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ORIGINAL ARTICLE

Clinical Implication of p16, Ki-67, and Proliferating Cell Nuclear Antigen Expression in Cervical Neoplasia: Improvement of Diagnostic Accuracy for High-grade Squamous Intraepithelial Lesion and Prediction of Resection Margin Involvement on Conization Specimen

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Background: Cervical intraepithelial neoplasia (CIN) grading is subjective and affected by substantial rates of discordance among pathologists. Although the use of p16INK4a (p16) staining has been proven to improve diagnostic accuracy for high-grade squamous intraepithelial lesion (HSIL), the clinical evidence for use of Ki-67 and proliferating cell nuclear antigen (PCNA) is insufficient to make an independent recommendation for use, alone or in combination. The primary objective was to evaluate clinical utility of Ki-67 and PCNA in combination with p16 in diagnosing HSIL. Also, we assessed the correlation between expressions of three biomarkers and resection margin status of conization specimen.
Methods: The expressions of p16, Ki-67, and PCNA were evaluated by immunohistochemical methods in 149 cervical tissues encompassing 17 negative lesion, 31 CIN 1, 25 CIN 2, 41 CIN 3, and 35 invasive squamous cell carcinoma. The immunohistochemical staining results were classified into four grades: 0, 1+, 2+ and 3+.
Results: The expression of three biomarkers was positively associated with CIN grade. Ki-67 immunostaining did not increase the accuracy of HSIL diagnosis when combined with p16 immunostaining compared with p16 immunostaining alone. In contrast, combining the staining results for p16 and PCNA (p16 = 3+ and PCNA ≥2+) increased its specificity (66.7% vs. 75.0%, P = 0.031) without decrease of its sensitivity (98.7% vs. 98.7%) for diagnosis of CIN 3 and more sever lesion. Subgroup analysis for conization specimen with CIN 2 and CIN 3 showed that positive Ki-67 immunostaining was an independent risk factor for predicting resection margin positivity (odds ratio = 6.52, 95% confidence interval 1.07-39.64).
Conclusions: We found that the combined use of p16 and PCNA immunostaining enhanced diagnostic accuracy for HSIL. Positive Ki-67 immunostaining was associated with incomplete excision.
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Key Words: Cervical intraepithelial neoplasia, p16INK4a, Ki-67, Proliferating cell nuclear antigen, Conization

INTRODUCTION

Successful screening program has decreased the incidence and mortality of cervical cancer during last decades.[1] The initial purpose of those screening tests is to select patients who are likely to have precancerous lesions and refer them to colposcopy and biopsy. If the colposcopy and biopsy confirms high-grade cervical lesion, those lesions are recommended to be excised by

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conization. Therefore, the final goal of clinical management is to identify and treat high-grade cervical lesion in order to decrease the risk of developing invasive cancer.[2] Recently, the Lower Anogenital Squamous Terminology (LAST) project recommended a two-tiered classification system of low-grade squamous intraepithelial lesion (LSIL) and high-grade squamous intraepithelial lesion (HSIL) for the histopathologic diagnosis.[3] HSIL refers to cervical intraepithelial neoplasia (CIN) 2 and more severe lesion (CIN 2+) and is regarded as a threshold for treatment. Therefore, the exact diagnosis of CIN 2+ is very important in cervical cancer screening program. However, reproducibility of the histopathologic diagnosis in cervical biopsy specimens has often been shown to be limited.[4,5]

Numerous studies have investigated the correlation between various biomarkers and the presence of CIN. p16INK4a (p16) protein indicates over-expression of the viral oncogenes E6 and E7 and hence transformation induced by human papillomavirus (HPV) infections.[6] Several studies have also suggested that adding p16 to the morphologic interpretation of cervical histology can achieve accuracy comparable to an expert panel diagnosis.[7,8] Recently, the LAST project recommended using p16 immunohistochemistry staining in equivocal CIN 2 cases to guide the decision for LSIL or HSIL.[3] Ki-67 is a nuclear protein that is associated with cellular proliferation and has been suggested as a sensitive biological indicator or progression in CIN lesions.[9,10] However, there is still debate on whether the combining p16 and Ki-67 increase diagnostic performance for CIN 2+ compared with p16 alone.[9,11] Proliferating cell nuclear antigen (PCNA) is 29 kDa protein and one of the cyclin family of proteins. It is essential for nucleic acid metabolism as a component of the replication and repair machinery and used as a surrogate marker for cell proliferation.[12] Its expression has been reported to be associated with severity and progression of cervical neoplasia.[13-15]

