RESEARCH COMMUNICATION

Interobserver Reproducibility in Determining p16 Overexpression in Cervical Lesions : Use of a Combined Scoring Method

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Abstract

Objectives : To evaluate interobserver reproducibility of a combined scoring method for immunohistochemical interpretation of p16 overexpression in cervical lesions. Materials and methods : p16 immunostaining was performed in cervical samples from 183 patients, including 69 normal, 42 low grade squamous intraepithelial lesions(LSIL), 36 high grade SIL (HSIL), and 36 squamous cell carcinomas(SCCAs). Each case was evaluated by a combined scoring method based on the percentage of positive cells (score 0-3), the intensitiy of staining (score 0-3), and the distribution pattern (score 0-2). Immunoexpression for p16 was considered as positive when the combined score was 4-8 and negative with a score of 0-3. Ten pathologists with varied experience in interpretating p16 immunostains evaluated each slide independently. Results : All normal cervical squamous epithelia (69/69) were uniformly negative for p16. All HSILs (36/36), all SCCAs (100/100), and all but one of the LSILs (40/41, 97.62%) showed positive expression. In 172 of 183 cases (93.99%), p16 interpretation was concordant with all pathologists. Eleven cases with discordant results included 10 LSILs and 1 normal mucosa sample. Percentage of agreement of each pathologist pair ranged from 96.7-100% (mean 98.1 ± 0.96%) with mean kappa value of 0.96 ± 0.0201 (range 0.93-1.000). Conclusion : The proposed combined scoring method shows good reproducibility among the participating pathologists and good correlation with the histologic diagnosis. This method may be a useful guide in the interpretation of p16 expression in cervical epithelial lesions.

Key Words: Human papillomavirus - cervical cancer - p16 overexpression - combined scoring method

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Introduction

Cervical cancer is one of the most common cancers contributed to cancer-related morbidity and mortality among women worldwide. Prevention is essentially based on cytological identification, colposcopic examination, histology confirmation, and treatment of its precursors. Human papillomavirus (HPV) is the major causative agent in cervical carcinogenesis (Bosch et al.,2002; Munoz et al.,2003; Walboomers et al.,1999) The virus is detected in almost all preneoplastic and neoplastic lesions of the cervix (Ostor,1993). There are more than 100 different genotypes of which about 30 can infect the uterine cervical epithelium (Bosch et al., 2002). Each genotype has been classified into high-risk(HR) and low-risk(LR) group according to carcinogenetic potential on cervical epithelial cells. The HR-HPV comprises 20genotypes (16, 18, 26, 31, 33, 35, 39, 45, 51, 52, 53, 55, 56, 58, 59, 66, 68, 73, 82, and 83) (Burchell et al., 2006). HPV16 is the most prevalent and has also been demonstrated to be the single most important factor linked with disease progression(Clifford et al.,2005).

HPV is a double-stranded circular DNA virus. The genome can be divided in to 3 regions: the upstream regulatory region(URR), the early(E) region, and the late(L) region (Clifford et al., 2005). The URR regulates viral replication and transcription of down stream sequences in the E region., which encodes viral proteins (E1-E8) taking part in viral replication. The L region encodes viral structural proteins (L1, L2) which encounter in capsid formation. E6 and E7 are the principal transforming proteins of HPV (Wright et al.,2002) The oncoprotein encoded by the E6 has the ability to degradation of p53 protein which prevents cells from

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undergoing apoptosis. E7 oncoprotein inactivates hypophosphorylated retinoblastoma protein (pRb) resulting in hyperproliferation (Sano et al.,1998) .The cyclin-dependent kinase inhibitor-2A p16 is regulated by a pRb-dependent negative feed back loop. Continuous inactivation of pRb by oncoprotein E7 results in increased p16INK4a level. Hence, increased p16 expression may reflect HPV-induced dysplasia with deregulated E7 expression. (Snikders et al.,2006) p16 has been reported to be highly overexpressed in dysplastic epithelial cells of the uterine cervix and absent in normal epithelium and benign lesions such as inflammation (Agoff et al., 2003; Wang et al., 2005; Benevolo et al., 2006; Kalof et al., 2006; Hariri et al., 2007).

p16 overexpression is usually detected by immunochemical study in both tissue sections and cytological preparation (Leong et al., 2006). Western blot is an alternative detection system (Murphy et al.,2005). In immunohistochemical study, the antibody to p16 shows nuclear and cytoplasmic localization. Various criteria for interpretation of the immunostains have been proposed. These criteria include nuclear with and without cytoplasmic staining, degree of intensity, basal and parabasal involvement, pattern of staining (focal or diffuse), and percentage of positive staining dysplastic cells. (Murphy et al., 2003; 2005; Lin et al., 2005; Wang et al., 2005; Benevolo et al., 2006; Carozzi et al., 2006; Queiroz et al., 2006; Focchi et al., 2007; Hariri et al., 2007; Yidiz et al., 2007). To address the issue of p16 immunoreactivity interpretation in cervical lesion, a group of Thai pathologists working in gynecologic pathology from 9 different institutes propose a combined scoring method for interpretation of p16 immunoreactivity and evaluate the interobserver reproducibility of this method. This study is a part of research project entitled 'Role of Human papillomavirus(HPV) in cervical carcinogenesis in Thai women'.

