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GENERAL GYNECOLOGY

Human papillomavirus DNA, HPV L1 capsid protein and p16^{INK4a} protein as markers to predict cervical lesion progression

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Abstract

Objectives Cervical cancer is the most common malignant tumors in women leading to serious morbidity and mortality worldwide, especially among developing countries. A main cause of the disease is the high-risk human papillomavirus (HR-HPV) infection. HSIL usually progress to cervical cancer, and low-grade lesions, including LSIL and ASCUS, mostly turn to normal or benign lesions, but there are still a small number of patients who will progress to HSIL. Up to now there is no efficient biomarker clinically available to predict people with high risk to progress into HSIL. This study was conducted to evaluate the value of human papillomavirus (HPV) DNA, p16^{INK4a} protein, and HPV L1 capsid protein in predicting HSIL and minimizing unnecessary colposcopy treatments.

Methods 1222 patients with HR-HPV infection or with abnormal Thinprep cytologic test (TCT) were chosen to conduct colposcopy in the cervical out-patient clinic of Shanghai First Maternity and Infant Hospital affiliated to Shanghai Tongji University from June 2014 to January 2017. TCT, cervical biopsy, HPV DNA and HPVL1 were performed on all patients. 110 patients were selected to detect p16^{INK4a} protein. Hybrid capture 2 (HC-2) was used to detect HPV DNA, and their subgroups using gene typing system. Immunohistochemical technology was used to detect HPV L1 and p16.

Results HPV DNA was positive in 1097 cases, with the positive rate of 89.7% (1097/1222). In particular, the positive expression rates of HPV DNA were 82.3, 95.7, 96.6 and 100% in Normal/CC, LSIL, HSIL and cervical cancer groups, respectively ($p < 0.001$). HPV L1 was negative in 781 cases with HR-HPV infection, and the overall negative rate is 71.1%. In patients with Normal/CC, LSIL and HSIL, the negative expression rates of HPV L1 were 91.3, 40 and 81.2%, respectively (p value < 0.001). In the 110 patients, HPV L1 was negative in 98.1% (53/54) of Normal/CC, 42.9% (12/28) of LSIL and 85.1% (23/27) of HSIL (p value $= 0.0043$). P16-positive rates in patients with Normal/CC, LSIL and HSIL were 33.3% (18/54), 75% (21/28) and 96.2% (26/27), respectively (p value < 0.001). 18 out of 28 cases express low positive (+) in LSIL, 25 out of 27 cases express strong positive (3+) in HSIL. Patients with L1(−) p16(+) including 18.5% (10/54) of normal/cervicitis, 60.7% (17/28) of LSIL and 85.1% (23/27) of HSIL (p value < 0.005). Furthermore, patients with L1(−) p16(1+) included 37% (10/27) of normal/cervicitis 59.3% (16/27) of LSIL and 3.7% (1/27) of HSIL; patients with L1(−) p16(2+) consisted of 0% of normal/cervicitis/LSIL and 100% (1/1) of HSIL; patients with L1(−) p16(3+) were composed of 0% of normal/cervicitis, 4.5% (1/22) of LSIL and 95.5% (21/22) of HSIL (p value < 0.005) (Table 6).

Conclusion With the increase in the degree of the cervical lesions, the expression of HPV DNA and p16 is up-regulated while HPV L1 protein is down-regulated. HPV DNA, HPV L1 and p16 are useful markers for the prediction of HSIL. Combined detection of these three markers has important potential to predicting HSIL and minimizing unnecessary colposcope examination.

Keywords HPV DNA · HPV L1 capsid protein · p16 · LSIL · HSIL

Abbreviations

CIN	Cervical intraepithelial neoplasia
CC	Chronic cervicitis
LSIL	Low-grade squamous intraepithelial lesion
HSIL	High-grade squamous intraepithelial lesion
SCC	Squamous cell carcinoma

Huiyan Hu and Jingjing Zhao contributed equally to this article.

