

Correlation Between ER, PR, p53, Ki-67 Expressions and High-Risk HPV Infection in Patients with Different Grades of Cervical Intraepithelial Neoplasia

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ABSTRACT

Objective: To explore the correlations of high-risk human papillomavirus (HPV) infection with the expressions of estrogen receptor (ER), progesterone receptor (PR), p53 and Ki-67 in patients with different grades of cervical intraepithelial neoplasia (CIN).

Methods: A retrospective study was conducted on 140 CIN specimens preserved in our hospital from June 2016 to June 2018, including 40 cases in grade 1, 50 cases in grade 2, and 50 cases in grade 3. The expressions of ER, PR, p53 and Ki-67 were determined using immunohistochemistry, and the high-risk infection was detected by fluorescence quantitative polymerase chain reaction. Besides, the correlation analysis was performed.

Results: Among the 140 specimens, low-grade squamous intraepithelial lesion specimens showed an HPV16-positive rate of 27.5% and an HPV18-positive rate of 25.0%, high-grade squamous intraepithelial lesion a (HSILa) specimens exhibited an HPV16-positive rate of 64.0% and an HPV18-positive rate of 60.0%, and HSILb specimens displayed an HPV16-positive rate of 90.0% and an HPV18-positive rate of 92.0%, with statistically significant differences ($P < 0.05$). The positive rates of HPV16 and HPV18 were not significantly correlated with the age and tissue differentiation degree of patients ($P > 0.05$). With the increase in CIN grades, the positive expression rates of ER, PR, p53 and Ki-67 in the tissue specimens were also significantly elevated, showing statistically significant differences ($P < 0.05$). The correlation analysis results revealed that the positive rates of HPV16 and HPV18 were significantly positively correlated with the positive expression rates of ER, PR, p53 and Ki-67 ($P < 0.05$).

Conclusion: The positive rate of HPV infection is raised with the increase in CIN grade, which is also accompanied with the elevation of the positive expression rates of ER, PR, p53 and Ki-67, and there are positive correlations among them.

KEYWORDS: cervical intraepithelial neoplasia; HPV infection; correlation; p53; Ki-67

INTRODUCTION

Cervical cancer is one of the common malignant tumors in women, which develops from cervical intraepithelial neoplasia (CIN). New cases of cervical cancer in China account for 1/3 of the world's total number, ranking second in the world for a long time [1,2]. Cervical cancer can be triggered by many pathogenic factors, which is closely related to human papillomavirus (HPV) infection, and HPV deoxyribonucleic acid (DNA) can be detected in more than 99% of cervical cancer

tissues [3]. HPV is a circular DNA virus that can specifically infect human skin and mucosa. It can be divided into low-to-medium-risk types and high-risk types according to relationship between HPV subtypes and tumorigenesis. HPV16, 18, 31 and 33 are high-risk types of HPV, which can participate in the occurrence of CIN and cervical cancer to varying degrees [4,5]. Modern studies have shown that the occurrence and development of CIN is a multi-step process involving gradual accumulation and changes of multiple genes including oncogenes, tumor suppressor genes and growth factor genes, in which normal cervical squamous epithelial cells show inflammation and further induce tumor formation [6,7]. The estrogen receptor (ER) and progesterone receptor (PR), existing on the surface of hormone target cells, are mainly distributed in

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the target organs such as the uterus and cervix and can specifically bind to corresponding hormones to regulate the occurrence and development of CIN [8,9]. Ki-67 is a relatively certain nuclear proliferation marker at present, and is also an important reference marker for predicting the occurrence and development of CIN and cervical cancer, whose expression level can reflect the biological behavior of tumor cells [10,11]. P53 gene is an important cancer suppressor gene located on human chromosome 17p13.1, and its encoding product p53 protein plays a vital role in the processes of cell division and differentiation. The normal expression of p53 can induce cell apoptosis and cell cycle arrest, and mutation of p53 protein can lead to cell transformation and excessive proliferation, resulting in tumor behaviors [12,13]. In this study, the correlations of the expressions of ER, PR, P53 and Ki-67 in patients suffering from different grades of CIN with high-risk HPV infection were specifically explored, aiming at providing a reference for the study of the pathogenesis of CIN, and further clarifying the correlations of ER, PR, P53 and Ki-67 protein expressions with HPV infection.

