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Original Article

Relationship of P16 and Ki67 in recurrence of HPV infection and cervical intraepithelial neoplasia

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Abstract: This study aimed to investigate the association of P16 and Ki67 expression in cervical conization with postoperative HPV reinfection and cervical intraepithelial neoplasia. This study retrospectively enrolled patients from January 2012 to December 2013. Patients with negative margins were followed up for 2 years to evaluate the correlation between Ki67 and p16 expression levels in the conization of patients with HPV persistence encountering infection or re-infection and CIN recurrence. The positive expression of p16 and Ki67 was significantly correlated with disease progression (P<0.05). p16 and Ki67 expression was chosen, and results showed that positive expression of p16 and ki67 proteins was a risk factor of disease progression (OR=5.3, 95% CI 1.177~24.365, P=0.042; OR=5.1, 95% CI 1.162~22.387, P=0.031, respectively). Results indicated that routine staining for p16 and Ki67 has clinically significant meaning in guiding disease progress and prognosis at follow-up.

Keywords: Cervical conization, P16, KI67, HPV reinfection, cervical intraepithelial lesion recurrence

Introduction

Cervical cancer is one of the most common gynecologic malignancies in women and ranks second among female cancer. The cervical cancer incidence and mortality in China account for about 1/3 of cases in the world [1]. Epidemiologic studies have confirmed that more than 90% of cervical cancer is associated with infection by human papilloma virus (HPV) [2]. The occurrence and development of cervical cancer are not directly associated with HPV infection, but associated with the cervical epithelium status. High-grade Cervical Intraepithelial Neoplasia (CIN) or untreated CIN (CIN2 or CIN3) shows a high risk of cervical cancer, but after treatment the risk is significantly decreased [3, 4]. Therefore, timely diagnosis and treatment of CIN, especially high-grade, should reduce the incidence and mortality of cervical cancer.

Epidemiologic and molecular studies have demonstrated 99.7% of the HPV host genome sequences [2, 5], and HPV has been identified to have more than 100 subtypes, including high-risk type (such as type 16, 18, 31, 33) and low-risk type (such as type 6, 11, 42, 43). High-risk HPV (HR-HPV) infection is the main cause of the development of high-grade CIN [6]. In fact, among young patients, most HPV infection is temporary, and only HR-HPV-related infections actively expressed oncogene E6 and E7. Therefore, long-term and persistent infection of HR-HPV is closely related with occurrence of CIN [7]. Carcinogenesis of HPV is mainly due to HPV E6 and E7 encoding proteins, which can be combined to tumor suppressor gene P53 protein, leading to P53 protein degradation and cell transformation.

P16 is a tumor suppressor gene, located at 9p21, with a total length of 8.5 kb, and is composed of three exons and two introns. It encodes protein which contains 148 amino acids, and works as a cell cycle-dependent kinase inhibitor in G1-S phase of the cell differentiation cycle to negatively regulate cell proliferation and division. P16INK4a, the expression product of CDKN2A gene, is overexpressed in cervical cancer and precancerous lesions with high risk of HPV infection. After HPV DNA inte-

