Biomarker expression in cervical intraepithelial neoplasia: potential progression predictive factors for low-grade lesions

- TITLE PAGE -

Biomarker expression in cervical intraepithelial neoplasia: potential progression predictive factors for low-grade lesions

Satoru Ozaki [a,*], Yoh Zen [a], Masaki Inoue[b]

[a]Division of Pathology, Kanazawa University Hospital, Kanazawa, Japan; [b]Department of Obstetrics and Gynecology, Kanazawa University, Graduate School of Medical Science, Kanazawa, Japan.

***Corresponding author:** Satoru Ozaki, PhD; Division of Pathology, Kanazawa University Hospital, 13-1 Takaramachi, Kanazawa 920-8641; Tel: +81 (0)76 265 2027; Fax: +81 (0)76 234 4278; E-mail: s-ozaki@med.kanazawa-u.ac.jp (S. Ozaki)

Abbreviations: CIN, cervical intraepithelial neoplasia; HPV, human papilloma virus; p16, p16INK4a; MCM2, minichromosome maintenance protein 2; TPO2A, DNA topoisomerase IIα; HCII, hybridcapture II: LSIL, low-grade squamous intraepithelial lesion; HSIL, high-grade squamous intraepithelial lesion.

Key words: Cervical cancer; p16; ProEx C; HPV; carcinogenesis.

Running title: ProEx C and p16 expressions in CIN

Summary

The aim of this study was to reveal whether three biomarkers (p16, ProEx C, and HPV DNA) are useful in the diagnosis of cervical intraepithelial neoplasia and whether they could predict disease progression of cervical intraepithelial neoplasia-1. We analyzed 252 cervical specimens: non-dysplastic mucosa (n=9), cervical intraepithelial neoplasia (n=229), and squamous cell carcinoma (n=14). Immunostaining for p16 and ProEx C, and the hybridcapture II assay for HPV DNA were performed. Expression of p16 and staining for ProEx C were significantly higher in intraepithelial neoplasia-2/3 (96% to 100%) than in non-dysplastic mucosa (11%) or intraepithelial neoplasia-1 (40% to 53%). HPV DNA was detected in 69% of intraepithelial neoplasia-1, 95% of intraepithelial neoplasia-2, and 100% of intraepithelial neoplasia-3. Of 99 patients with intraepithelial neoplasia-1 for whom follow-up data was available, 62 (73%) showed spontaneous regression, 17 (20%) demonstrated persistent low-grade lesion, and 7 (7%) progressed to intraepithelial neoplasia-2/3. Expressions of p16 and staining with ProEx C were significantly higher in the progression group than in the regression group. Testing for p16 and ProEx C were sensitive (86%) and moderately specific (60% and 61%, respectively) in predicting the progression of cervical intraepithelial neoplasia-1. HPV DNA testing was highly sensitive (100%) but less specific (37%). In conclusion, this study revealed that p16 and ProEx C are useful biomarkers for the diagnosis of cervical intraepithelial neoplasia, and have potential as predictors of progression of low-grade lesions.

1. Introduction

It is well established that most cervical cancers develop from non-invasive dysplastic lesions known as cervical intraepithelial neoplasia (CIN) [1,2]. There are three categories of CIN (CIN-1, -2, and -3) based on the degree of dysplasia [3], and infection with human papilloma viruses (HPV) is closely involved in the development and progression of these lesions [4]. Most developed countries have national or public screening systems to detect cervical dysplastic lesions at an early stage by cytology or HPV testing [5]. Precise diagnosis and prediction of progression risk are important issues in the clinical management of patients with CIN.

Histology is the gold standard in the diagnosis of CIN. However, the small size of biopsy specimens, potential sampling error, and heterogeneous distribution of dysplastic lesions can result in over- and under-diagnosis. Another difficulty is the existence of several CIN mimics such as immature squamous metaplasia [6,7]. Several biomarkers suitable for use on paraffin sections have been established to avoid these diagnostic inaccuracies. The protein p16INK4a (p16) is a cyclin-dependent kinase inhibitor which down-regulates progression through the G1-S transition checkpoint of the cell cycle [8]. Immunostaining for p16 has revealed overexpression in cervical squamous epithelium infected with high-risk HPV [9,10]. Two newer biomarkers include the minichromosome maintenance protein 2 (MCM2) and DNA topoisomerase IIα (TOP2A) [11], both of which are involved in DNA replication in the S phase of the cell cycle. ProEx C is a cocktail of two monoclonal antibodies that targets the expression of these two proteins [12].