MATERIALS AND METHODS

Subjects

This study was approved by the Ethics Committee of our hospital, and 140 CIN specimens preserved in Department of Pathology of our hospital from June 2016 to June 2018 were selected. Inclusion criteria: (1) Patients with complete clinical and pathological data, (2) those pathologically diagnosed with different grades of CIN, (3) those receiving no radiotherapy, chemotherapy or hormone therapy before specimen operation, and (4) those aged 20-80 years old. Exclusion criteria: (1) Patients with a previous history of cervical cancer, (2) those with immunodeficiency disease, or (3) those with incomplete clinical and pathological data. All the enrolled patients were aged 26-78 years old, with a mean of (47.49 ± 3.39) years old, and there were 90 patients with an age ≥ 40 years old. In terms of CIN grades (the images of all the sections were read independently by three pathologists with an intermediate or above professional title based on the pathologic and genetic classification criteria of female reproductive system tumors), there were 40 cases of grade 1 CIN, 50 cases of grade 2 CIN, and 50 cases of grade 3, and with regard to histological differentiation, there were 20 cases of low differentiation, 30 cases of moderate differentiation and 90 cases of high differentiation.

Immunochemical assay

Mouse anti-human p53 monoclonal antibodies, mouse anti-human PR monoclonal antibodies, mouse anti-human Ki-67 monoclonal antibodies, and mouse anti-human ER monoclonal antibodies were purchased from Santa Cruz Biotechnology, and universal immunohistochemical staining kits, phosphate buffered saline (PBS), and DAB development kits were obtained from Beijing Zhongshan Bio-Tech Co., Ltd.

All the tissue specimens were embedded in paraffin blocks and serially sliced into 2 μm -thick sections, and then the sections were transparentized and dehydrated in xylene and ethanol, respectively. The resulting tissue sections were counter-stained with hematoxylin for 15-30 s and sealed using neutral resin. Finally, the results were observed under a light microscope. The cervical cancer tissues and PBS, rather than primary antibodies, were set as positive control and negative control, respectively, for each piece of section. Each piece of section was observed in 5 randomly selected high-power fields of view. Brownish yellow particles in the nucleus indicated positive expressions of ER and PR, Ki-67 was mainly positively expressed in the nucleus, and p53 staining-positive signals were yellow and strictly located in the nucleus. The grade of CIN was determined by pathologists using immunohistochemistry with reference to the combined criteria for scoring and pathological diagnosis and grading. Negative staining intensity, the staining slight but more intensive than that of negative control, clear staining, and intensive staining were given 0 points, 1 point, 2 points, and 3 points, respectively, while 0 points, 1 point, 2 points, and 3 points represented the proportions of positive cells of $<10\%$, $10\text{-}30\%$, $31\text{-}60\%$, and $>60\%$, respectively. According to the sum of the above two scores, 0-1 point indicated negativeness (-), 2 points weak positiveness (+), 3-4 points moderate positiveness (++), 5-6 points strong positiveness (+++), and (+), (++) and (+++) suggested positive expression.

High-risk HPV DNA detection

The tissue specimens were taken, rinsed with sterile water, and fixed with 95% ethanol for 15 minutes, and the cervical secretions on the specimens were then dissolved with lysis solution. The tissues were fully cut using sterile ophthalmic scissors to obtain a size of 1 mm^3 , added with an appropriate amount of 0.25% trypsin and digested in a 37°C water bath. The resulting tissue suspension was filtered through a 200-mesh