Materials and Methods

Tissue samples

Six hundred and twenty formalin fixed and paraffin embedded cervical tissue blocks and corresponding H&E slides were collected from files of 8 institutes. The cervical tissues were obtained between 2004 and 2007 and were composed of 4 diagnostic categories: normal or benign 204 cases, low grade squamous intraepithelial lesion(LSIL) 174 cases, high grade squamous intraepithelial lesion (HSIL) 122 cases, and squamous cell carcinoma (SCCA) 120 cases. To be included in the study, cases with SIL must have the lesion size of at least 2 mm in greatest extent. Cases with invasive squamous cell carcinoma were at least stage Ib. The exclusion criteria included cervical lesions other than squamous lesion, size of lesion less than 2 mm, and the presence of more than one type of epithelial lesion. The normal or benign cervical tissues were obtained from hysterectomy specimens without preneoplastic and neoplastic gynecologic lesions. All slides were reviewed simultaneously by 6-10 pathologists using a multi-headed microscope to confirm the histologic diagnosis.

Table 1. p16 Scoring Criteria

Features		Score
Percentage of positive cells	<5%	0
	5-49%	1
	50-80%	2
	>80%	3
Intensity of the reaction	No reaction	0
	Weak	1
	Variable	2
	Strong	3
Cellular reaction pattern	No reaction	0
	Focal	1
Diffuse		2

Total score: 0-3 =Negative, 4-8 = Positive

Immunohistochemical method

Three-micron thick tissue sections were mounted on positive-charged slides, deparaffinized with xylene and passed through graded alcohols before successively in deionized water and phosphate-buffered saline (PBS). Endogenous peroxidase was blocked with 0.3% hydrogen peroxide –deionized water for 30 min. The sections were subjected to epitope retrieval by placing the deparaffinized and rehydrated sections in a closed plastic container filled with Tris-EDTA pH9.0. They were heated in a pressure cooker 120˚C, 15 psi (Pascal electric pressure cooker for 3 minutes. After cooling the sections to room temperature (15 minutes), they were incubated with 5% non-immune horse serum to block non-specific staining. They were then incubated with primary antibodies (p16INK4a, dilution 1:500, clone JC8, DBS, CA, USA) at room temperature for 2 hours. After rinsing in phosphate-buffered solution, the sections were incubated with Biogenex detection system (Cat.No HK518-06K) for 40 minutes. The sections were then incubated with DAB (diaminobenzidine) chromogen. A light Mayer hematoxylin was applied as a counterstain. A positive control using colonic cancer tissue known to express the antigen was done at the same time.

Interpretation method

The antibody to p16 showed reaction to nucleus, cytoplasm, or both. Interpretation is based on percentage of positive cells, intensity of the reaction, and distribution pattern. Each parameter is graded and a combined score is used to determined positive or negative result based on criterias proposed earlier (Kong et al.,2007; Queiroz et al.,2006; Yidiz et al.,2007) (Table 1). The percentage of positive cells were scored in the highest expression area (hot spot) The intensity of the reaction is divided into weak, variable (containing weak and strong areas of intensity), and strong (Figure 1). The distribution pattern is interpreted as focal and diffuse. The latter was defined as continuous staining of areas of cells larger than X10 field area.

Pathologists

The ten pathologists practiced in 8 different institutions with special interest in gynecologic pathology. All had at least ten years experience in this field. However, they have varied experience in interpretation of p16 immunostains.