Extended author information available on the last page of the article

HPV Human papilloma virus
IHC Immunohistochemistry

Introduction

Carcinoma of the uterine cervix is the most common cancer among women worldwide [1, 2]. In recent years, through effective screening programs and management of precursor lesions, a marked decline has been seen in the number of the mortality and morbidity of cervical cancer. However, in developing countries, because of limited resources and screening capability, cervical cancer remains the most common cause of cancer mortality among women. Fortunately, it has a long period of time from cervical precancerous to cervical cancer. It is known that the high-risk human papillomavirus infection (HR-HPV) is the main cause in the evolution of the disease [3]. HPV DNA is detected in 93–100% of the cervical cancer cases and its precursor lesions [4, 5]. Persistent HPV infection is a necessary, but not sufficient condition for the development of the cervical cancer [6]. Ninety percent of HPV infections disappear spontaneously within 2 years [7, 8]. Therefore, it is vital to find prognostic markers that can differentiate patients who will experience this progression from those who will not.

Many researches have carried out on finding possible prognostic markers in cervical lesion, such as HPV L1 capsid protein and $p16^{INK4a}$ (henceforth referred to as p16) protein [9–18], while their studies lack sufficient sensitivity and specificity.

L1 is the major capsid protein of the HPV, which is one major protein of the eight known HPV-specific proteins (E1, E2, E4, E5, E6, E7, L1 and L2). L1 is only produced at the early stage of the natural viral life cycle during a productive phase, is produced within the cytoplasm and translocated into the nucleus. The existence of HPV L1 capsid protein is a proof of a completed HPV life cycle, it can reflect the state of cervical HPV virus replication in cells, and by detecting the expression of HPV L1 capsid protein can predict cervical lesion progression and regression.

Recently, Lisang Zheng et al. [19] performed a screening on HPV DNA and HPV L1, among 596 patients with cervical lesions and proposed that combined detection of both HPV DNA and L1 had important values in diagnosing of cervical lesion and predicting progressive risk, it is worth of clinical promotion.

p16 is a cyclin-dependent kinase inhibitor that regulates the activity of cell cycle, and its expression is tightly controlled in normal cells. In normal cells, p16 protein is expressed at a very low level which makes it difficult to be detected by immunohistochemistry. The overexpression of p16 in cervical dysplasia has been shown to be associated with the transforming activity of the E7 oncoprotein of high-risk HPV types. RB gene is the first tumor-suppressor gene to be discovered and encode proteins that inhibit cellular proliferation by regulating the cell cycle. When cervical cells are infected by HPV, HPV produces E7, the E7 proteins bind and inactivate the tumor-suppressor function of the retinoblastoma protein (pRb), p16 overexpression as a negative feedback mechanism for the inactivation of pRbin dysplastic cervical cells [20, 21]. Phaik-Leng Cheah et al. [22] made a study including 99 patients to assess the correlation between HR-HPV and p16 immunoexpression in cervical squamous intraepithelial lesions and squamous carcinoma, underscoring the possible use of p16 expression to further subcategorise equivocal and early premalignant cervical squamous lesions in which HR-HPV is detected on screening.

In this study, we evaluated the value of the combination detection of HPV DNA, HPV L1 and p16 expression in biopsies of normal tissue as well as precancerous and cancerous lesions of uterine cervix, trying to find more effective surrogate markers for the prediction of HSIL, for the colposcope biopsy examination provides a more precise indication.

Materials and methods

Patients

Totally 1222 patients with HR-HPV infection or with abnormal TCT were chosen in the cervical out-patient clinic of our hospital from June 2014 to January 2017. Cases with cervical surgical therapy 6 months before this study or pregnancy were excluded. TCT, cervical biopsy, HPV DNA and HPV-L1 were performed on all patients.

HPV DNA and genotyping

All specimens' HPV DNA was detected with HC-2 technology. HPV genotyping detection used 21 (HPV) type detection kit (Cape Biotechnology Company, Guangdong, China). The kit targeted common HR-HPV types, i.e., 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, 68 and 6 low-risk HPV types (6, 11, 42, 43, 44, CP8304).

Immunocytochemistry for HPV L1 and p16

Immunohistochemical staining was performed on 4-umthick serial sections of formalin-fixed, paraffin-embedded tissue section. In brief, the paraffin-embedded sections were mounted on glass slides and dried by microwave for 15 min. The tissues were deparaffinized and rehydrated with xylene and ethanol, endogenous peroxidase was blocked with 3% H_2O_2 for 20 min, citrate buffer was used to pretreat the

sections, then in a microwave for 13 min at pH 6.0 and incubated in protein blocking solution for 10 min.

Immunocytochemistry for the presence of HPV L1

It was performed using the monoclonal antibody HPV L1 (Advanced Medical Science Company, CytoReact, America) according to the manufacturer's instructions [23]. The antibody recognized the major L1 including HPV-6, HPV-11, HPV-16, HPV-18, HPV-31, HPV-33, HPV-42, HPV-51, HPV-52 and HPV-58.

