RESEARCH ARTICLE

Expression of the p16 and Ki67 in Cervical Squamous Intraepithelial Lesions and Cancer

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Abstract

Purpose: To evaluate the expression of p16 and Ki67 in cervical intraepithelial neoplasia (CIN) and cancer. Materials and Methods: We performed a immunohistochemical study of p16 and Ki67 in 243 cervical tissues - 53 non-dysplastic lesions, 106 CIN1, 61 CIN2/3 and 23 squamous cell carcinomas. The expression of p16 and Ki67 was interpreted independently by 2 researchers and the sensitivity and specificity to detect clinically significant lesions (≥ CIN2) were determined. Results: The overall agreement results of positive or negative immunostaining of intra-inter observer variability were 0.659 for p16 and 0.808 for Ki67. p16 expression was demonstrated in 91.3% of invasive carcinomas, 78.7% of CIN2/3, 10.4% of CIN1 and 9.4% of non-dysplasic lesions. The corresponding Ki67 expression was: 100% of all invasive carcinomas, 75.4% of CIN2/3, 22.6% of CIN1, and 11.3% with non-dysplasia. The expression was significantly different between CIN2/3 vs CIN1 for both p16 and Ki67 (p-values <0.001 both), and cancer vs CIN2/3 for Ki67 (p-value 0.008). The differences were not significant between CIN1 vs non-dysplasia (p-values 1.000 for p16 and 0.130 of Ki67), and cancer vs CIN2/3 for p16 (p value 0.219). The sensitivity and specificity to detect > CIN2 were 84.5% and 90.5% by p16 and 82.1% and 88.6% by Ki67. Conclusions: The rates for 16 and Ki67 expression were directly associated with the severity of cervical lesions. Significant differences in these markers expression may be useful in cases with equivocal histologic features among cervical intraepithelial lesions, but not between CIN1 and non-dysplastic lesions. The two markers had high sensitivity and specificity in determining >CIN2.

Keywords: p16 - Ki67 - immunohostochemical study - cervical intraepithelial neoplasia - cancer

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Introduction

Cervical cancer is the fourth most common cancer in women worldwide after breast, colorectal and lung cancer. The estimated new cases and deaths in 2012 were 528,000 and 266,000 respectively (Ferlay et al., 2012). The majority of cervical cancer occurred in less developed regions where most patients are in advanced stage disease at diagnosis (Moore et al., 2010). In Thailand, cervical cancer is the second common cancer after breast cancer with 8,184 new cases and 4,513 deaths in 2012 (Ministry of public health, 2015). One effective means to decrease cervical cancer incidence and death is an early detection of cancer and its precancerous lesions or cervical intraepithelial neoplasia (CIN) (Nam et al., 2008).

Cervical squamous intraepithelial neoplasia is classified into CIN1, CIN2 and CIN3 by the extent of epithelial involvement. The progression rates of CIN1 to CIN3 and to invasive carcinoma were 10% and 1% respectively. The corresponding progression rates of CIN2 were 20% and 5%, and of CIN3 to invasive cancer was greater than 12% (Ostor, 1993; Kim et al., 2011).

Human papilloma virus (HPV) is a DNA virus from the papillomavirus family that is well recognized as a cause of cervical cancer and its precursor lesions, especially the high-risk types. The carcinogenic activity is mediated through two viral oncoproteins, E6 and E7. The E6 and E7 proteins have ability to bind host cell regulatory proteins. The tumor suppressor gene p53 dysfunction caused by E6 will inhibit program cell death while the retinoblastoma protein (pRb) inactivation by E7 leads to uncontrolled cell mutation (Srisomboon, 2004; Lambert et al., 2006). These pre-clinical cellular dysregulation could be evidenced clinically by immunohistochemical study of some proteins, such as, p16 and Ki67.

p16 is a cell-cycle regulatory protein. Its function is to regulate cell proliferation in G1-S phase and negatively influences cell proliferation through a reciprocal relationship with another tumor suppressor protein, pRb. The overexpression of p16 could be found in cells with inactive pRb which is commonly present in HPV infection (Lambert et al.,2006).

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Ki67 is a nuclear and nucleolar protein expressed only in active phases of cell cycle (G1, S, G2, and M phases) but not in resting phases (G0 and early G1). Overexpression of Ki67 correlates with high cellular proliferation. Since HPV infection leads to increased epithelium cell proliferation in infected tissue, increased Ki67 staining can be an indicator of HPV (Evanthia et al., 2012; Brown et al., 2012).