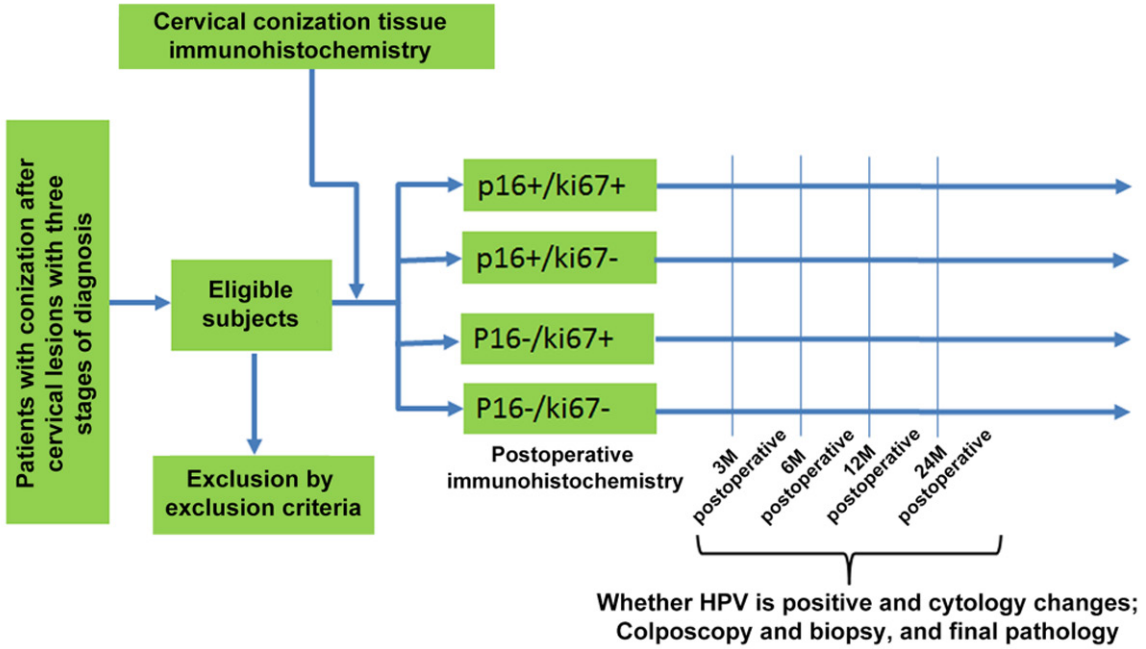

Figure 1. Research program implementation flow chart.

grates into the host cell genome, the overexpression of HPV E7 led to tumor suppressor gene product Rb inactivation, thus leading to inhibition of Rb. E2F-1 level was increased, leading to excessive expression of P16 [8, 9].

Ki67 is a marker of cell proliferation, and is expressed in G1, S, G2 and M phases of cell cycle, but not in G0 phase. Ki67 proliferation index is closely related to tumor differentiation, invasion, metastasis, and prognosis. It is widely used to determine proliferation and malignancy of various malignant tumor cells [10].

In recent years, many studies focused on the expression of p16 and Ki67 in CIN and cervical cancer diagnosis. Positive rate of P16 level in cervical squamous epithelial non-neoplastic lesions was very low, but was extremely high in CIN, especially in high-grade CIN. As an auxiliary diagnostic marker, it can effectively tell CIN lesions apart [11-13]. Ki67 gradually increased in diverse CIN to cervical cancer, and has a strong predictive value in predicting whether low-level CINs can develop into high-level CINs [14]. However, these studies examined the expression of p16 and Ki67 through cervical smear, a cervical liquid-based cell smear [12, 15, 16], and this will lead to a high false negative rate. In addition, the expression of p16 and Ki67 during occurrence and development of CIN is limited to assess diagnosis and progression of first occurrence of CIN, but not to investigate the relationship between the expression of p16 and Ki67 and the recurrence of HPV and CIN after cervical conization.

Thus, in the current study we combined these two indicators to analyze their relationship with the prognosis of conization and to guide clinical follow-up management.

Materials and methods

Patient enrollment

This study is a retrospective observational cohort study. This study enrolled patients from January 2012 to December 2013 in the Department of Obstetrics and Gynecology, Liaocheng People's Hospital. Patients were confirmed to have high-grade cervical lesions or high-level gland involvement after pathology evaluation and to have negative margins. Patients were followed up for 2 years to evaluate the correlation between Ki67 and p16 expression levels in the conization of patients with HPV persistent infection, re-infection, or CIN recurrence. The follow-up time points are shown in the **Figure 1**. The study protocol was approved by the hospital ethics committee.

Patients who met all the following criteria were included in this study: 1) the patient is over 18 years; 2) consistent with cervical incision surgery indications and without surgery contraindications; 3) patients with cervical conization due to diagnostic or therapeutic purposes; 4) postoperative pathology confirmed high grade cervical lesions or high levels involving the glands, margins are negative; 5) have complete clinical data and experimental data. Exclusion criteria were: 1) Hysterectomy patients; 2) Having history of CIN treatment within 5 years; 3) Having malignant tumor in other parts; 4) With pregnancy; 5) Any other reason that precludes participation.

Cervical HPV examination

HR-HPV DNA was detected by HC2 method using Digene HR-HPV detection kit and gene hybrid capture device, which can detect 13 common high-risk HPVs at one time including type 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59 and 68. The criteria for positive results were HPV DNA≥1.0 RLU/CO.