Immunocytochemistry for the presence of p16

It was performed using a CINtec Histology Kit (Biogen/Roche, Germany) according to the manufacturer's instructions. The percentage of positive cells was classified into four classes as follows: negative (below 5%), weak positive (1+, 5–25%), moderate positive (2+, 26–50%), and intense positive (3+, greater than 50%).

Statistical analysis

The data were analyzed using IBM SPSS ver. 19.0 for windows (IBM Co., Armonk, NY, USA). The association between groups was analyzed using χ^2 test or Fisher's exact test. p value < 0.05 was considered having statistical significance.

Results

The basic information

The patients aged from 20 to 71 years (mean 40.6 years). According to histopathological results, patients were divided into Normal or CC (565 cases), LSIL (condyloma/CIN1, 380 cases), HSIL (CIN2/3, 267 cases), and cervical cancer (SCC/adenocarcinoma, 10 cases). In addition, 110 cervical biopsy tissue samples were collected in this study, including 28 LSIL (condyloma/CIN1), 27 HSIL (CIN2–3), 1 adenocarcinoma and 54 normal cervical tissues as a control group. All of the patients signed the informed consent. The research protocol was approved by the Ethics Committee of our hospital.

HPV DNA and genotyping results

HPV DNA was positive in 1097 cases, with the positive rate of 89.7% (1097/1222). The positive rates of HPV DNA in patients with Normal/CC, LSIL, HSIL and SCC/adenocarcinoma were 82.3%, 95.7%, 96.6% and 100%, respectively. The difference between Normal/CC groups and other groups was statistically significant ($p < 0.05$), while in the LSIL and HSIL groups, the difference was not significant (Table 1). Among them, 171 cases were of specific types (including 111 cases of single kind of HPV infection, 38 cases of 2 types of HPV infection, 21 cases of 3 types of HPV infection, 1 case of 5 types of HPV infection), high-risk type 16, 52, 58 were the most common, with HPV11 and CP8034 type being the most common low-risk types.

HPV L1 and histological results

The HPV L1 was identified as positive when there was a clear nuclear staining, even if only one positive nucleus was found. One stained cell in the specimen was interpreted as positive case (Fig. 1). HPVL1 were negative in 781 cases, with the overall negative rate of 71.1% (781/1097). In normal or CC, LSIL, HSIL and cervical cancer, the negative expression rates of HPV L1 were 91.2, 40, 81.2 and 100%, respectively. Difference in HPV L1 protein-negative expression rate between LSIL, HSIL and cervical cancers was statistically significant ($p < 0.05$) (Table 2 and Fig. 3).

Among the 110 cases, the immunostaining for HPV L1 was found in 1 of 54 (1.8%) Normal/CC cases, 16 of 28 (57.1%) LSIL cases, 4 of 27 (14.9%) HSIL cases. However, the SCC/adenocarcinoma ($n = 1$) case showed negative expression of HPV L1 (Table 3). There was a statistically significant difference in HPV L1 expression between normal and lesional groups and also among other lesional groups of cases (LSIL, HSIL, SCC/adenocarcinoma) ($p < 0.005$).

Table 1 HPV DNA compared with histological results

HPV DNA	Histological results				
	Normal/CC	LSIL	HSIL	SCC/adenocarcinoma	Total
Positive	465 (82.3%)	364 (95.7%)	258 (96.6%)	10 (100%)	1097 (89.7%)
Negative	100 (17.7%)	16 (4.3%)	9 (3.4%)	0 (0%)	125 (10.3%)
Total	565 (100%)	380 (100%)	267 (100%)	100 (100%)	1222 (100%)

CC chronic cervicitis, *LSIL* low-grade squamous intraepithelial lesion, *HSIL* high-grade squamous intraepithelial lesion, and *SCC* squamous cell carcinoma