In certain cases, the reactive changes, immature metaplasia or atrophic changes of cervix may show similar morphologic features as intraepithelial lesion or discretion between low grade lesion and high grade lesion is not possible by the routine hematoxylin and eosin stain of tissue, the study of these 2 molecular biomarkers may be useful (Evanthia et al., 2012). The correct diagnosis will certainly reduce an inappropriate surgical intervention, overtreatment, and psychological distress from unnecessary follow up.

Our study aimed to evaluate p16 and Ki67 expression in normal cervical tissue, CIN1, CIN2/3, and invasive carcinoma.

Materials and Methods

The study was approved by the institutional Ethics Committee. Inclusion criteria were women who came to gynecologic clinic of the Faculty of Medicine Vajira Hospital, Navamindradhiraj University during March 2012 and April 2015, had abnormal cytology or high-risk HPV as an indication for colposcopy and biopsy, or direct tissue biopsy from gross cervical lesion. The histology of benign squamous epithelium, CIN 1, CIN 2, CIN 3 and invasive carcinoma were selected. Exclusion criteria were those who had inadequate subsequent cervical tissue for histologic diagnosis or unavailable tissue blocks to process with the immunohistochemical study.

Hematoxylin and Eosin stained pathological slides of all cases included in the study were reviewed by one author who is a pathologist experienced in gynecologic pathology (N.P.). Immunohistochemical staining was performed on 3-□m sections of formalin-fixed, paraffin-embedded tissue section. In brief, the paraffin-embedded sections were mounted on slides and dried by microwave for 15 minutes. The tissues were deparaffinized and rehydrated with xylene and ethanol, blocked endogenous peroxidase with 3% H_2O_2 for 20 minutes. The sections were pretreated with citrate buffer, pH 6.0 in a microwave for 13 minutes and incubated in protein blocking solution for 10 minutes. All slides were incubated with a 1:10 dilution of primary p16 (Ventan. a, Medical Systems, USA), 1:200 dilution of primary Ki67 (Dako, Denmark) for 120 minutes at room temperature followed by secondary antibody (Envision kit, Novocastra, Newcastle, UK) for 30 minutes and finally with diaminobenzidine for 6 minutes. All samples were counterstained with Mayer's hematoxylin for 2 minutes and mounted in coated glass.

Expression of immunostaining slides was interpreted independently by two authors (K.K. and N.P.) under a transmission light microscope. Positive p16 expression was interpreted with a diffuse staining in both nuclear and cytoplasm of basal, parabasal, with or without superficial cells (Figure 1a). Unstained, focal or sporadic epithelial staining was considered as negative (Figure 1b) (Hariri and Oster, 2007; Kostopoulou el al., 2011; Aslani et al., 2013). Positive Ki67 expression was diagnosed with nuclear stain in the intermediate and superficial cells (Figure 2a). Ki67 staining in basal or parabasal cell was considered as negative (Figure 2b) (Evanthia et al., 2012; Aslani et al., 2013). Negative control was performed in the same tissue without primary antibodies.

The results of positive or negative immunostaining among the first 30 cases were compared between the two authors for inter-observer and intra-observer reliability. For any discordant interpretation, the two authors would study the immunostaining slides together for the adjustment. After this, all cases would be interpreted independently. Inter-observer reliability was analyzed again.

The Kappa values of intra-observer reliability of the first 30 cases were 0.733 and 0.667 for p16 expression and 0.862 and 0.796 for Ki67 expression. The corresponding inter-observer Kappa values of the first 30 cases were 0.634 for p16 and 1.000 for Ki67. From the total cases studied, the Kappa values of inter-observer were 0.659 for p16 and 0.808 for Ki67. A few cases with discordant result were studied together until reaching a consensus for a final result of each stain.

Data collected were age, types of abnormal cervical cytology and histopathology. Data were analyzed by descriptive statistics. Number and percentage were used to describe categorical variables, and mean and range were used for continuous variables. The expression of p16 and Ki67 staining in association with the histopathologic results were compared using Chi-square test. A P value < 0.05 was regarded as significant. Sensitivity, specificity, accuracy, PPV and NPV of each marker to detect ≥ CIN2 were also determined. Data were analyzed were performed using IBM SPSS statistics version 22.

Results

Among 243 cases included in the study, mean age of the women was 40.4 ± 11.0 years. The review of pathologic slides of all 243 cases revealed consistent histopathologic results with the primary diagnoses: 53 non-dysplastic lesions, 106 CIN2, 61 CIN2/3 and 23 invasive carcinomas. The histopathology of cervical tissues according to the type of preceding cervical cytology is shown in Table 1.