Numerous studies have revealed that incomplete excision of CIN is a risk factor for treatment failure.[16-19] Therefore, resection margin status on conization specimen is one of major concern for treating physicians. Age, tumor size, disease severity, and depth of conization, and the training level of the gynecologic surgeon have been reported to be associated with margin positivity.[20-24]

In the present study, we conducted immunohistochemical staining for p16, Ki-67, and PCNA in specimens of normal cervix, CIN, and invasive squamous cell carcinoma (SCC). The primary objective was to identify whether the combination of those markers increase clinical performance in detecting CIN 2+ or CIN 3+. In addition, we evaluated correlation between expression of biomarkers and resection margin status of conization specimen with CIN 2+.

MATERIALS AND METHODS

1. Patients and tissue samples

Formalin-fixed paraffin-embedded-samples of cervical lesions, collected from January 2006 to December 2006 and for which sufficient material was left for further analysis, were selected from the files of the Pathology Departments in Seoul National University Hospital. A total of 149 cervical tissues were analyzed for this study. There were 17 negative (11.4%), 31 CIN 1 (20.8%), 25 CIN 2 (16.8%), 41 CIN 3 (27.5%), and 35 invasive SCC (23.5%) (Table 1). CIN samples were selected from the specimen of colposcopy-directed biopsy or conization or simple hysterectomy. All invasive SCC samples were selected from radical hysterectomy specimens. Normal cervical tissues were selected from the specimens of hysterectomy which were performed to treat benign uterine fibroid. The diagnosis of all specimens was reviewed by an experienced gynecologic pathologist (E.S.) only using H&E stained slides. Patient median age was 47 years (range 24-80 years). The use of the tissues for this study was approved by the institution review board of the Seoul National University Hospital.

2. Conization and evaluation of margin status

Conization was performed only with large loop excision of the

Table 1. Frequency of diagnostic criteria according to diagnosis established on H&E stained slides

Specimen type	Diagnosis by H&E					
	Negative	CIN 1	CIN 2	CIN 3	Invasive SCC	Total
Punch biopsy	0 (0)	8 (47.1)	8 (47.1)	1 (5.9)	0 (0)	17 (100.0)
Conization	0 (0)	23 (30.3)	17 (22.4)	36 (47.4)	0 (0)	76 (100.0)
Simple hysterectomy	17 (81.0)	0 (0)	0 (0)	4 (19.0)	0 (0)	21 (100.0)
Radical hysterectomy	0 (0)	0 (0)	0 (0)	0 (0)	35 (100.0)	35 (100.0)
All cases	17 (11.4)	31 (20.8)	25 (16.8)	41 (27.5)	35 (23.5)	149 (100.0)

Values are presented as number (%). CIN, cervical inraepithelial neoplasia; SCC, squamous cell carcinoma.

transformation zone (LLETZ) method and all women had no previous cervical surgery. The indications for conization were abnormal Pap smear and/or colposcopy. LLETZ procedure was performed by one of faculty members at the Seoul National University Hospital. The transformation zone was excised by cutting and coagulation using an Ellman Surgitron (Ellman International Inc., Hewlett, NY, USA) unit with an appropriately sized wire loop and its generator set. Following the excision, additional hemostasis was achieved with a diathermy ball. No endocervical curettage in any patient was performed. Margin positivity of conization specimen was evaluated only in cases of CIN 2+. The ectocervical and endocervical margins were classified as either positive. Margins were reported as involved if the distance between the lesion and the resection surface was < 1 mm.