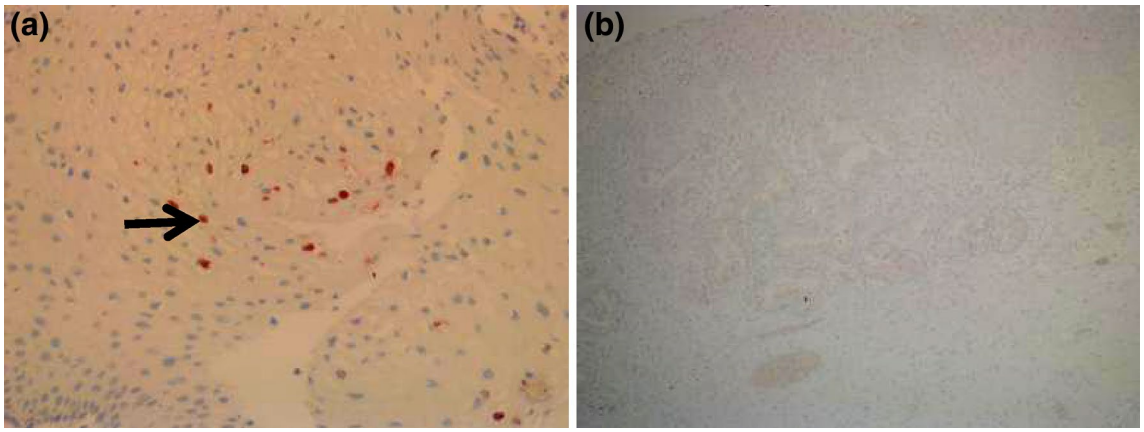

Fig. 1 **a** HPV L1 immunohistochemical expression in low-grade cervical intraepithelial neoplasia (×200) (a clear nuclear staining with red color was identified as HPV L1 positive), **b** without HPV L1 immunohistochemical expression in normal tissue (×200)

Table 2 The correlation between HPV L1 expression and histological results

HPV L1	Histological results				
	Normal/CC	LSIL	HSIL	SCC/adenocarcinoma	Total
Negative	402 (91.3%)	152 (40%)	217 (81.2%)	10 (100%)	781 (73.1%)
Positive	38 (8.7%)	228 (60%)	50 (18.8%)	0 (0%)	316 (26.9%)
Total	440 (100%)	380 (100%)	267 (100%)	10 (100%)	1097 (100%)

CC chronic cervicitis, *LSIL* low-grade squamous intraepithelial lesion, *HSIL* high-grade squamous intraepithelial lesion, and *SCC* squamous cell carcinoma

Table 3 The correlation between HPV L1 expression and histological results

HPV L1	Histological results				
	Normal/CC	LSIL	HSIL	SCC/adenocarcinoma	Total
Negative	1 (11.9%)	16 (57.1%)	4 (14.9%)	0 (0%)	21 (19.1%)
Positive	53 (98.1%)	12 (42.9%)	23 (85.1%)	1 (100%)	89 (80.9%)
Total	54 (100%)	28 (100%)	27 (100%)	1 (100%)	110 (100%)

CC chronic cervicitis, *LSIL* low-grade squamous intraepithelial lesion, *HSIL* high-grade squamous intraepithelial lesion, and *SCC* squamous cell carcinoma

p16 and histological results

p16 immunostaining was considered positive when the cervical cell appeared as brown color in nucleus, cytoplasm, or both (Fig. 2). Immunohistochemical results revealed that the whole rate of positive staining for p16 was 60% (66/110), the positive rates in Normal/CC, LSIL and HSIL were 33.3, 75 and 96.3%, respectively. p16 expression significantly increased with disease progression ($p < 0.005$) (Table 4 and Fig. 4). 18 out of 28 cases express low positive (+) in LSIL, and 25 out of 27 cases express strong positive (3+) in HSIL; we can find that with the severity of the cervical lesion, the intensity of positive expression of p16 increased (Table 5). However, there was one patient with HSIL whose p16 expression was negative (Table 5).

The intensity of positive expression of p16 and histological results in L1-negative patients

All patients with L1(−) p16(+) included 18.5% (10/54) of normal/cervicitis, 60.7% (17/28) of LSIL and 85.1% (23/27) of HSIL (p value< 0.005). Furthermore, patients with L1(−) p16(1+) include 37% (10/27) of normal/cervicitis, 59.3% (16/27) of LSIL and 3.7% (1/27) of HSIL; patients with L1(−) p16(2+) include 0% of normal/cervicitis/LSIL and 100% (1/1) of HSIL; patients with L1(−) p16(3+) include 0% of normal/cervicitis, 4.5% (1/22) of LSIL and 95.5% (21/22) of HSIL (p value< 0.005) (Table 6).