Our study demonstrated P16 and Ki67 expression in 85 cases (35.0%) and 99 cases (40.7%) respectively. The rates of expression were in descending order from invasive carcinoma, CIN2/3, CIN1, and non-dysplastic lesions (Table 2). The expression of p16 was 91.3% in invasive

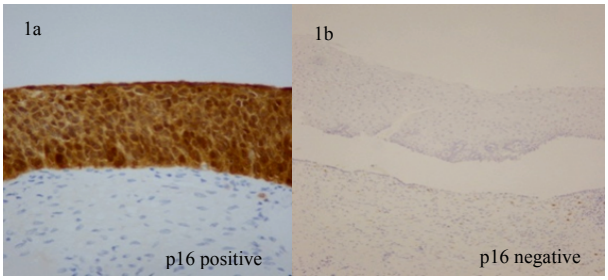

Figure 1. Immunostaining of p16

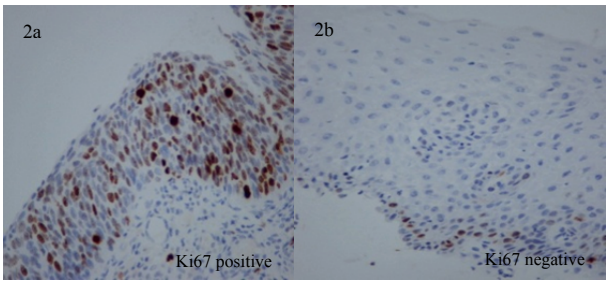

Figure 2. Immunostaining of Ki67

carcinoma, 78.7% in CIN2/3, 10.4% in CIN1, and 9.4% in non-dysplastic lesions. In the same direction, Ki67 expression was found in all invasive carcinoma, 75.4% in CIN2/3, 22.6% CIN1, and 11.3% non-dysplasia. We found the expression of p16 and Ki67 of were significantly different between CIN2/3 vs CIN1 (p values <0.001 both), and of only Ki67 between invasive carcinoma vs CIN2/3 (p value 0.008). The expression of both p16 and Ki67 were not significant different between CIN1 vs non-dysplasia (p values 1.000 and 0.130, respectively), and p16 expression of invasive carcinoma vs CIN2/3 (p value 0.219). We also studied the expression of p16 in combination Ki67. The results are shown in Table 3.

We founded 23 cases of ≥ CIN 2 having negative immunohistochemical expression (false negative): 2 cases of invasive carcinoma and 6 cases of CIN2/3 had negative p16; 8 cases of CIN2/3 had negative Ki67; and 7 cases

Table 1. Histopathology According to the Cytology Distribution (N=243)

Cytology	Histopathology			
	Non-dysplasia	CIN1	CIN2/3	Invasive carcinoma
	n=53 (%)	n=106 (%)	n=61 (%)	n=23 (%)
Negative cytology, positive HR-HPV (n= 22)	11 (50.0)	11 (50.0)	-	-
ASC-US (n=35)	16 (45.7)	14 (40.0)	5 (14.3)	-
LSIL (n=117)	23 (19.7)	78 (66.7)	16 (13.7)	-
HSIL (n=40)	-	1 (2.5)	33 (82.5)	6 (15.0)
ASC-H (n=11)	3 (27.3)	2 (18.2)	5 (45.5)	1 (9.1)
AGC-NOS (n=1)	-	-	1 (100)	-
AGC-FN (n=1)	-	-	-	1 (100)
CIS (n=1)	-	-	-	1 (100)
SCC (n=1)	-	-	1 (100)	-
Adenocarcinoma (n=1)	-	-	-	1 (100)
No cytology (only biopsy) (n=13)	-	-	-	13 (100)

CIN, cervical intraepithelial neoplasia; HR-HPV, high risk human papilloma virus; ASC-US, atypical squamous cells of undetermined significance; LSIL, low grade squamous cell intraepithelial lesion; HSIL, high grade squamous cell intraepithelial lesion; ASC-H, atypical squamous cells cannot exclude HSIL; AGC-NOS, atypical glandular cells not otherwise specified; AGC-FN, atypical glandular cells favor neoplastic; CIS, carcinoma in situ; SCC, squamous cell carcinoma

Table 2. Results for p16 and Ki67 expression according to histopathology (N=243)

Histopathology/Cytology	Positive p16 expression	Positive Ki67 expression	P values
	n (%)	n (%)	
Non-dysplasia	5 (9.4)	6 (11.3)	
CIN1	11 (10.4)	24 (22.6)	<0.001
CIN2/3	48 (78.7)	46 (75.4)	
Invasive carcinoma	21 (91.3)	23 (100)	