3. Immunohistochemical analysis

Immunohistochemical analysis was performed on formalin-fixed, paraffin-embedded sections using commercially available antibodies against p16 (Abcam Ltd., Cambridge, UK; diluted 1 : 800), Ki-67 (Dako, Glostrup, Denmark; diluted 1 : 50) and PCNA (Dako; diluted 1 : 300) as primary antibodies, 4 μM paraffin-embedded slides were cut adjacent to H&E section with a microtome and dried overnight at 37°C on a silane coating slide. Samples were deparaffinized in Histoclear solution for 10 minutes and rehydrated with fraded ethanol (100%, 95%, and 90%) and distilled water. For antigen retrieval, sections were immersed in Tris-ethylenediaminetetraacetic acid buffer (pH 9.0) and incubated in a decloacking chamber to 125°C for 3 minutes. Endogenous peroxidase activity was quenched by incubating tissue sections in 3% H_2O_2 for 10 minutes. The sections were incubated with primary antibody for an hour at room temperature. After washing in Tris-buffered saline tween20 (TBST), the sections were treated with an Envision + peroxidase mouse kit (K4001; Dako) for 30 minutes, and then washed with TBST and treated with 3,3'-diaminobensine tetrahydrochloride as the chromofen for 5 minutes, followed by counterstaing with Mayer's hematoxylin.

4. Interpretation for p16/ Ki-67/proliferating cell nuclear antigen expression in biopsy tissues

The slides were evaluated by one experienced gynecologic pathologist (E.S.). All sections of three biomarkers were graded according to the followings scale: 0 (no positive staining of dysplastic cells or only staining in basal layer), 1+ (basal layer staining plus $<$ 10% positive staining of dysplastic cells), 2+ ($>$ 10% but $<$ 50% positive staining of dysplastic cells), and 3+ ($>$ 50% positive staining of dysplastic cells).

5. Statistical analysis

Linear by linear association was used to assess trends of staining intensity with the severity of the H&E diagnosis. Sensitivity, specificity, and Youden's index (YI) (YI = sensitivity + specificity − 100%), as a metric of accuracy, were calculated for H&E diagnoses of CIN 2+ and CIN 3+. McNemar χ^2 was used to test for differences in sensitivity and specificity. Univariate and multivariate logistic regression and the chi-squared test with Fisher's correction were used to determine the association between various clinicopathologic parameters and margin status in patients with CIN2+ receiving conization. The Statistical Package for the Social Sciences (SPSS) for Windows, version 12.2 (SPSS Inc., Chicago, IL, USA) was used to analyze all data. For all statistical tests, a value of $P < 0.05$ was considered significant.

RESULTS

Table 1 shows the source of specimens according to the H&E diagnosis. The immunohistochemical staining results are summarized in Table 2 and Figure 1. Both CIN 3 and invasive SCC tissues had diffuse staining for all markers, while varying degree

Table 2. Intensity of p16, Ki-67, and PCNA immunohistochemical staining to H&E diagnosis

Diagnosis by H&E	p16			Ki-67				PCNA			
	0	2+	3+	0	1+	2+	3+	0	1+	2+	3+
Negative	17 (100.0)	0 (0)	0 (0)	17 (100.0)	0 (0)	0 (0)	0 (0)	17 (100.0)	0 (0)	0 (0)	0 (0)
CIN 1	9 (29.0)	12 (38.7)	10 (32.3)	15 (50.0)	8 (26.7)	7 (23.3)	0 (0)	15 (48.4)	4 (12.9)	3 (9.7)	9 (29.0)
CIN 2	4 (16.0)	6 (24.0)	15 (60.0)	9 (36.0)	4 (16.0)	6 (24.0)	6 (24.0)	6 (25.0)	1 (4.2)	6 (25.0)	11 (45.8)
CIN 3	0 (0)	0 (0)	41 (100.0)	0 (0)	6 (15.0)	11 (27.5)	23 (57.5)	0 (0)	0 (0)	6 (14.6)	35 (85.4)
Invasive SCC	0 (0)	1 (2.9)	34 (97.1)	0 (0)	0 (0)	13 (37.1)	22 (62.9)	0 (0)	0 (0)	0 (0)	35 (100.0)

Values are presented as number (% row). PCNA, proliferating cell nuclear antigen; CIN, cervical inraepithelial neoplasia; SCC, squamous cell carcinoma.