stainless steel screen, transferred into a test tube, and centrifuged at 5,000 rpm/min for 5 min. With the supernatant discarded, the cell pellets were washed with Hank's solution for 2-3 times to harvest single-cell suspension. The predetermined protocol was implemented using an automatic nucleic acid extractor (Jiangsu Shuoshi Biotechnology Co., Ltd.). The HPV16- and HPV18-specific primers were designed and amplified with reference to the sequences from the National Center for Biotechnology Information (NCBI) BLAST databases: HPV16: forward 5'-GAATCCATATGCTGTATGTGATAA-3', reverse 5'-GATGATETGCAACAAGACATACAT-3', HPV18: forward 5'-CACGGEGAECCTACAAGCTACCTG-3', reverse 5'-TGCAGEACGAATGGCACTGGCCTG-3'. Besides, they were synthesized by Sangon Biotech (Shanghai) Co., Ltd. Polymerase chain reaction (PCR) fluorescence cycle conditions are as follows: 95°C for 5 min, 55°C for 30s, and 72°C for 1 min for 40 cycles, and 72°C for 10 min. Subsequently, PCR enzyme-digested products were sent to Sangon Biotech (Shanghai) Co., Ltd. to determine DNA sequences and compare them with the NCBI gene

sequences.

Statistical analysis

SPSS 22.00 software was used to analyze quantitative and numerical data. The normally distributed quantitative data were expressed as mean \pm standard deviation, while numerical data were represented as frequency. Comparisons were conducted using *t* test, χ^2 test, and analysis of variance. Pearson correlation analysis was performed. The test level was $\alpha=0.05$.

RESULTS

High-risk HPV-positive rates

Among the 140 specimens, low-grade squamous intraepithelial lesion (LSIL) samples showed an HPV16-positive rate of 27.5% and an HPV18-positive rate of 25.0%, high-grade squamous intraepithelial lesion a (HSILa) specimens exhibited an HPV16-positive rate of 64.0% and an HPV18-positive rate of 60.0% and HSILb specimens displayed an HPV16-positive rate of 90.0% and an HPV18-positive rate of 92.0%, with statistically significant differences ($P<0.05$) (Table 1).

Table 1. High-risk HPV-positive rates among different grades of CIN (n)

Group	n	HPV16		HPV18	
		Positive	Positive rate	Positive	Positive rate
LSIL	40	11	27.5%	10	25.0%
HSILa	50	32	64.0%	30	60.0%
HSILb	50	45	90.0%	46	92.0%
F			37.224		42.169
P			0.000		0.000
Total		88	62.9%	86	61.4%

Correlations between high-risk HPV-positive rate of CIN and clinical characteristics

The positive rates of HPV16 and HPV18 were not

significantly correlated with the age and tissue differentiation degree of patients ($P>0.05$) (Table 2).

Table 2. Correlations between high-risk HPV-positive rate of CIN and clinical characteristics (n=140)

Clinical characteristic	n	Positive rate of HPV16 (n=88)			Positive rate of HPV18 (n=86)		
			F or χ^2	P		F or χ^2	P
Age ≥ 40 years old	90	80(88.9%)	73.144	0.000	78(86.7%)	67.745	0.000
<60 years old	50	8(16.0%)			8(16.0%)		
Histological differentiation High	90	55(61.1%)	12.336	0.002	53(58.9%)	14.549	0.001
Moderate	30	14(46.7%)			15(50.0%)		
Low	20	19(95.0%)			20(100.0%)		

Positive expression rates of ER, PR, p53 and Ki-67 among different grades of CIN

With the increase in CIN grades, the positive expression rates of ER, PR, p53 and Ki-67 in the

tissue specimens were also elevated, showing statistically significant differences ($P<0.05$) (Table 3, Figure 1-3).

Table 3. Positive expression rates of ER, PR, p53 and Ki-67 among different grades of CIN (n=140)

Group	n	Positive rate of ER	Positive rate of PR	Positive rate of p53	Positive rate of Ki-67
LSIL	40	16(40.0%)	15(37.5%)	17(42.5%)	16(40.0%)
HSILa	50	35(70.0%)	34(68.0%)	37(74.0%)	41(82.0%)
HSILb	50	48(96.0%)	48(96.0%)	50(100.0%)	49(98.0%)
F		33.670	35.797	38.466	42.326
P		0.000	0.000	0.000	0.000

Correlation analysis results

According to the Pearson correlation analysis results, among the 140 specimens, the positive

rates of HPV16 and HPV18 were significantly positively correlated with the positive expression rates of ER, PR, p53 and Ki-67 ($P < 0.05$) (Table 4).