CIN2 and CIN3 have a considerable risk of progression to invasive cancer and are therefore usually treated by conization or other less invasive procedures. In contrast, approximately 80% of CIN1 lesions regress spontaneously [13], but they must be periodically followed up by cytology and biopsy to detect progression to higher grade lesions. At the moment, there is no consensus regarding the natural history of CIN1. Nonetheless, it seems important, not only for patient care but also from the viewpoint of medical economics, to establish a triage strategy for patients with CIN1.

In this study, we examined expressions of biomarkers in cervical premalignant lesions to determine which markers were useful in diagnosis and which could help in predicting the progression risk of CIN1.

2. Patients and Methods

2.1. Patients

A total of 252 cervical specimens, consisting of biopsy (n=205), conization (n=34), and hysterectomy (n=13), were retrieved from the histopathology file of Kanazawa University Hospital, from 2005 to 2008. They consisted of non-dysplastic mucosa (n=9), CIN1 (n=123), CIN2 (n=57), CIN3 (n=49), and invasive squamous cell carcinoma (n=14). Type of specimens and mean age of patients in each diagnostic category are shown in Table 1. Before starting this study, all cases were independently reviewed by two authors (S.O. and Y.Z.). When there were disagreements about classification, a consensus diagnosis was made through discussion with a multi-header microscope.

2.2. Immunohistochemistry

We performed p16 and ProEx C immunostaining using one representative section selected from each case using the EnVision+ system (Dako Cytomation, Glostrup, Denmark). The deparaffinized sections were microwaved twice in ethylenediaminetetraacetic acid (EDTA) buffer, pH 9.0, for 5 minutes at a 2-minute interval. After the blocking of endogenous peroxidase with REAL Peroxidase-Blocking Solution (Dako Cytomation), the deparaffinized sections were incubated for one hour at room temperature with primary monoclonal antibodies: anti-p16 (Dako Cytomation; clone E6H4; dilution 1:25) and ProEx C (BD, Franklin Lakes, NJ, USA; MCM2 clone 26H16.19 and 27C5.6; TOP2A clone SWT3D1; ready to use). The sections were then incubated at room temperature for 1 hour with goat anti-mouse immunoglobulins conjugated to peroxidase labeled-dextran polymer (EnVision+; Dako Cytomation). The reaction products were developed by immersing the section in a 3,3'-diaminobenzidine tetrahydrochloride (DAB) solution. Nuclei were lightly counterstained with hematoxylin.

The expression of p16 was evaluated as positive or negative according to the distribution of positive cells: positive, lower 1/4 of the epithelium or more; negative, less than lower 1/4. ProEx C staining was also assessed as positive (lower 3/4 of the epithelium or more) or negative (less than lower 3/4). Presence or absence of ProEx C staining in the basal layer was also recorded in each case. Expressions of ProEx C in basal and non-basal layer were separately examined. Positive basal staining meant the

expression in the basal layer with or without non-basal layer staining. Similarly, cases with basal positive / non-basal positive or basal negative / non-basal positive were determined as positive for non-basal staining. If staining was patchy rather than a complete band, the highest position of strong evident staining was evaluated based on the classification described above. Focal weak ambiguous staining was not considered.

2.3. HPV DNA testing

The hybridcapture II (HCII) assay for HPV DNA testing was performed with brushed sample taken just before the biopsy (biopsy cases) or at the time of cytology (surgical cases). The assay was performed using a HPV DNA HCII kit (Mitsubishi Chemical Medience, Tokyo, Japan), which is suitable for the detection of HPV types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and 68 (all medium or high risk subtypes for cervical cancer). HPV subtypes cannot be specified in this test. This assay was performed only for CIN and carcinoma cases.