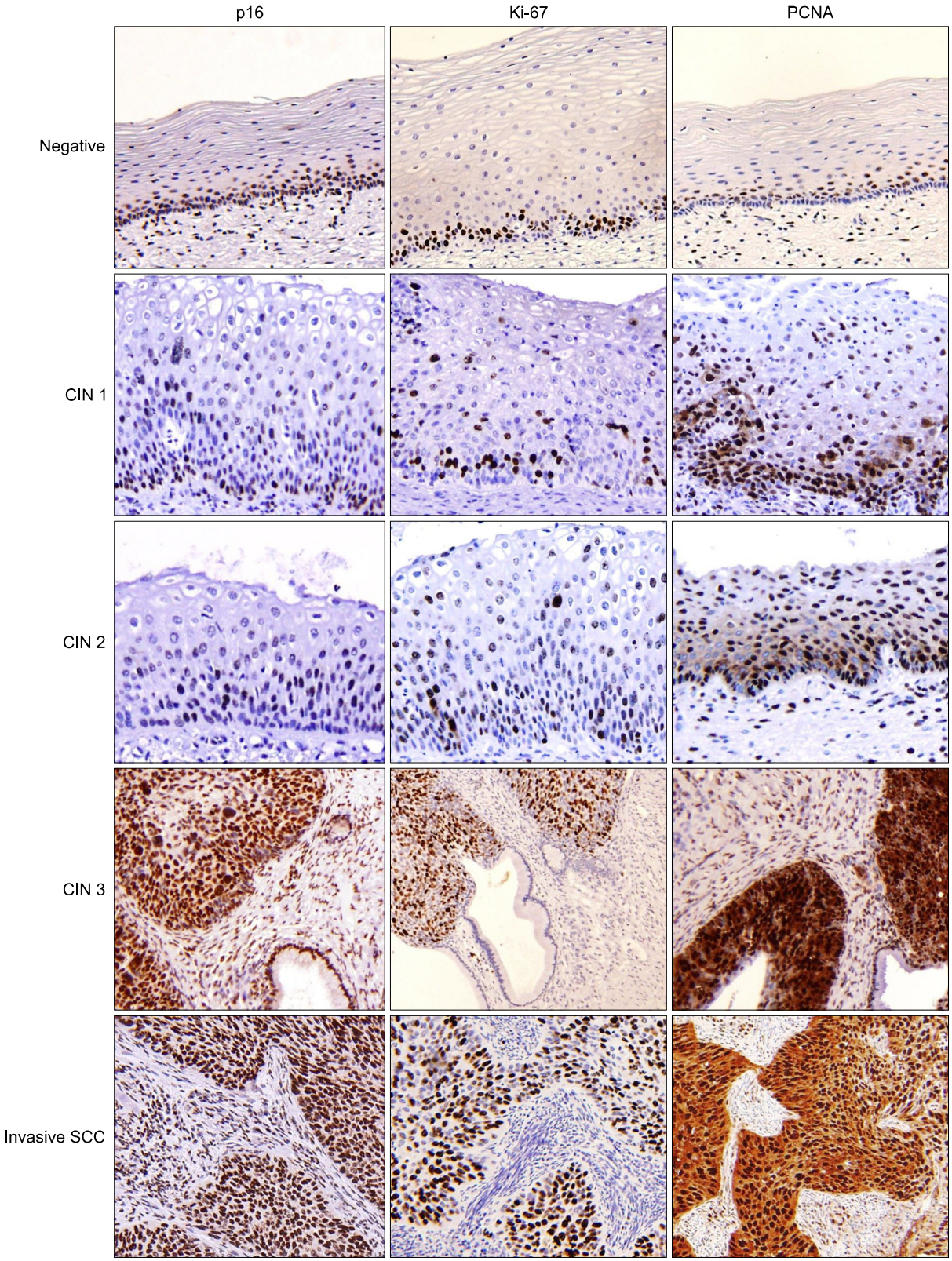

Figure 1. Representative picture of immunohistochemical analysis of p16INK4a (p16), Ki-67, and proliferating cell nuclear antigen (PCNA) expression in cervical tissue (× 100). In a tissue with negative lesion, all markers were stained only in basal layer. Varying degree of staining intensity was observed in cervical intraepithelial neoplasia (CIN) 1 and CIN 2 tissues. Both CIN 3 and invasive squamous cell carcinoma tissues had diffuse staining for all markers.