CIN, cervical intraepithelial neoplasia; P values < 0.05

Table 3. Results of Dual p16 and Ki67 Expression According to Histopathology (N=243)

	Immunostain of p16 and Ki67			
p16	Negative	Positive	Negative	Positive
Ki67	Negative	Negative	Positive	Positive
	n (%)	n (%)	n (%)	n (%)
Histology				
Non-dysplasia	44 (83.0)	3 (5.7)	4 (7.6)	2 (3.8)
CIN1	76 (71.7)	6 (5.7)	19 (17.9)	5 (4.7)
CIN2	7 (11.5)	8 (13.1)	6 (9.8)	40 (65.6)
Invasive carcinoma	0	0	2 (8.7)	21 (91.3)

CIN, cervical intraepithelial neoplasia

Table 4. Diagnostic Performance of p16 and Ki67 to Determine ≥ CIN 2

Immunostaining positive	Sensitivity (%) (95%CI%)	Specificity (%) (95%CI%)	Accuracy (%) (95%CI%)	PPV (%) (95%CI%)	NPV (%) (95%CI%)
p16	84.5 (76.3-92.6)	90.5 (83.1-97.8)	86.8 (81.1-92.4)	93.4 (88.1-98.7)	78.6 (67.6-89.6)
Ki67	82.1 (73.4-90.7)	88.6 (80.6-96.5)	84.6 (78.8-90.6)	92.0 (86.2-97.8)	75.8 (64.2-87.3)
Both p16 and Ki67	72.6 (61.6-83.6)	96.2 (91.8-100)	81.7 (75.2-88.1)	96.8 (93.0-100)	68.9 (56.5-81.3)
Either p16 or Ki67	91.6 (85.7-97.4)	83.0 (72.7-93.3)	88.3 (83.0-93.6)	89.5 (83.0-96.0)	86.2 (77.0-95.4)

CIN, cervical intraepithelial neoplasia; PPV, positive predictive value; NPV, negative predictive value; CI, confidence interval

Table 5. p16 and Ki67 Expression in Cervical Intraepithelial Lesion and Report in the Literature

Reference	n	Non-dysplasia	CIN1	CIN2/3	Invasive carcinoma	Criteria for positive staining
p16 Expression						
Volgareva et al., 2004	197	8.1%	37.2%	45.2%	96.9%	Nuclear and/or Cytoplasm
Wang et al., 2004	292	32.7%	72%	94.7%	NA	Any reaction of stain
Murphy et al., 2005	176	0	100%	98.7%	100%	Nuclear or cytoplasm
Benevolo et al., 2006	100	NA	31%	95.2%	100%	Nuclear and cytoplasm
Ishikawa et al., 2006	141	0	24.5%	87.5%	100%	Moderate and strong stain in neoplastic lesion
Queiroz et al., 2006	60	9.1%	66.6%	93.4%	100%	Nuclear and cytoplasm
Hariri & Oster, 2007	190	6.0%	71.4%	100%	NA	Nuclear and cytoplasm Continuous basal and parabasal
Aslani et al., 2013	77	1.8%	50%	100%	NA	Nuclear and cytoplasm Continuous basal and parabasal
Our study	243	9.4%	10.4%	78.7%	91.3%	Nuclear and cytoplasm Continuous basal and parabasal
Ki67 Expression						
Keating et al., 2001	74	7.7	71.4	83.3	NA	Nuclear stain above parabasal
Agoff et al., 2003	363	10.1	60.0	88.7	92.1	Nuclear stain above 1/3 of epithelium
Conesa et al., 2009	150	23.0	48.0	89.0	100	Nuclear stain above parabasal
Walts & Bose., 2009	136	0	39.1	98.0	NA	Nuclear stain > 25% of epithelium
Cavalcante et al., 2012	72	5.5	32.0	74.0	NA	Nuclear stain above 1/3 of epithelium
Jackson et al., 2012	91	9.0	23.9	83.3	NA	Nuclear stain above parabasal
Our study	243	11.3	22.6	75.4	100	Nuclear stain above parabasal

CIN, cervical intraepithelium neoplasia; NA, not available

were negative both markers. On the other hand, 39 cases of CIN1 or non-dysplasia had positive expression (false positive): 6 cases of CIN1 and 3 cases of non-dysplasia had positive p16; 19 cases of CIN1 and 4 cases of non-dysplasia had positive Ki67; 5 cases of CIN1 and 2 cases of non-dysplasia had positive both markers.

