reduced in cervical cancer patients compared with the healthy controls (Figure 4A). Quantification of the Western blot results also demonstrated significantly higher p16 expression in the healthy population compared with the cervical cancer patients (Figure 4B). The results from Western blot were consistent with the ELISA results.

Expression of p16 Protein Detected by Immunohistochemistry

Finally, we performed immunohistochemistry to examine p16 protein expression in the healthy cervix and cervical cancer lesions. p16 accumulated mainly in the cytoplasm in cervix tissues (Figure 5). The positive stained cell rate in the healthy cervix tissues (83.29%) was significantly higher than that in the cervical cancer lesions (10.18%) (Table IV).

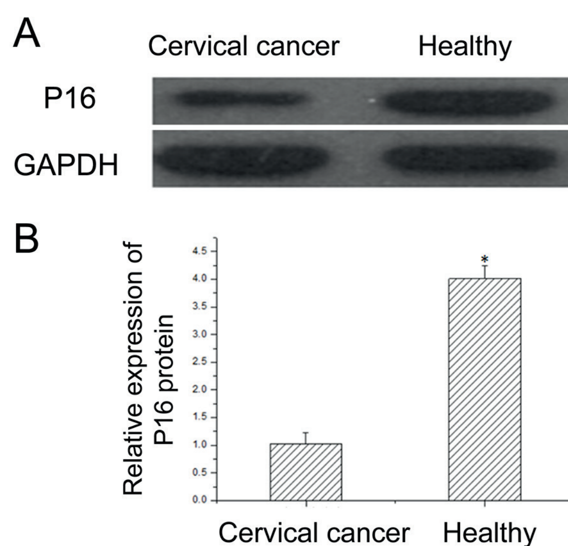

Figure 4. Detection of the p16 protein expression. ***A,*** Western-blotting for cervical cancer and healthy people; ***B,*** Quantification of the Western blotting results. *indicates a significant between-group difference.

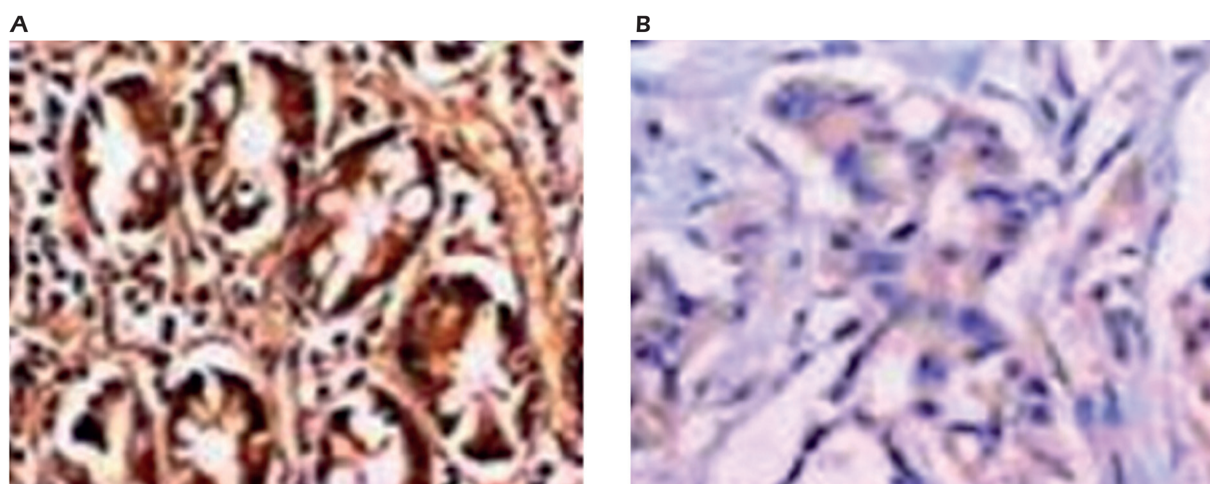

Figure 5. Immunohistochemical detection of the protein expression of p16 in different cervical samples. ***A*,** Healthy cervical tissues. ***B*,** Cervical cancer lesions. Purple indicates p16 negative cells and brown indicates p16 positive cells (100 x).

Discussion

Cervical cancer has become a major gynecological disease that threatens women health because of its high incidence and mortality rate[14]. The epidemiology of cervical cancer is complicated, involving environmental, genetic and other risk factors[15]. At present, surgery, radiotherapy, and chemotherapy are the gold standard for treating cervical cancer[16]. Since the pathogenic mechanisms of cervical cancer are not well understood at this time, no specific therapies have been developed for cervical cancer[17]. In recent years, researchers suggested that gene methylation plays an important role in the occurrence and development of many diseases. Gene methylation occurs mainly in GC-rich regions (i.e., CpG islands). DNA sequencing demonstrated that a mammalian genome contained about 40,000 to 50,000 CpG islands[18]. Intracellular methylase can easily induce cytosine methylation in these CpG islands, and methylated genes show varying degrees of suppressed gene expression. This methylation may be also relevant in the disease mechanisms. For example, methylation of p16 promoter was significantly higher in the pancreas of pancreatic cancer and control patients[19]. Also, p16 exhibited significantly lower mRNA and protein expression in the patients with pancreatic cancer. These data suggested that the methylation of the *p16* promoter could significantly reduce p16 expression and prevent its tumor suppressor activity in the pancreas[20]. Previous studies showed that p16 is mainly involved in the cell proliferation. Recent results indicated that the methylation of *p16* is associated with many tumors and cancers, including breast, colon, and stomach cancer[20].

Conclusions

We firstly discovered the close correlation between methylation of the *p16* promoter and cervical cancer. We found that the methylation of the *p16* promoter significantly reduced *p16* mRNA and protein expression. But we did not explore how the methylation affects the expression of *p16*, and how p16 protein functions mediate the development of cervical cancer. These questions will be the focus of our future research.

Conflict of interest

The authors declare no conflicts of interest.

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