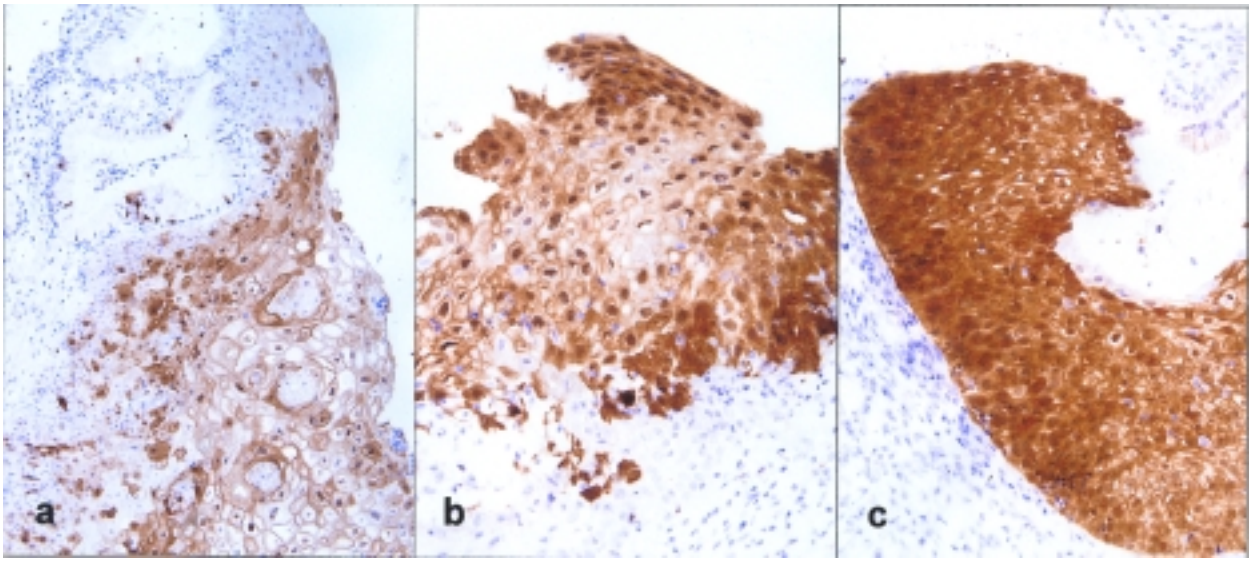

Figure 1. The Intensity of the Immunohistochemical p16 reaction: a) weak; b) variable; c) strong

Assessing procedure

The first set of immunostains (183 cases) were drawn out for an analysis of interobserver reproducibility. These included normal 69 cases, LSIL 42 cases, HSIL 36 cases, and SCCA 36 cases. After a discussion on the interpretation criteria, all 10 pathologists (designated as P1, P2, P3, P4, P5, P6, P7, P8, P9, and P10) evaluated and scored each immunostained slide independently.

Statistic analysis

Intercooled Stata 8.0 for Windows (Stata Corporation, College Station, TX, USA) were used for data summarization and analysis. The data were presented as number (percentage) or mean (SD). The interobserver reproducibility between each pair of pathologists was analyzed using kappa statistics. Degree of agreement were obtained from the kappa values as follows; <0 = poor, 0-0.2 = slight, 0.2-0.4 = fair, 0.4-0.6 = moderate, 0.6-0.8 = substantial, 0.8-1 = almost perfect. For any disagreement case, the majority score were recorded as the consensus score. Chi square test was used for examining the difference of p16 expression in cervical lesions. The significant level was set at 0.05.

Results

The p16 scores for each pathologist are demonstrated in Table 2. The highest number of p16 positive cases was interpreted by pathologist P1, while the lowest number of those positive cases was assigned by pathologist P10. Percentage of agreement of each pathologist pair ranged from 96.72-100% (mean 98.09 ± 0.96%). Kappa statistics of each pathologist pair were shown in Table 3. These kappa values ranged from 0.9310 (95% CI; 0.7864, 1.0756) to 1.000 (95%CI; 0.8552, 1.1448) with mean kappa value of 0.9600 ± 0.020. The lowest kappa values were from the pathologists P1 and P8. The highest kappa values were from the pathologists P6 and P7.

Uniform agreement by all pathologists for p16 immunoexpression was observed in 172 of 183 cases (94.0%). Eleven cases with discordant interpretation included 10 LSILs and 1 normal mucosa (Table 4).

Consensus results of p16 expression in all 183 cases were normal cervical squamous epithelium (69/69)

Table 2. Interpretation of p16 immunoexpression by Each Pathologist

Pathologist	p16 negative	p16 positive
P1	69 (37.70)	114 (62.30)
P2	70 (38.25)	113 (61.75)
P3	71 (38.80)	112 (61.20)
P4	72 (39.34)	111 (60.66)
P5	74 (40.44)	109 (59.56)
P6	70 (38.25)	113 (61.75)
P7	70 (38.25)	113 (61.75)
P8	73 (39.89)	110 (60.11)
P9	71 (38.80)	112 (61.20)
P10	75 (40.98)	108 (59.02)

Table 3. Pair-wise κ Statistics for p16 Expression Evaluated by the 10 Pathologists