Colposcopic cervical biopsy

Cervix was fully exposed with the speculum, cervical secretions were cleaned with sterile cotton, and electronic colposcopy was used to observe the cervix and vaginal condition. Form, color and vascular changes of the junction area were observed after using of 5% cotton acetate balls to coat cervix. Cervical coloring was observed after staining with 5% iodine solution. Score and diagnosis were performed according to the colposcopy system. Multipoint biopsy was done in the colposcopy positive area, and biopsy of routinely taken points of 3, 6, 9, 12 in the normal transformation zone was done. Unsatisfactory colposcopy (colposcopy cannot see the boundaries of the lesion, colposcopy without scaly column junction) was followed by cervical scraping surgery, and biopsy specimens were sent for pathologic diagnosis.

Cervical cold knife conization

Cervix was fully exposed with the speculum, cervical secretions were cleaned with sterile cotton, and 5% Lugol's iodine cervical staining was performed to show the lesion. Lidocaine (2%) with adrenaline (1:50000) was used for local infiltration anesthesia at points 3, 6, 9, and 12, with a knife blade cone resection of cervical lesions. The width of the lesion should be outside the lesion for 0.5 cm, with a 2 to 2.5 cm cone height to the neck, and the junction should also be excised. Wound should be fully sutured to hemostasis. Cone specimens were fixed in formalin, paraffin-embedded, 12 points drawn, continuous sections stained, and read by experienced pathologists to make a diagnosis. Results of the diagnosis were recorded and whether the disease was involved was noted.

Immunohistochemical staining

The expression of P16INK4a and KI67 in the conization tissue was detected by immunohistochemistry PV-6000 two-step method. P16-INK4a and KI67 monoclonal antibodies were ready-to-use working fluids.

Slices: Paraffin-embedded tissue was placed on a paraffin-slicer. The slicer adjusts the tissue by adjusting the upper, lower, left and right tissues. Slice thickness was 4 μm, and they were placed in 40°C warm water, unfolded, dried and placed on a slide shelf.

Baking sheet: Slides were placed in a 58°C electric heating oven overnight.

Dewaxing, hydration: At room temperature, the sample slices were soaked in xylene solution for dewaxing for 5 minutes × 2 times. Then the sections were sequentially immersed in anhydrous ethanol, anhydrous ethanol, 95% ethanol, 85% ethanol, 1 minute each time. The sections were then rinsed under running water for 1 minute, rinsed with ethanol and immersed in distilled water for 1 minute.

Antigen retrieval: Antigen retrieval solution EBTA was inserted in the antigen retrieval box and placed in a pressure cooker to boil. The slices were placed in a box containing pH=9.0 solution so that the tissue part of the slice was below the level and the antigen box was placed in the autoclave. It was heated to produce a jet for 1 minute and 40 seconds. After natural cooling to room temperature, the slide was then rinsed with distilled water 3 times.

3% H_2O_2 blocking endogenous peroxidase: 30% H_2O_2 was mixed with distilled water at 1:10,

and the slide was allowed to soak for 10 minutes at room temperature to inactivate the endogenous enzyme.

Primary antibody: At room temperature and after washing using PBS (PH=7.2~7.6) buffer for 4 times × 1.5 minutes, mouse anti-human KI67, P16INK4a protein monoclonal antibody working solution 50 μL was added.

Secondary antibody: The sections with primary antibody were set into a 37°C constant temperature water bath for 1 hour and then washed with PBS (pH=7.2~7.6) buffer 4 times × 1.5 minutes at room temperature. Horseradish enzyme labeled goat anti-rabbit IgG polymer was added and incubated for 20 minutes in a 37°C constant temperature water bath.

DAB staining: At room temperature and after washing using PBS (p=7.2~7.6) buffer for 4 times × 1.5 minutes, DAB color kit was used to stain.

Hematoxylin staining: Slides were soaked in the hematoxylin for 1 minute 30 seconds, rinsed with tap water. The slices were put into the hydrochloric acid acidification, and then rinsed with ammonia again.

Slices were put into xylene solution after becoming transparent, neutral gum sealed, and observed by microscopy.

Establishment of immunohistochemical controls

Negative control was set as 0.01 mol/L PBS instead of primary antibody. Other procedures were all the same with the normal immunohistochemical staining steps.

Immunohistochemical results standard: Squamous cell cytoplasm and nucleus with brown particles were considered to be positive cells, and positive cells' diffuse distribution in the entire lesion epithelium was seen as positive. Lesion epithelium not colored or only focal cytoplasm and/or nucleolus coloring was seen as negative.