2.4. Follow-up study

Follow-up data of the patients with CIN1 were retrospectively examined. Of 123 patients, 5 (4%) underwent conization. After the diagnosis of CIN1, 19 patients (27%) did not undergo cytologic examination or biopsy at our hospital. The remaining 99 patients enrolled in the follow-up study. All follow-up samples including biopsy, cytology, and surgical specimens were reviewed. The starting point was defined as the day that CIN1 was diagnosed in a biopsy, and outcome at the most recent examination

was classified into 3 groups as follows: (1) regression, never diagnosed as CIN2/3 and no dysplastic lesion at the most recent examination; (2) persistent CIN1, never diagnosed as CIN2/3 and remaining as CIN1 on biopsy or low-grade squamous intraepithelial lesion (LSIL) on cytology at the most recent examination; (3) progression to CIN2/3, diagnosed as CIN2/3 on biopsy or high-grade squamous intraepithelial lesion (HSIL) on cytology at least once. In our hospital, a fixed interval for follow up is 6 months, but patients were followed in less than 3 months if there were concerns in colposcopic examination.

2.5. Statistical analysis

Statistical analyses were performed using the Mann-Whitney U or chi-square test for two groups or Tukey's test for more than two groups. Correlations among different biomarker expressions were evaluated according to the concordance rate (%). A probability of $p<0.05$ was considered statistically significant.

3. RESULTS

3.1. Immunostaining

3.1.1. p16

The results of immunohistochemistry are summarized in Table 2. The expression of p16 was seen in one of 9 cases (11%) of non-dysplastic mucosa, and in 54 of 123 cases (44%) of CIN1. Of note was that all but one case of CIN2/3 or carcinoma expressed p16 (Fig. 1). Expression of p16 in CIN2/3 and carcinoma was significantly

more frequent than in non-dysplastic mucosa and CIN1 ($p<0.001$). CIN1 also expressed p16 more frequently than did non-dysplastic mucosa ($p=0.037$).

3.1.2. ProExC

Positive staining for ProEx C was seen in only one case of non-dysplastic mucosa and in 40% of CIN1. Similarly to p16, ProEx C staining was commonly detected in high-grade lesions, and was almost consistent in CIN2/3 and carcinoma (96% to 100%) (Fig. 2A, B). Interestingly, ProEx C staining in the basal layer was also characteristic for high-grade lesions (Fig. 2C, D); it was noted in all but one case of CIN2/3 and carcinoma, 65 of 123 cases (53%) of CIN1, and only one case (11%) of non-dysplastic mucosa. ProEx C staining in either the basal or non-basal layer was noted significantly more frequently in CIN2/3 and carcinoma than in non-dysplastic mucosa and CIN1 (Table 2).

3.2. HPV testing

HCII for HPV was positive in 82 of 119 cases (69%) of CIN1. Positive results were more commonly obtained for CIN2 (95%) and CIN3 (100%) than for CIN1 (both $p<0.001$). All cases of carcinoma were also positive for HPV.

3.3. Correlations among the three biomarkers

As shown in Table 3, the results of immunohistochemistry for p16 and ProEx C were closely correlated with concordance rates of 84% to 87%. The agreement between

HCII and immunohistochemistry was poorer with concordance rates of 70% or less. Forty eight of 123 cases (39%) of CIN1 were positive for both basal ProEx C staining and HCII.

3.4. Follow-up study

Of 99 patients with CIN1 for whom follow-up data was available (follow-up period: range, 42 to 1781 days; median, 511 days), 76 (77%) showed spontaneous regression as evidenced by negative cytology or histology. Among them, 46 (46%) and 27 (27%) patients were persistently negative for more than 1 and 2 years, respectively. Sixteen (16%) patients showed persistent CIN1, and 7 (7%) were found to have histological or cytological progression to CIN2/3. None of the patients with CIN1 in this study progressed to invasive squamous cell carcinoma during the follow-up period.

As shown in Table 4, expression of p16 and staining for ProEx C (non-basal and basal) were significantly higher in the progression group than in the regression group. The predictive value of each biomarker is shown in Table 5. Among patients with p16 expression, 6 (14%) showed histological progression, whereas the progression rate was only 2% in the negative cases (p=0.019). Similarly, patients with ProEx C (both non-basal and basal) staining showed significantly higher progression rate than negative cases (both p=0.016). The staining of p16 or ProEx C (non-basal) was highly sensitive (86%) and moderately specific (60% or 61%, respectively) to predict disease progression. ProEx C staining (basal) and HCII were highly sensitive (100%), but their specificity was less than 50%. Multiple biomarker positivity (both p16/ProEx C [basal],

both p16/ProEx C [non-basal], both ProEx C [basal]/ProEx C [non-basal], and all three markers) showed 86% sensitivity and 61 to 68% specificity.