	P2	P3	P4	P5	P6	P7	P8	P9	P10
P1	0.963	0.977	0.965	0.943	0.988	0.988	0.931	0.977	0.931
P2		0.965	0.954	0.954	0.977	0.977	0.943	0.965	0.943
P3			0.966	0.943	0.989	0.989	0.931	0.977	0.932
P4				0.954	0.977	0.977	0.966	0.989	0.943
P5					0.954	0.954	0.943	0.943	0.966
P6						1.000	0.943	0.989	0.943
P7							0.943	0.989	0.943
P8								0.954	0.932
P9									0.932

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P2		0.965	0.954	0.954	0.977	0.977	0.943	0.965	0.943
P3			0.966	0.943	0.989	0.989	0.931	0.977	0.932
P4				0.954	0.977	0.977	0.966	0.989	0.943
P5					0.954	0.954	0.943	0.943	0.966
P6						1.000	0.943	0.989	0.943
P7							0.943	0.989	0.943
P8								0.954	0.932
P9									0.932

uniformly negative for p16. All HSILs (36/36) and SCCAs (100/100) showed positive p16 expression. All but one (40 cases, 97.6%) of the LSILs were p16-positive. The expression of p16 in cervical lesions (LSIL, HSIL, SCCA) was significantly greater than that in normal cervix ($p<0.0001$).

Discussion

In normal cell cycle, the progression of cells through various phases is orchestrated by cyclins and cyclin-dependent kinases(CDK), and by their inhibitors. Cyclin D is the first cyclin to increase in the cell cycle (G1 phase). It binds and activate CDK4, forming a cyclin D/CDK4 complex which has a critical role in the cell cycle by phosphorylated retinoblastoma protein(pRb). The hypophosphorylated pRb prevents cells from replicating by forming a tight inactive complex with the transcription factor E2F. Phosphorylation of pRb dissociated the complex and releases the inhibition on E2F transcriptional activity. This result in forming cyclin E/CDK2, which stimulating DNA synthesis (S phase) and promotes cell replication. (Kumar et al., 2005)

The p16 protein contains 156 amino acids and was first discovered in a yeast two-hybrid system to detect proteins that interact with human cyclin-dependent kinase. (Ruas et al.,1998) P16 is a negative regulartory protein, whereas cyclin D1 and CDK 4 are positive regulators. P16 binds competitively to the CDK4, which inhibits the interaction of CDK4 with cyclin D1 and leads to the inhibition of the G1 phase of the cell cycle. (Serrano, 1997) Besides cell cycle control, p16 has been implicated in other processes such as senescence and apoptosis. In addition, p16 has shown to reduce cell invasion, cell spreading, and angiogenesis. (Nilsson et al., 2004) The inactivation of the p16 gene is involved in the pathogenesis of many types of human malignant tumors. Promoter hypermethylation, in addition to gene deletion and point mutation of p16 locus, has been found to be one of the main mechanism of p16 inactivation (El-Naggar et al., 1997; Heinzel et al.,1996), resulting in reduced expression of the p16.

In HR-HPV infection, overexpression or presence of high levels of p16 is the main finding. This due to the binding of viral E7 oncoprotein to pRb which releases the bond between pRb and E2F, resulting in cell replication. In addition, cyclin D/CDK4 is overproduced in an attempt to phosphorylate the bound pRb. To counteract the amplification of cyclin D/CDK4, the production of p16 is also increased. Thus, overexpression of p16 can be considered an indirect marker for the presence of altered HR-HPV and growth cycle transformation (O'Connor, 2007).

Detection of p16 overexpression is best done by immunohistochemical study. It is superior to HPV in situ hybridization for detection of HR-HPV (Kong et al.,2007). There is no consensus criterias for p16 immunostaining interpretation in the literatures such as cellular staining (nuclear, cytoplasmic, or nuclear and cytoplasmic staining), percentage, distribution of positive cells (rare, basal, full thickness), intensity (weak, moderate, strong), and pattern of staining (focal, diffuse). Based on the previously reported criterias, we develop the combined scoring system used in this study. Using this method, we find that the interpretation of p16 expression has a good correlation with the histologic diagnoses with an excellent interobserver reproducibility. One case of LSIL displays negative result. This case may be infected by non HR-HPV infection. The other LSIL lesions that showed discordant interpretation among the pathologists have small limited size of lesions and need careful interpretation regarding the percentage of positive cells and distribution pattern.

This combined scoring method may be a good tool in the interpretation of p16 expression. It will be useful in differentiating benign from dysplastic or malignant processes that are otherwise difficult to evaluated morphologically. These include differentiating markedly reactive squamous metaplasia or atrophic squamous epithelium from high-grade cervical intraepithelial neoplasia.

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