Statistical analysis

The statistical analysis was performed using SPSS software (V21). Data were shown as mean ± s.d. Continuous variable comparisons of baseline data were done using ANOVA followed by Bonferroni method as post hoc tests. Comparison between qualitative variables and percentage was performed using chi-square test, and the subsequent comparison between the two groups was done to do *p* value correction. Multiple logistic regression analysis was done as needed. Taking the outcome as the dependent variable, the possible influencing factors were independent variables, and the independent influencing factors were obtained by stepwise method. $P<0.05$ was considered significant.

Results

Baseline data

A total of 2500 patients were included in this study at first. Among them, 100 patients were not treated with CIN within 5 years, 10 patients were pregnant, and 24 patients were excluded because of tumors in other parts. So, of all these patients, 1893 patients finished the follow-up, and 1,800 patients completed the study and were included in statistical analysis. According to positive or negative status for p16 and Ki-67, all the patients were divided into four groups. We conducted a detailed statistical analysis of the detailed clinical data, and expression of P16 and ki67 in tissue samples. According to the expression of p16 and ki67 in cervical intraepithelial neoplasia, it was divided into p16+/ki67+, p16+/ki67-, p16-/ki67+, and p16-/ki67- groups. There were no statistically significant differences in age and body mass index between the four groups. Marital status and maternal history also showed no statistical significance, indicating that p16 and ki67 expression had no significant relationship with the patient's age, weight, or reproductive history.

The following data were recorded for the analysis including: 1) Demographic information of age, gender, height, weight, BMI, marital status, maternal history, smoking and drinking and 2) Gynecologic history of cervical conization before the diagnosis and examination results and treatment. 3) Cervix cytology pathologic examination, cervical HPV examination (including qualitative and typing), colposcopy results and pathological results, cervical conization postoperative pathology, P16 and Ki-67 immunohistochemical staining.

Table 1. Baseline data comparison

Number	group	case	age	BMI	marriage	history of gestation
1	p16+/ki67+	509	45.13±6.54	22.01±3.57		
2	P16+/ki67-	601	44.33±8.91	21.89±4.22		
3	P16-/ki67+	403	40.87±9.67	22.63±5.16		
4	P16-/ki67-	387	42.34±8.66	22.11±9.11		
	Statistical index		T=0.648	T=0.846	X^2=7.640	X^2=10.342
			P=0.463	P=0.399	P=0.131	P=0.954

Note: Age, body mass index, marital status, and maternal history all showed no statistical significance in four groups of patients.

Table 2. Primary end point data comparison

Number	group	case	Follow-up time/ recurrence case			
			3 months	6 months	12 months	24 months
1	p16+/ki67+	509	3 (0.59%)	16 (3.1%)	20 (3.9%)	50 (9.8%)
2	P16+/ki67-	601	3 (0.50%)	10 (1.66%)	11 (1.83%)	53 (8.82%)
3	P16-/ki67+	403	0 (0)	7 (1.74%)	8 (1.99%)	35 (8.67%)
4	P16-/ki67-	387	0 (0)	5 (1.29%)	6 (1.55%)	11 (2.84%)
	Statistical index					

Note: The number of recurrent cases changed with the prolongation of follow-up time in all four groups. The recurrence rate of group 1 was higher than that of other 3 groups. In comparison with group 4, the 24 month recurrence rate in group 1 was significantly higher than that in group 4 (P<0.05). In the comparison between group 1, 2, group 1, 3, the *P* values were all >0.05, but the recurrence rate increased.

Follow-up and outcome indicators

For the follow-up of this study, the deadline was Septmeber 2015. The follow-up interval was 3, 6, 12, and 24 months. During each follow-up, cervical HPV examination, colposcopy, and pathology examination were performed. In this study we set the primary end point to be cervical intraepithelial lesion recurrence according to biopsy results and set the secondary end point as HPV persistent infection or re-infection, which was determined according to follow-up HPV test or final pathologic results.

Results indicated that age, body mass index, marital status and maternal history all showed no statistical significance in all four groups (**Table 1**).

For primary end point results, the number of recurrences was changed with the prolongation of follow-up time in all four groups. The recurrence rate of group 1 was higher than that of the other 3 groups. In comparison with group 4, the 24 month recurrence rate in group 1 was significantly higher (P<0.05). From comparison between groups 1 and 2, and groups 1 and 3, the *P* values were all >0.05, but the recurrence rate showed an increase (**Table 2**).