Discussion

Cervical cancer screening tests that use conventional cytology have demonstrated obvious reductions in both cervical cancer morbility and mortality in the past decades [24]. However, conventional cytology lacks enough sensibility and specificity [25]. Liquid-based cytology (LBC) potentially

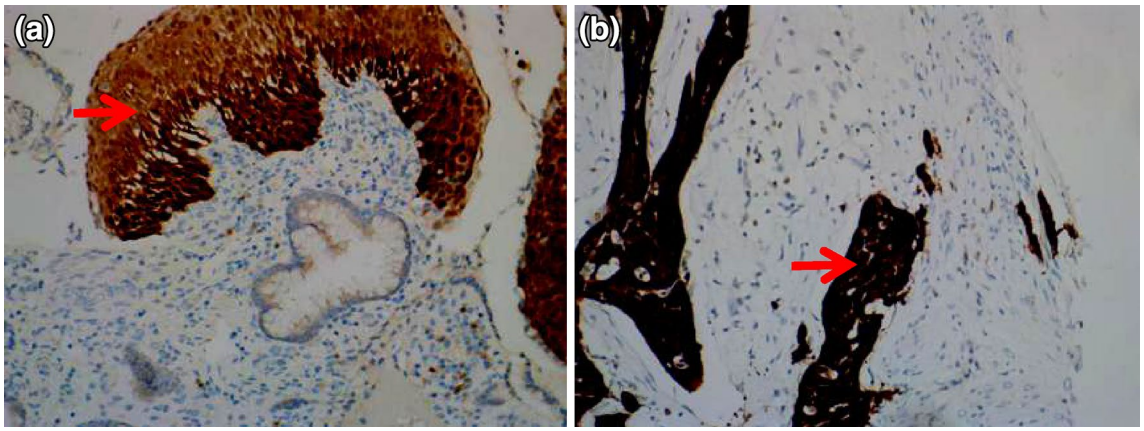

Fig. 2 **a** p16 immunohistochemical expression in high-grade cervical intraepithelial neoplasia (×200), **b** p16 immunohistochemical expression in low-grade cervical intraepithelial neoplasia (×200) (the lesional cells appeared a brown color innucleus or cytoplasm was considered p16 positive)

Table 4 The correlation between p16 expression and histological results

p16	Histological results				
	Normal/CC	LSIL	HSIL	SCC/adenocarcinoma	Total
Negative	18 (33.3%)	21 (75%)	26 (96.3%)	1 (100%)	66 (60%)
Positive	36 (66.7%)	7 (25%)	1 (3.7%)	0 (0%)	44 (40%)
Total	54 (100%)	28 (100%)	27 (100%)	1 (100%)	110 (100%)

CC chronic cervicitis, *LSIL* low-grade squamous intraepithelial lesion, *HSIL* high-grade squamous intraepithelial lesion, and *SCC* squamous cell carcinoma

Table 5 Correlation between the intensity of positive expression of p16 and histologic results

p16 ($n=110$)	Histological results				
	Normal	LSIL	HSIL	SCC	*p* value
+	18	18	0	0	0.00
++	0	0	1	0	
+++	0	3	25	1	
Neg	36	7	1	0	

LSIL low-grade squamous intraepithelial lesion and *HSIL* high-grade squamous intraepithelial lesion

Table 6 Correlation between the intensity of positive expression of p16 and histologic results in L1-negative patients

L1(−) p16	Histological results ($n=50$)			
	Normal	LSIL	HSIL	*p* value
+	10	16	1	< 0.005
++	0	0	1	
+++	0	1	21	

LSIL low-grade squamous intraepithelial lesion and *HSIL* high-grade squamous intraepithelial lesion

improved conventional cytology test that can support co-testing (HPV plus cytology), but its effect remains uncertain [25]. Then histology screening tests were suggested, but the interobservers' various points in the histological diagnosis of cervical biopsy specimens are still a potential problem [26, 27]. Improving screening tests is a priority task for the early diagnosis of cervical lesion.

Moreover, the current screening tests for cervical cancer (histology and cytology) are unable to distinguish the lesions which have high risk progressing to HSIL and which have not [28], leading to unnecessary colposcope examination and overtreatment.

Many researches have carried out on finding possible specific biomarkers in cervical lesion, such as HPV DNA, L1 and p16 [9–18].