4. Discussion

The present study can be summarized as follows: (1) Immunostaining for p16 and ProEx C was almost consistently positive in CIN2 and CIN3. (2) Staining for ProEx C at the basal layer was also characteristic for CIN2/3. (3) p16 and ProEx C were potential biomarkers to predict the progression of CIN1. (4) HPV testing was less specific for the progression risk of CIN1.

Like previous reports [14-19], our study confirmed the diagnostic utility of p16 and ProEx C immunostaining for CIN. These tests are especially useful to distinguish between non-dysplastic epithelium and CIN. However, given the high positive rate even in CIN1, they seem less helpful for differentiation between CIN1 and CIN2/3. Another important issue is the expression of these markers in CIN mimics. Immature squamous metaplasia was not examined in this study, but Pinto et al. presented two cases of diagnostically problematic immature metaplasia, both of which were negative on ProEx C staining [15]. Larger studies with many cases of CIN mimics seem necessary to conclude this issue.

There is no consensus on how to evaluate ProEx C staining. Some groups consider the cases with staining of lower 1/3 of the epithelium or more as positive [19], whereas others use a threshold of 1/2 of the epithelial layer [17]. Evaluation is not easy, particularly when positive staining is seen in approximately the lower 1/3 to 1/2 of the

epithelium, which can commonly happen in CIN1. In our experience, ProEx C staining is easier to assess in the basal layer. We therefore suggest that staining in the basal layer might be helpful for the evaluation of borderline cases. The biological nature of MCM2/TOP2A expression detectable by ProEx C staining in the basal layer is unknown. Considering the cancer stem cell theory [20,21], the following theory may be reasonable. In the non-dysplastic cervical mucosa, MCM2 and TOP2A are not usually expressed in the basal layer, suggesting that stem cells in this layer are stable. In contrast, the basal expression of MCM2 and TOP2A may indicate activation or neoplastic transformation of stem cells. Among the 99 CIN 1 with follow-up, 7 (7%) showed ProEx C (basal) positivity and disease progression.

The issue of how to predict the progression of CIN1 is critical. Patients with CIN1 must be periodically followed up because they are at risk of progression to higher grade lesions or squamous cell carcinoma. However, it should be noted that 70% to 80% of low grade lesions regress spontaneously [13]. Hence alternative less expensive and equally effective management strategies could yield enormous cost savings and reduce patient anxiety. A large clinical trial was performed in the USA to assess whether or not HPV DNA testing is useful as a triage strategy for patients with LSIL/CIN1 [22]. That study concluded that HPV DNA testing has a limited potential for the clinical management of patients with LSIL because a very high percentage of women with this lesion were positive for HPV DNA [22]. The present study provided the interesting data that p16 and ProEx C could be potential markers to predict progression of CIN1. If CIN1 expressing these biomarkers had a higher progression risk, we could change the

follow-up strategy of CIN1 patients based on the results of these biomarkers.

In conclusion, this study revealed that p16 and ProEx C are useful biomarkers for the diagnosis of CIN, and have potential as predictive factors for progression risk of CIN1.

References

[1] Snijders PJ, Steenbergen RD, Heideman DA, Meijer CJ. HPV-mediated cervical carcinogenesis: concepts and clinical implications. J Pathol 2006; 208:152-164.

[2] Thomison J, 3rd, Thomas LK, Shroyer KR. Human papillomavirus: molecular and cytologic/histologic aspects related to cervical intraepithelial neoplasia and carcinoma. Hum Pathol 2008; 39:154-66.

[3] Solomon D, Davey D, Kurman R, Moriarty A, O'Connor D, Prey M, Raab S, Sherman M, Wilbur D, Wright T, Jr., Young N. The 2001 Bethesda System: terminology for reporting results of cervical cytology. JAMA 2002; 287:2114-9.

[4] Crum CP, Mitao M, Levine RU, Silverstein S. Cervical papillomaviruses segregate within morphologically distinct precancerous lesions. J Virol 1985; 54:675-81.

[5] Canfell K, Barnabas R, Patnick J, Beral V. The predicted effect of changes in cervical screening practice in the UK: results from a modelling study. Br J Cancer 2004; 91:530-6.