Table 4. Correlations of ER, PR, p53 and Ki-67 expressions with high-risk HPV infection in patients with different grades of CIN (n=140)

Index	ER	PR	p53	Ki-67
HPV16-r	0.563	0.610	0.544	0.395
P	0.000	0.000	0.000	0.013
HPV18-r	0.433	0.594	0.388	0.410
P	0.012	0.000	0.016	0.010

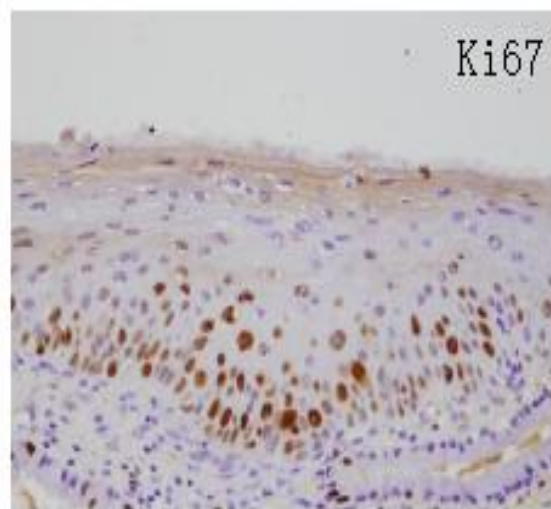
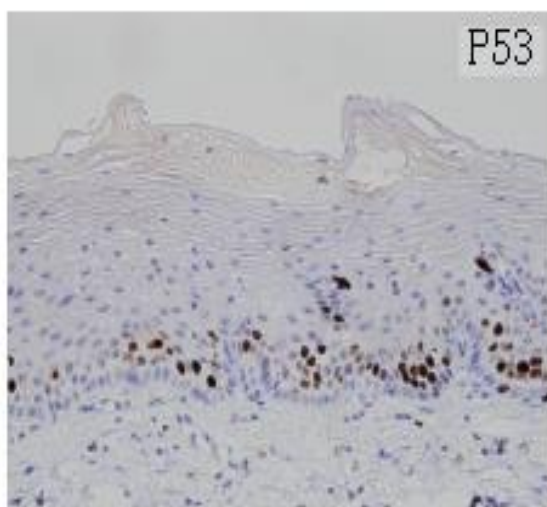
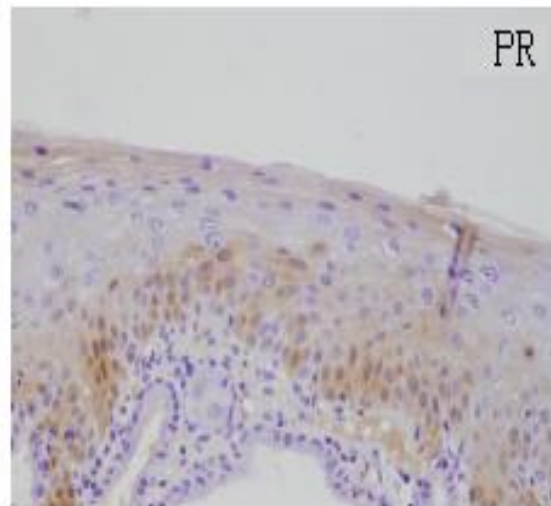
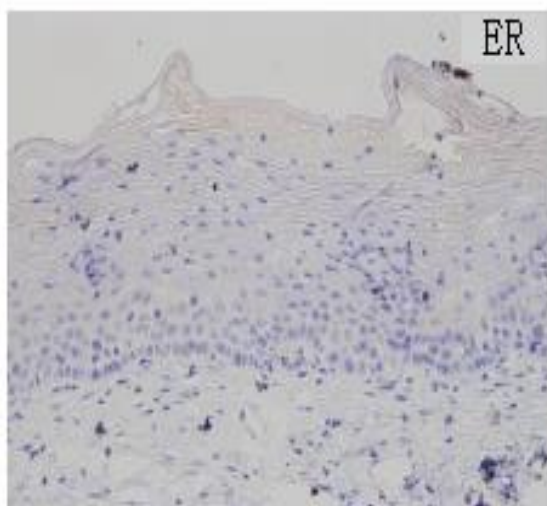