In this study, the HPV DNA was detected in 89.7% (1097/1222) of all the cases. The rate was higher than other researches reported previously. In this study, the positive rates of HPV DNA in patients with Normal/CC, LSIL, HSIL and SCC/adenocarcinoma were 81.9%, 95.7%, 96.6% and 100%, respectively. We found a significant difference between HPV DNA positive rates in normal/cervicitis and other groups ($p < 0.001$). HPV16 and HPV18 were the most common types.

This result was consistent with other reports and highlighted that HPV16 and HPV18 were the most high-risk types leading to cervical cancer [29–31].

Recently, some researches have reported that HPV L1 capsid protein can be a new potential useful biomarker to

show the status of productive HPV infections and predict the cervical lesion progression [13, 32, 33].

L1 capsid protein is a major capsid protein of HPV, highly conserved, is the main specific antigen, is the major target for the body's immune response to the HPV infections, and can be detected during the productive stage of HPV disease. It is progressively lost in the later phases [33]. It indicated that L1 capsid protein expression tends to decline with increasing severity of the lesion ($p < 0.001$). This can explain why the positive rate in LSIL is higher than HSIL and cancers, and the positive rate in HSIL is higher than cancers. Griesser et al. did a research about 84 CIN patients with high-risk-type HPV infection, they were followed up for 22.8 months (6–46), the results showed that in HPV L1 capsid protein-negative cases 76.4% progressed; in HPV L1 capsid protein-positive cases, 69.0% showed spontaneous regression [34]. In another international multicenter joint research [12], 908 cases of high-risk-type HPV infection were included, they were followed up for 54 months, the results showed that in HPV L1-negative groups, 84% cases progressed to CINIII, while in HPV L1-positive groups, 20% progressed to CINIII [35]. In this study, HPV L1 was negative in 40% of LSIL, 81.2% of HSIL and 100% of cervical cancer while 91.3% of normal/cervicitis; this differs from the previously reported results [32, 33, 36]. Difference in HPV L1 protein-negative expression rate between LSIL, HSIL and cervical cancers was statistically significant ($p < 0.05$) (Table 2 and Fig. 3). With the increase of degree of the cervical lesions, the negative expression of HPV L1 protein is raised.

The p16 is a cyclin-dependent kinase inhibitor that blocks the phosphorylation of various cyclins. When cervical cells infect HPV, HPV produces E7, the E7 proteins bind and inactivate the tumor-suppressor function of the pRb, p16 overexpression as a negative feedback mechanism for the inactivation of pRbin dysplastic cervical cells [19–21].

In the previous studies, p16 has been proposed as a potential biomarker for the identification of the dysplastic cervical cells. In this study, the positive rates of p16 in Normal/CC, LSIL, HSIL and invasive cancer were 33.3%, 75%, 96.3% and 100%, respectively (Table 4 and Fig. 4). This study had shown a positive correlation between p16 and the degree of the cervical neoplasia, which was consistent with other researches [37–39]. In addition, we also found that with the severity of the cervical lesion, the intensity of positive expression of p16 increased (Table 5). The immunohistochemical detection of p16 expression could be a useful indicator of progression in cervical dysplasia.

Based on the combination of the p16 and L1 immunohistochemical detection, we have found patients with L1(−) p16(+) might be defined as high-risk cases. This means the virus that already inhibited the RB protein pathway resulted in the integration of HPV DNA into the host genes, which is typically found in high-grade lesions of the cervix. In our study, this pattern was expressed in 50 cases (50/110, 45.5%) with cervical lesion, including 18.5% (10/54) of Normal/cervicitis, 60.7% (17/28) of LSIL and 85.1% (23/27) of HSIL (p value < 0.005). Furthermore, patients with L1(−) p16(1+) included 37%