When we studied the performance of p16 and Ki67 in determining the clinical significant lesions or ≥ CIN 2, the results are shown in Table 4. The sensitivity was highest at 91.6% (95%CI, 85.7%-97.4%) when either p16 or Ki67 was positive. On the other hand, the specificity was highest when both markers were positive, 96.2% (95%CI, 91.8%-100%).

Discussion

Histopathology is a gold standard for diagnosis of squamous intraepithelial lesions and invasive carcinoma. However, the pathologist may be reluctant to make a diagnosis in some cases with equivocal pathologic features.

Previous studies have evaluated immunohistochemical expression of biomarkers in cervical intraepithelial lesions as an adjunct for a diagnosis of cervical squamous intraepithelial lesion and invasive carcinoma. Our study found p16 expression in 91% of invasive cancer, 78% CIN2/3, only 10% in CIN1 and 9% in non-dysplasia. Other previous studies reported p16 in 80% to100% in invasive carcinoma, 45% to 100% in CIN2/3, and 0% to 15% in non-dysplasia (Volgareva et al., 2004; Wang et al.,2004; Murphy et al., 2005; Benevolo et al., 2006; Ishikawa et al., 2006; Queiroz et al., 2006; Hariri & Oster, 2007; Aslani et al., 2013). The variation of expression rates may partly depend on the criteria defining positive expression.

Most of other studies which found higher rate of p16 expression than our study used non-rigid criteria in defining positivity compared to our study. Three studies used criteria of nuclear or cytoplasmic staining as positive (Wang et al.,2004; Murphy et al., 2005; Queiroz et al., 2006) while our study required both nuclear and continuous diffuse cytoplasmic staining of the cells in basal and parabasal as positive. However, other studies which used the same criteria as our study still demonstrated higher p16 expression than our study, 50% or 70% compare to 10%, respectively (Hariri & Oster, 2007; Aslani et al., 2013). One of the two studies had follow-up data which showed persistent or progressive CIN lesions in a large number of patients (23 cases progressed and 6 cases persisted). The authors even remarked that their high rate of p16 expression in CIN1 might be due to an underestimation of CIN2/3 to CIN1 at the beginning (Hariri & Oster, 2007). One study which found higher rate of p16 expression in CIN and invasive cancer also found higher rate of expression in non-dysplasia (32%) than other studies (0% to 15%) or our study (9%) (Wang et al., 2004). This might lie on the criteria used in that study.

Immunopositivity for Ki67, marker for cell proliferation, linearly increase as the CIN grade is higher (Nam et al., 2008; Kim et al., 2011). Our study found Ki67 expression in 100% of invasive cancer, 75% CIN2/3, only 22% in CIN1 and 11% in non-dysplasia. These were concordant with previous studies which found Ki67 in 90% to100% in invasive carcinoma, 20% to 70% in CIN2/3, 70% to 90% in CIN1, and 0% to 20% in non-dysplasia (Keating et al., 2001; Agoff et al., 2003; Walts & Bose., 2008; Conesa et al., 2009; Cavalcante et al., 2012; Jackson et al., 2012).

The expression of p16 and Ki67 in our study was significant different between CIN2/3 vs CIN1 and Ki67

between invasive carcinoma vs CIN2/3. The rates of 16 and Ki67 expressions were directly associated with the severity of cervical lesions but should be interpreted result with caution because in our study had false positive in CIN1 or non-dysplasia may be result of inflammation or infection of HR-HPV. The possible reason for lower expression (false negative) in cases of ≥ CIN 2 may be caused by low risk-HPV because the affinity of E7 protein of low risk-HPV is much lower than of HR-HPV or the tumor extensively necrotic and decrease detection of HPV.

Sensitivity was highest at 91.6% when either p16 or Ki67 positive and specificity were highest 96.2% when both markers were positive. The improvement of sensitivity and specificity when both stains were used together was also demonstrated in previous study which showed high sensitivity and specificity or 94% and 90% respectively using both tests (Van et al., 2007).

This study had some limitations. Being a retrospective study, the specimens were taken from samples remote in the past. Another limitation was our study had no information of HPV infection. These data would certainly assist further in interpretation for the correlation of especially the low- or high-risk groups and immunohistochemical findings. A further prospective study with more appropriate and accurate data should be conducted.

In conclusion, p16 and Ki67 expressions were directly associated with the severity of cervical lesions. The highest expression of both markers was found in invasive carcinoma and CIN2/3 and lower in descending order for CIN1. The significant differences in these markers expression may be useful in equivocal histologic features among the cervical intraepithelial lesions.

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