Table 3. Clinical performance of p16, Ki-67, and PCNA immunostaining in relation to H&E diagnosis of cervical intraepithelial neoplasia grade 2 or more severe (CIN 2+) and CIN 3+

Cutpoint	CIN 2+			CIN 3+		
	Sensitivity	Specificity	YI	Sensitivity	Specificity	YI
p16 ≥2+	96.0	54.2	50.2	100.0	41.1	41.1
p16 = 3+	89.1	79.2	68.3	98.7	65.8	64.4
Ki-67 ≥1+	91.0	68.1	59.1	100.0	56.9	56.9
Ki-67 ≥2+	81.0	85.1	66.1	92.0	73.6	65.6
Ki-67 = 3+	51.0	100.0	51.0	60.0	91.7	51.7
PCNA ≥1+	94.0	66.7	60.7	100.0	52.8	52.8
PCNA ≥2+	93.0	75.0	68.0	100.0	59.7	59.7
PCNA = 3+	81.0	81.3	62.3	92.1	72.2	64.3
p16 = 3+ and Ki-67 ≥2+	79.0	89.4	68.4	90.7	77.8	68.4
p16 = 3+ or Ki-67 ≥2+	91.0	74.5	65.5	100.0	61.1	61.1
p16 = 3+ and PCNA ≥2+	87.0	87.5	74.5	98.7	75.0	73.7
p16 = 3+ or PCNA ≥2+	95.0	66.7	61.7	100.0	51.4	51.4

Values are presented as percent. PCNA, proliferating cell nuclear antigen; CIN, cervical inraepithelial neoplasia; YI, Youden's index (sensitivity + specificity−100%).

of staining intensity was observed in CIN 1 and CIN 2 tissues (Fig. 1). There was a linear relationship between the severity of cervical lesion and the intensity of p16, Ki-67, and PCNA expression ($P < 0.001$ in all markers by Linear by linear association). Strong and diffuse staining (3+) for p16 was observed in 0 (0%) of 17 negative cases, 10 (32.3%) of 31 CIN 1 cases, 15 (60.0%) of 25 CIN 2 cases, 41 (100%) of 41 CIN 3 cases, and 34 (97.1%) of 35 invasive SCC cases. Both Ki-67 and PCNA staining was not detected in all negative cases and varies according to the severity of cervical lesions (Table 2).

We next examined the clinical performance (sensitivity, specificity, and YI) of different positive cutpoints for p16, Ki-67, and PCNA staining, and the various combinations of 2 markers, in relationship to the H&E diagnoses of CIN 2+ and CIN 3+ (Table 3). Increasing the positive cutpoint for p16 staining increased its specificity and accuracy for CIN 2+ and CIN 3+. Therefore, 3+ was used as a cutpoint for p16. Increasing the positive cutpoint for Ki-67 increased its specificity and decreased its sensitivity. Similar pattern was observed for PCNA. Consequently, 2+ was used as a cutpoint for both Ki-67 and PCNA. In comparison to p16 alone (= 3+), combining the staining results for p16 and Ki-67 (p16 = 3+ and Ki-67 ≥2+) decreased its sensitivity (89.1% vs. 79.0%; $P = 0.002$) and increased its specificity (79.2% vs. 89.4%; $P = 0.063$) for CIN 2+. Similarly, combining the staining results for p16 and Ki-67 (p16 = 3+ and Ki-67 ≥2+) decreased its sensitivity (98.7% vs. 90.7%; $P = 0.031$) and increased its specificity (65.8% vs. 77.8%; $P = 0.004$) for CIN 3+. The difference of YI was minimal (68.3% vs. 68.4% for CIN 2+, 64.4% vs. 68.4% for CIN 3+).

By comparison, combining the staining results for p16 and PCNA (p16 = 3+ and PCNA ≥2+) revealed to enhance the clinical performance. Compared with p16 staining alone, combining the staining results for p16 and PCNA (p16 = 3+ and PCNA ≥2+) had higher specificity (79.2% vs. 87.5%; $P = 0.125$) and similar sensitivity (89% vs. 87%; $P = 0.5$) for CIN 2+. Moreover, combining the staining results for p16 and PCNA (p16 = 3+ and PCNA ≥2+) increased its specificity (66.7% vs. 75.0%; $P = 0.031$) without decrease of its sensitivity (98.7% vs. 98.7%) for CIN 3+, resulting in increase of YI (64.4% vs. 73.7%).