[6] Duggan MA, Akbari M, Magliocco AM. Atypical immature cervical metaplasia: immunoprofiling and longitudinal outcome. Hum Pathol 2006; 37:1473-81.

[7] Geng L, Connolly DC, Isacson C, Ronnett BM, Cho KR. Atypical immature metaplasia (AIM) of the cervix: is it related to high-grade squamous intraepithelial lesion (HSIL)? Hum Pathol 1999; 30:345-51.

[8] Keating JT, Cviko A, Riethdorf S, Riethdorf L, Quade BJ, Sun D, Duensing S, Sheets EE, Munger K, Crum CP. Ki-67, cyclin E, and p16INK4 are

complimentary surrogate biomarkers for human papilloma virus-related cervical neoplasia. Am J Surg Pathol 2001; 25:884-91.

[9] Klaes R, Friedrich T, Spitkovsky D, Ridder R, Rudy W, Petry U, Dallenbach-Hellweg G, Schmidt D, von Knebel Doeberitz M. Overexpression of p16(INK4A) as a specific marker for dysplastic and neoplastic epithelial cells of the cervix uteri. Int J Cancer 2001; 92:276-84.

[10] Agoff SN, Lin P, Morihara J, Mao C, Kiviat NB, Koutsky LA. p16(INK4a) expression correlates with degree of cervical neoplasia: a comparison with Ki-67 expression and detection of high-risk HPV types. Mod Pathol 2003;16:665-73.

[11] Chen Y, Miller C, Mosher R, Zhao X, Deeds J, Morrissey M, Bryant B, Yang D, Meyer R, Cronin F, Gostout BS, Smith-McCune K, Schlegel R. Identification of cervical cancer markers by cDNA and tissue microarrays. Cancer Res 2003; 63:1927-35.

[12] Kelly D, Kincaid E, Fansler Z, Rosenthal DL, Clark DP. Detection of cervical high-grade squamous intraepithelial lesions from cytologic samples using a novel immunocytochemical assay (ProEx C). Cancer 2006; 108:494-500.

[13] Follen M, Richards-Kortum R. Emerging technologies and cervical cancer. J Natl Cancer Inst 2000; 92:363-5.

[14] Shi J, Liu H, Wilkerson M, Huang Y, Meschter S, Dupree W, Schuerch C, Lin F. Evaluation of p16INK4a, minichromosome maintenance protein 2, DNA topoisomerase IIalpha, ProEX C, and p16INK4a/ProEX C in cervical squamous intraepithelial lesions. Hum Pathol 2007; 38:1335-44.

[15] Pinto AP, Schlecht NF, Woo TY, Crum CP, Cibas ES. Biomarker (ProEx C, p16(INK4A), and MiB-1) distinction of high-grade squamous intraepithelial lesion from its mimics. Mod Pathol 2008; 21:1067-74.

[16] Halloush RA, Akpolat I, Jim Zhai Q, Schwartz MR, Mody DR. Comparison of ProEx C with p16INK4a and Ki-67 immunohistochemical staining of cell blocks prepared from residual liquid-based cervicovaginal material: a pilot study. Cancer 2008; 114:474-80.

[17] Badr RE, Walts AE, Chung F, Bose S. BD ProEx C: a sensitive and specific marker of HPV-associated squamous lesions of the cervix. Am J Surg Pathol 2008; 32:899-906.

[18] Conesa-Zamora P, Domenech-Peris A, Orantes-Casado FJ, Ortiz-Reina S, Sahuquillo-Frias L, Acosta-Ortega J, Garcia-Solano J, Perez-Guillermo M. Effect of human papillomavirus on cell cycle-related proteins p16, Ki-67, Cyclin D1, p53, and ProEx C in precursor lesions of cervical carcinoma: a tissue microarray study. Am J Clin Pathol 2009; 132:378-90.

[19] Sanati S, Huettner P, Ylagan LR. Role of ProExC: a novel immunoperoxidase marker in the evaluation of dysplastic squamous and glandular lesions in cervical specimens. Int J Gynecol Pathol; 29:79-87.

[20] Zhang S, Balch C, Chan MW, Lai HC, Matei D, Schilder JM, Yan PS, Huang TH, Nephew KP. Identification and characterization of ovarian cancer-initiating cells from primary human tumors. Cancer Res 2008; 68:4311-20.