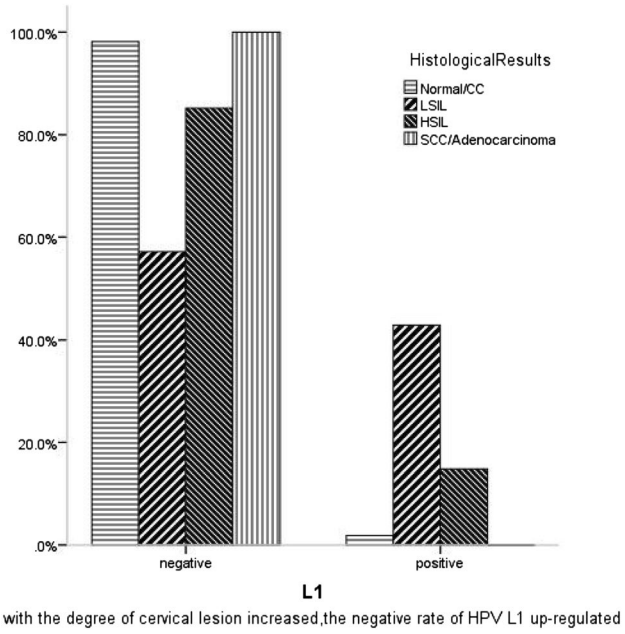

Fig. 3 Results of HPV L1 expression and pathology

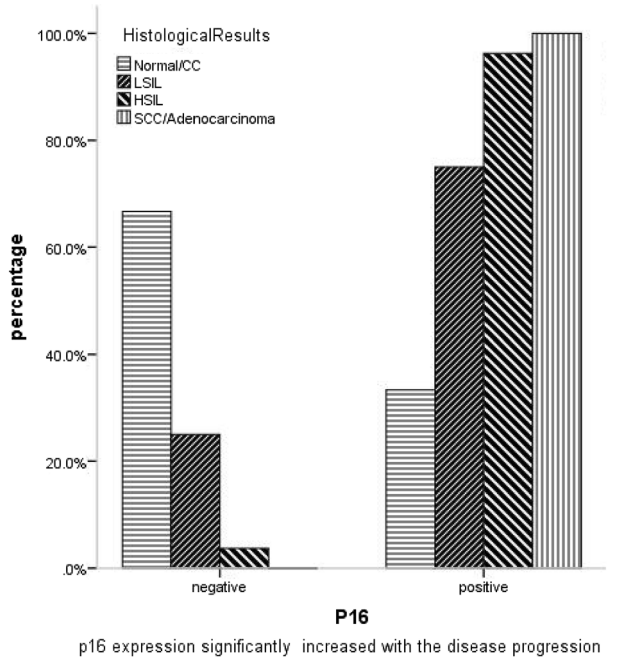

Fig. 4 Imunohistochemical results of $p16^{INK4a}$ protein in groups

Table 7 Correlation between expression of p16 and HPV L1 and histologic results

	Histological results ($n=110$)				
	Normal/CC	LSIL	HSIL	SCC/adenocarcinoma	Total
L1(−) P16(−)	36	6	1	0	43
L1(+) P16(−)	0	1	0	0	1
L1(+) P16(+)	1	11	4	0	16
L1(−) P16(+)	17	10	23	1	51
Total	54	28	27	1	110

LSIL low-grade squamous intraepithelial lesion and *HSIL* high-grade squamous intraepithelial lesion

(10/27) of normal/cervicitis, 59.3% (16/27) of LSIL and 3.7% (1/27) of HSIL; patients with L1(−) p16(2+) include 0% of normal/cervicitis/LSIL and 100% (1/1) of HSIL; with L1(−) p16(3+) include 0% of normal/cervicitis, 4.5% (1/22) of LSIL and 95.5% (21/22) of HSIL (p value <0.005) (Table 6). In patients with HPV L1(−), the correlation between the intensity of p16 and the severity of cervical lesion is obvious. Cases with this pattern need further examination or even treatment.

The L1/p16 expression is related to the severity of cervical lesions, can improve the histopathological diagnosis of precancerous cervical lesions, and may serve as useful biomarker for predicting the progression of cervical lesion and giving an early intervention or determining a follow-up strategy (Table 7).

Besides, we found two strange phenomenons, one is that L1 expression in normal/cervicitis cases was lower than other researches; for another, p16 was negative in one case diagnosed as adenocarcinoma. Detecting error may explain, or persistent HR-HPV infection leading to, before it lead to cervical epithelial cell lesion's occurring; HPV type may not have been included in this HPV panel used for detection, or there is potential pathogeny that we do not know yet, further research is needed for us.

In conclusion, HPV DNA, HPV L1 and p16 are useful markers for the early diagnosis of cervical lesions. With the increase of degree of the cervical lesions, the expression of HPV DNA and p16 is up-regulated while HPV L1 protein is opposed. Combined detection of these three targets has important value in diagnosing cervical lesion and predicting progressive risk; it can also reduce unnecessary colposcope examination and overtreatment.

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Compliance with ethical standards

Conflict of interest The authors declare no potential conflicts of interest.

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