Next, we tried to evaluate whether these biologic markers have clinical implication for predicting resection margin involvement on conization specimen with CIN 2+. Among 53 patients who received conization for CIN2+, margin status could be obtained from the pathologic report of conization in 52 patients (98.1%). Twenty-eight of 52 patients (53.8%) had positive margin involvement by CIN 2+ on conization specimen. Eight patients (15.4%) had exocervical margin involvement and 22 patients (42.3%) had endocervical margin involvement. Age and histology were statistically different according to resection margin status (Table 4). The p16 and Ki-67 staining scales were different according to overall resection margin status (Table 4). In multivariate logistic regression analysis adjusting age and histology, high Ki-67 immunostaining (more than 2+) was associated with risk of positive resection margin involvement (odds ratio = 6.52, 95% confidence interval 1.07-39.64).

Table 4. Clinicopathologic risk factors for overall (endocervical + ectocervical) resection margin positivity on conization (n = 52)

	Negative margins (n=24)	Positive margins (n=28)	*P*-value	Univariate analysis, OR (95% CI)	Multivariate analysis, OR (95% CI)
Age (yr)	38 (24-75)	45.5 (28-67)	0.040[a]	1.05 (0.99-1.10)	1.06 (1.00-1.13)
Cytology			0.438[a]		
Low grade	12 (52.2)	11 (47.8)		1.00 (reference)	(–)
High grade	12 (41.4)	17 (58.6)		1.55 (0.51-4.64)	(–)
Histology			0.029[a]		
CIN 2	11 (68.8)	5 (31.3)		1.00 (reference)	1.00 (reference)
CIN 3	13 (36.1)	23 (63.9)		3.89 (1.11-13.68)	1.49 (0.31-7.08)
Glandular extension			0.115[a]		
No	10 (62.5)	6 (37.5)		1.00 (reference)	(–)
Yes	14 (38.9)	22 (61.1)		2.62 (0.78-8.82)	(–)
p16			0.016[b]		
0, 1+, 2+	5 (100)	0 (0)		(–)	(–)
3+	19 (40.4)	28 (59.6)		(–)	(–)
Ki-67			0.020[a]		
0, 1+	10 (71.4)	4 (28.6)		1.00 (reference)	1.00 (reference)
2+, 3+	13 (35.1)	24 (64.9)		4.62 (1.21-17.66)	6.52 (1.07-39.64)
PCNA			0.162[b]		
0, 1+	4 (80.0)	1 (20.0)		1.00 (reference)	(–)
2+, 3+	19 (41.3)	27 (58.7)		5.68 (0.59-54.94)	(–)

Values are presented as median (range) or number (%). OR, odds ratio; CI, confidence interval; CIN, cervical inraepithelial neoplasia; PCNA, proliferating cell nuclear antigen. [a]Chi-squared test. [b]Fisher's exact test.

DISCUSSION

Various numbers of potential biomarker has been evaluated for diagnostic usefulness in the evaluation of cervical cancer and its precursors. In the present study, we compare and contrast the expression patterns of p16, Ki-67, and PCNA in 17 patients with normal epithelium, 97 patients with CIN, and 35 patients with invasive SCC and assess their diagnostic usefulness as biomarkers of cervical neoplasia. Our study found that all three biomarkers showed a linear correlation according to disease severity and combining p16 and PCNA had higher specificity without compromising sensitivity compared with p16 alone for diagnosing CIN 2+. In addition, subgroup analysis for conization specimen with CIN 2+ showed that positive Ki-67 expression was an independent risk factor for predicting resection margin positivity.

Patient management is highly contingent on histopathologic diagnosis.[2] Since lack of reproducibility was substantially high especially in diagnosis CIN 2+ when using H&E morphology alone, there has been much effort to increase diagnostic accuracy and reproducibility using various biomarkers. Recently, a systematic review and meta-analysis concluded that improved interobserver agreement of the diagnosis of CIN 2+ with the conjunctive use of H&E morphology with p16 immunostaining compared with H&E morphology alone.[5]

Recently, the College of American Pathologists and the American Society for Colposcopy and Cervical Pathology included p16 immunohistochemistry in their revised nomenclature for lower genital tract lesions.[3] They concluded that only p16, a biomarker that is recognized in the context of HPV biology to reflect the activation of E6/E7 driven cell proliferation, had sufficient evidence upon which to marker recommendations regarding use in lower anogenital tract squamous lesions. They also recommended that strong and diffuse block positive p16 results support a categorization of precancerous disease. In our study, 3+ staining for p16 was defined as the cutpoint, which is accordant with their recommendation.