For secondary end point results, in all four groups, there was a significant change in HPV infection or re-infection rate with prolonged follow-up time. Although there was no significant regression in the period of 3 months to 1 year, in the case of more than one year, significantly lower trends were observed in group 4. At 24 months' follow-up time, changes of HPV persistent infection or re-infection rate in each group were statistically significant, all *P* values <0.05 (**Table 3**).

Multivariate analysis

Five non-specific factors including age, high-risk HPV DNA load, initial TCT results, p16, and Ki67 expression were selected for analysis. Univariate analysis results showed that there was no significant correlation between age, high-risk HPV DNA load, initial TCT outcome, and disease progression (P>0.05), but positive expression of p16 and Ki67 were significantly correlated with progression of the lesion (P<0.05).

Table 3. Second end point data comparison

Number	group	case	Follow-up time/HPVpersistent infection or re-infection cases			
			3 months	6 months	12 months	24 months
1	p16+/ki67+	509	267 (52.45%)	198 (38.90%)	114 (22.40%)	99 (19.45%)
2	P16+/ki67-	601	454 (75.54%)	381 (63.40%)	135 (22.46%)	83 (13.81%)
3	P16-/ki67+	403	201 (49.88%)	198 (49.13%)	66 (16.38%)	50 (12.41%)
4	P16-/ki67-	387	200 (51.68%)	164 (42.38%)	86 (22.22%)	34 (8.79%)
	Statistical index					

In all four groups, there was a significant change in HPV infection or re-infection rate with prolonged follow-up time. Although there was no significant regression in the period of 3 months to 1 year, in the cases of more than one year, significantly lower trends were observed in group 4. At 24 months follow-up time, cases of HPV persistent infection or re-infection rate of each group were statistically significant, all *P* values were <0.05.

Discussion

Cervical conization is a major treatment for cervical intraepithelial lesions, but even in patients with negative margin, there is still a recurrence or re-infection rate of 2-29.5%. Therefore, identification of a biologic marker for guiding CIN cone postoperative follow-up is of high importance.

In a previous study, P16 and ki67 expression focused on the differential diagnosis of high-grade cervical lesions. Prior studies of p16 and Ki-67 expression indicated that these biomarkers may be preferentially expressed in cervical neoplasia. Ki-67, cyclin E, and p16 are complementary surrogate biomarkers for HPV-related preinvasive squamous cervical disease. Because cyclin E and p16 are most sensitive for LSIL and HSIL including high-risk HPV, respectively, combined application of these biomarkers for resolving diagnostic problems, with an appreciation of background staining, is recommended [17].

The performance of p16 (INK4a) was more sensitive (P<0.001), less specific (P<0.001), and of similar overall accuracy for CIN2(+) compared with the combined performance (sensitivity =68.9%, specificity =97.2%). Ki-67 was also strongly associated with CIN2(+) diagnosis, but its performance at all staining intensities was inferior to p16 immunostaining, and did not increase the accuracy of CIN2(+) diagnosis when combined with p16 (INK4a) immunostaining in comparison with p16 (INK4a) immunostaining alone. We found no utility for L1 immunostaining in distinguishing between CIN and non-CIN. We found immunohistochemical staining for p16 to be a useful and reliable diagnostic adjunct for distinguishing biopsies with and without CIN2(+) [18].

Thus, Ki67 expression can discriminate high-grade and low-grade lesions. It has also been reported that in the diagnosis of high-grade cervical lesions, p16 sensitivity is higher than HR-HPV. During the follow-up, we found that the recurrence rate was higher in the p16+/Ki67+ group than the other three groups at 3, 6, 12, and 24 months postoperatively, and the recurrence rate increased with follow-up time.

This study has some limitations. All patients in our study originated from only one medical center, and this was a retrospective observational cohort study, thus the data might not be adequate for a reliable conclusion. Therefore, further prospective analyses and multicentral studies with larger sample size are warranted.

Through logistic regression, multivariate analysis showed that p16 and ki67 positive expression were risk factors of progression of the lesion. Therefore, for cervical conization specimens, routine p16 and ki67 protein staining has clinical significance in guiding the progression of disease and prognosis of follow-up.

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Disclosure of conflict of interest

None.

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