Figure 1. Expressions of ER, PR, p53 and Ki-67 in grade 1 CIN samples.

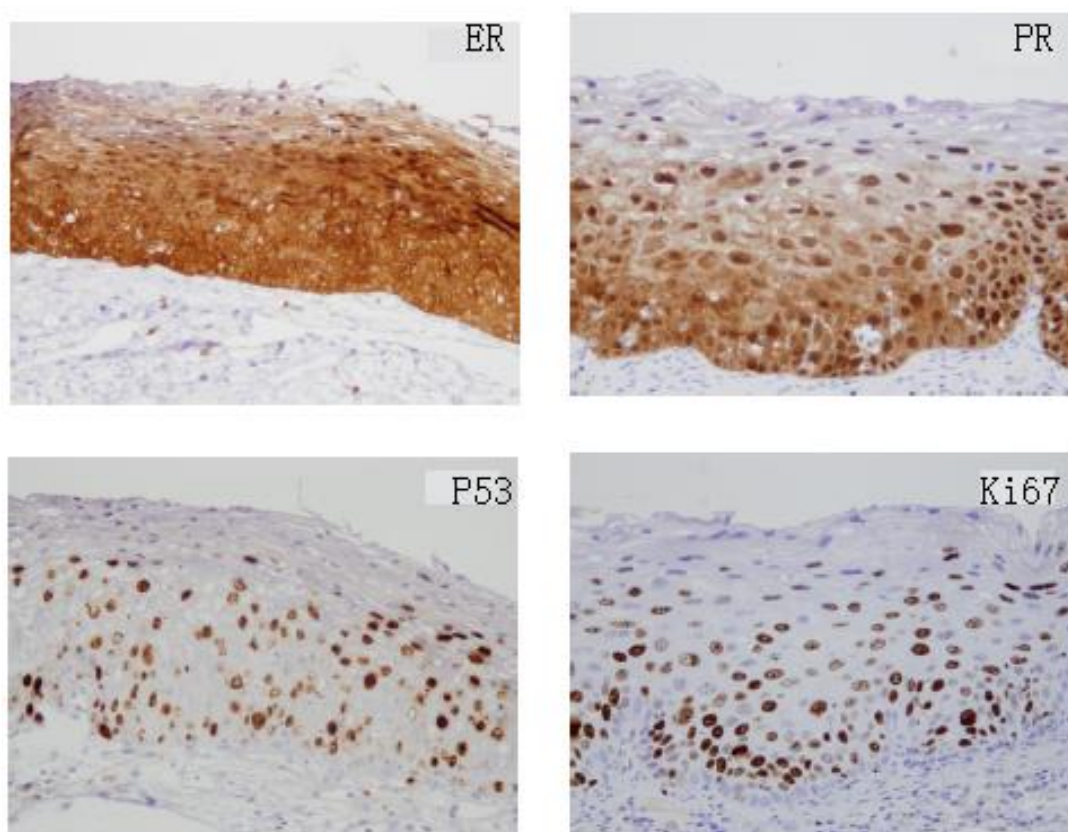


Figure 2. Expressions of ER, PR, p53 and Ki-67 in grade 2 CIN samples.