Several studies have been evaluated clinical performance of combination of biomarkers in cervical neoplasia. In 2007 Van Niekerk et al.[9] reported that each p16 and Ki-67 had sensitivities and specificities for the diagnosis of HSIL versus LSIL and normal of approximately 85% to 90% and this improved by 5% for both sensitivity and specificity when used together (p16 sensitivity 90%, specificity 85%; Ki-67 sensitivity 89%, specificity 87%; together sensitivity 94%, specificity 90%). In 2010, Galgano et al.[11] conducted a community-based and population-based evaluation in almost 1,500 consecutive cervical biopsies in order to evaluate the utility of HPV L1, p16, and Ki-67 immunohistochemical staining for improving diagnostic accuracy. Consensus diagnosis was achieved strictly by three pathologist using H&E

stains only. This well designed study showed that the addition of p16 to H&E increased sensitivity for a consensus diagnosis of both CIN 2+ and CIN 3+. Specificity was decreased with the addition of p16. When combining p16 and Ki-67, the overall improvement of performance (sensitivity and specificity) was minimal when compared with the p16 result alone. Our result is accordant with the result of Galgano et al.'s study.[11] Combining p16 and Ki-67 did not improve diagnostic performance compared with p16 alone in terms of YI (Table 3). Non-significant increase of specificity may be attributed to decrease of sensitivity. In accordance with these result, the LAST project stated that the routine use of Ki-67 to p16 immunostaining is not recommended in diagnosis of CIN 2+.[3]

In contrast to Ki-67, the additional use of PCNA to p16 statistically significantly increased its specificity for CIN 3+ without any decrease of its sensitivity in present study. Also for CIN 2+, there was non-significant trend of increase of its specificity. As indicated in previous study,[11] the use of p16 accompanies decrease of specificity for consensus diagnosis. In our study, substantial number of CIN 1 had p16 positivity, which refers to false positive. Combining PCNA with p16 decreased false positive rate, resulting in improvement of overall accuracy.

Very recently, these biomarkers have been also evaluated for use in liquid-based cytology samples to increase diagnostic accuracy of Pap cytology for detecting CIN 2+.[25-29] In a large prospective diagnostic screening study, p16/Ki-67 dual-stained cytology showed higher sensitivity than Pap cytology for detecting CIN 2+, with a comparable specificity.[29] Considering findings of current study, it would be interesting to evaluate whether p16/PCNA dual-stained cytology is superior to p16 stained cytology alone in detecting CIN 2+.

The additional analysis for resection margin status of conization specimen suggested another clinical implication of these biomarkers. Although p16 staining was statistically associated with margin positivity, its predictive value is limited because most specimens with CIN 2+ showed p16 positivity (Table 4). Positive Ki-67 staining has predictive value for resection margin positivity independent of age and disease severity. It has been reported that large tumor size is associated with margin positivity.[24] Although we could not check the tumor size, it can be inferred that large tumor may have strong Ki-67 staining, which stands for enhanced proliferation.

The major limitation of this study is the absence of consensus diagnosis of biopsy specimen as well as the retrospective nature and small number of cases. Since only one experienced gynecologic pathologist reviewed all H&E slides, there exists the substantial chance of misclassification in diagnosing CIN. However, our study demonstrated for the first time that the use of PCNA was superior to it of Ki-67 when it was combined with p16. The problem of low specificity of p16 staining in diagnosis of CIN 2+ may be solved by additional use of PCNA staining.

Both Ki-67 and PCNA are regarded as biomarker reflecting cellular proliferation. PCNA is known to be associated with HPV induced carcinogenesis.[12,30] The use of PCNA in addition to p16 may enhance diagnostic accuracy for CIN 2+. However, further large study with consensus diagnosis is warranted to demonstrate it. In addition, positive Ki-67 immunostaining was associated with incomplete excision.

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CONFLICTS OF INTEREST

No potential conflicts of interest were disclosed.

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