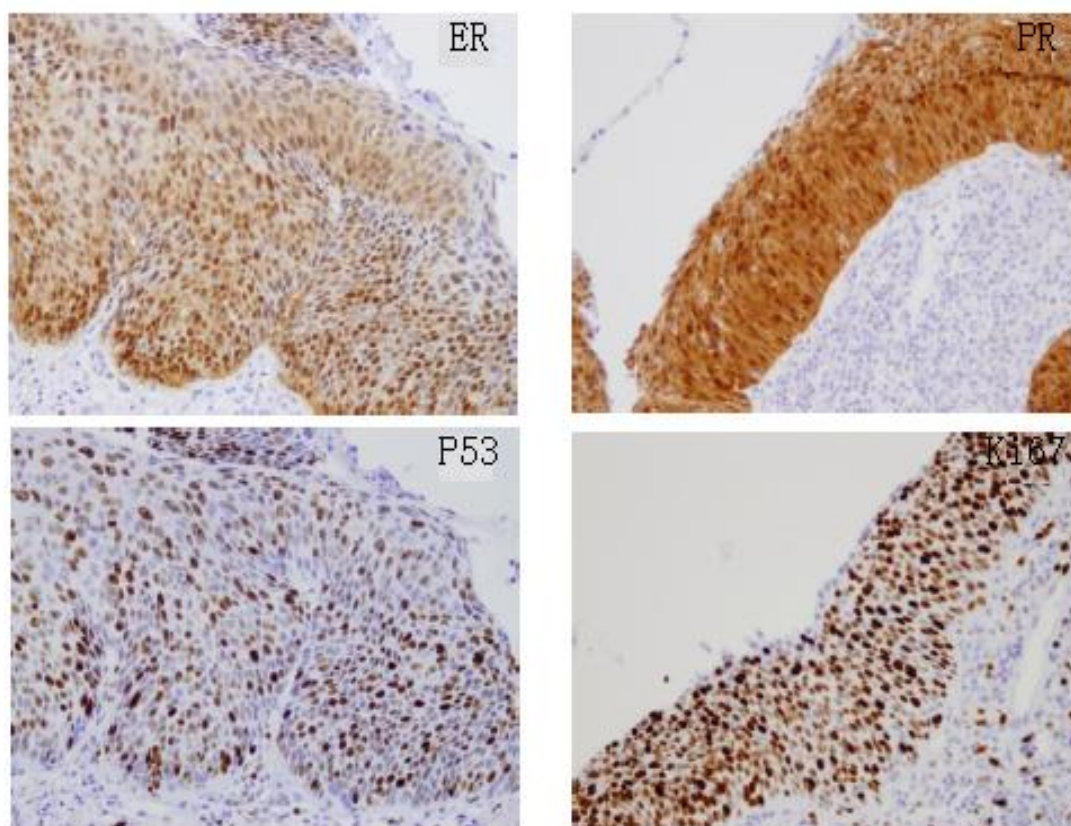


Figure 3. Expressions of ER, PR, p53 and Ki-67 in grade 3 CIN samples.

DISCUSSION

CIN is a malignant tumor that seriously threatens women's health and whose etiology has not yet been fully elucidated. Relevant studies have shown that the onset of this disease is associated with early marriage, sexual life disorder, multiple parturitions, economic status, premature sexual life, and ethnicity. Moreover, persistent infection by high-risk HPV is also an important factor [14-16].

Nearly 200 types of HPV, a common pathogen of female reproductive tract infections, have been discovered so far. An epidemiological investigation showed that HPV16 and HPV18 are found in about 50% and 15% of cervical squamous cell carcinomas, respectively [17]. Among the 140 specimens in this study, the HPV16-positive rate and HPV18-positive rate were 27.5% and 25.0% in LSIL specimens, 64.0% and 60.0% in HSILa specimens, and 90.0% and 92.0% in HSILb specimens, respectively, and the differences were statistically significant ($P < 0.05$). The positive rate of HPV16 and HPV18 had no significant associations with the age and histological differentiation ($P > 0.05$). The above results prove the dominating role of CIN in lesions. Current studies have demonstrated that the overexpression of E6 and E7 proteins in HPV can promote the release of p53 and pRB proteins and immortalize the host cells, thereby leading to CIN and even carcinogenesis [18].

Although HPV is necessary for the development of CIN, not all HPV infections cause CIN. Besides, HPV infections belong to subclinical infections or latent viral infections in most patients [19]. HPV infections alone do not suffice to cause CIN or cervical cancer, and the synergy of other factors is responsible for the progression of HPV infections to CIN [20]. According to a current study, the development of CIN is a multi-stage and multi-factorial complex process, in which the proliferation and differentiation of cells and their apoptosis remain unbalanced. Both ER and PR are located in the nucleus and mainly present in the tissue cells of the female reproductive system, and the binding of receptors and hormones can activate genes, thereby regulating cell proliferation and apoptosis [21]. Existing studies have shown that estrogen can induce the production of ER and PR at the DNA replication and transcription levels during the proliferation of endometrial cells, while progesterone, as an antagonist against estrogen, can make abnormally proliferating cells differentiate to be mature and down-regulate estrogen at the transcriptional and post-transcriptional levels [22]. A study revealed that ER and PR have the highest expression levels in normal

endometrial tissues, but lower expression levels in cancer tissues, and they may be involved in abnormal proliferation and inhibit normal cell apoptosis [23]. Abnormalities in p53 gene are closely correlated with the carcinogenesis of cervical epithelial cells, and p53 gene mutation occurs in CIN tissues. P53 is an important cancer suppressor gene, and the crucial role of its functional inactivation in the development and progression of CIN has been corroborated. High-risk HPV E6 proto-oncogene-encoded proteins can bind to p53 protein and degrade it. Wild-type p53 can participate in the regulation of multiple functions, such as cell growth, development and differentiation by the p53-dependent or p53-independent pathway [24]. At present, some studies have also demonstrated that p53 can prevent cells in G1 phase from entering S phase, thereby inhibiting cell proliferation and regulating cell cycle. Overexpression of p53 has been proven to be significantly associated with the initiation of HP-HPV infection [25,26]. Ki-67 is now relatively definite nuclear proliferation marker, and it has a short half-life and can be rapidly degraded once evading the cell cycle, which is an index for determining the viability of tumor cells. HPV infections can accelerate cell proliferations to substantially increase Ki-67-positive cell nuclei, and they rise as the grade of cervical lesions increases [27,28]. According to the present study, the positive expression rates of ER, PR, p53 and Ki-67 in the tissue specimens were significantly elevated with the increase in the grade of CIN, with statistically significant differences ($P < 0.05$). These results suggest that ER, PR, p53 and Ki-67 have important value in predicting the development and progression of CIN and can guide the treatment of tumors and the judgment of the prognosis.

CIN results from the interaction between environmental factors and genetic factors, in which cell atypia tends to occur based on the abnormalities in gene expression and viral infections [29]. In particular, HPV infection is not the only etiology of CIN, and the functional changes of oncogenes and tumor suppressor genes play pivotal roles in the development and progression of CIN [30]. In the present study, the Pearson correlation analysis results showed that among the 140 specimens, the positive rates of HPV16 and HPV18 were significantly positively correlated with the positive expression rates of ER, PR, p53 and Ki-67 ($P < 0.05$). Current studies have shown that the HPV16 and HPV18 E6 proteins can bind to wild-type p53 proteins, and promote their degradation in the presence of E6-related proteins, ultimately

weakening the p53-induced G1 cell cycle arrest, completing DNA synthesis, and promoting cell proliferation [31,32]. HPV infections also destroy normal functions of sex hormones, thereby weakening the antagonistic effect of progesterone against estrogen in diseased endometrial tissues and facilitating the malignant transformation of cells [33]. HPV can induce the cycle activity of Ki-67-positive cells, and its copy number notably rises with the increase in Ki-67 expression level [34,35]. The present study had certain shortcomings. For example, relatively few experimental cases made this study relatively limited, and no healthy controls were set, which could lead to certain study biases. Therefore, in-depth analysis will be performed in the subsequent study.

In summary, as the CIN grades increase, the positive high-risk HPV infection rate rises, with the increases in the positive expression rates of ER, PR, p53 and Ki-67, and the two increases have a positive correlation.

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