[21] Maliekal TT, Bajaj J, Giri V, Subramanyam D, Krishna S. The role of Notch

signaling in human cervical cancer: implications for solid tumors. Oncogene 2008; 27:5110-4.

[22] Human papillomavirus testing for triage of women with cytologic evidence of low-grade squamous intraepithelial lesions: baseline data from a randomized trial. The Atypical Squamous Cells of Undetermined Significance/Low-Grade Squamous Intraepithelial Lesions Triage Study (ALTS) Group. J Natl Cancer Inst 2000; 92:397-402.

Figure legends

Figure 1. Diffuse expression of p16 in a case of CIN3. p16 immunostaining, ×200.

Figure 2. ProEx C expression (A) in lower 1/2 of squamous epithelium in a case of CIN 2; (B) in almost the full thickness in a case of CIN2; (C) in the basal layer in a case of CIN2; (D) not observed in the basal layer in a case of non-dysplastic epithelium. ProEx C immunostaining, ×200 (A, B) and ×400 (C, D).

Table 1. The number of cases, average age (years), and type of specimen (biopsy, conization, and hysterectomy) used in this study.

	n	Average age (years)	Biopsy/conization or hysterectomy
Non-dysplastic mucosa	9	47	8/1
CIN1	123	39	119/4
CIN2	57	36	45/12
CIN3	49	40	19/30
Squamous cell carcinoma	14	55	14/0

Table 2. The results of biomarker expression.

	Non-dysplastic mucosa (n=9)	CIN1 (n=123)	CIN2 (n=57)	CIN3 (n=49)	Squamous cell carcinoma (n=14)
p16	1 (11%)	54 (44%)*	56 (98%)*†	49 (100%)*†	14 (100%)*†
ProEx C (non-basal)‡	1 (11%)	49 (40%)	55 (96%)*†	49 (100%)*†	14 (100%)*†
ProEx C (basal)□	1 (11%)	65 (53%)*	56 (98%)*†	49 (100%)*†	14 (100%)*†
HCII	NA	82 (69%)	53 (95%)†	49 (100%)†	14 (100%)

NA, not analyzed; *, $p<0.05$ vs. non-dysplastic mucosa; †, $p<0.05$ vs. CIN1;. ‡, including basal negative / non-basal positive and both positive; □, including basal positive / non-basal negative and both positive.

Table 3. Correlation of different biomarker expression. These data includes all cases irrespective of morphologic diagnosis.

	p16	ProEx C (non-basal)*	ProEx C (basal)†
p16			
ProEx C (non-basal)*	87%		
ProEx C (basal)†	84%	86%	
HCII	63%	66%	70%

*, including basal negative / non-basal positive and both positive; †, including basal positive / non-basal negative and both positive.

Table 4. Biomarker expression and follow-up of CIN 1 (n=99).

	n	p16	ProEx C (non-basal)‡	ProEx C (basal)□	HCII
Progression	7	6 (86%)	6 (86%)	7 (100%)	7 (100%)
Persistent	16	11 (69%)	11 (69%)	11 (69%)	16 (100%)
Regression	76	26 (34%)*	25 (33%)*	37 (49%)*	43 (57%)
Regression (>1 year†)	46	19 (41%)	16 (35%)*	25 (54%)	29 (63%)
Regression (>2 years†)	27	8 (30%)*	10 (37%)	13 (48%)	17 (63%)

*, $p<0.05$ vs. the progression group; †, follow-up periods; ‡, including basal negative / non-basal positive and both positive; □, including basal positive / non-basal negative and both positive.

Table 5. Progression predictive values of biomarkers for CIN1 patients.

	Result	n	Progression rate	Progression predictive value	
				Sensitivity	Specificity
p16	(+)	43	6 (14%)	86%	60%
	(□)	56	1 (2%)		
ProEx C (non-basal)*	(+)	42	6 (14%)	86%	61%
	(□)	57	1 (2%)		
ProEx C (basal)†	(+)	56	7 (13%)	100%	47%
	(□)	43	0		
HCII	(+)	65	7 (11%)	100%	37%
	(□)	34	0		

*, including basal negative / non-basal positive and both positive; †, including basal positive / non-basal negative and both positive.

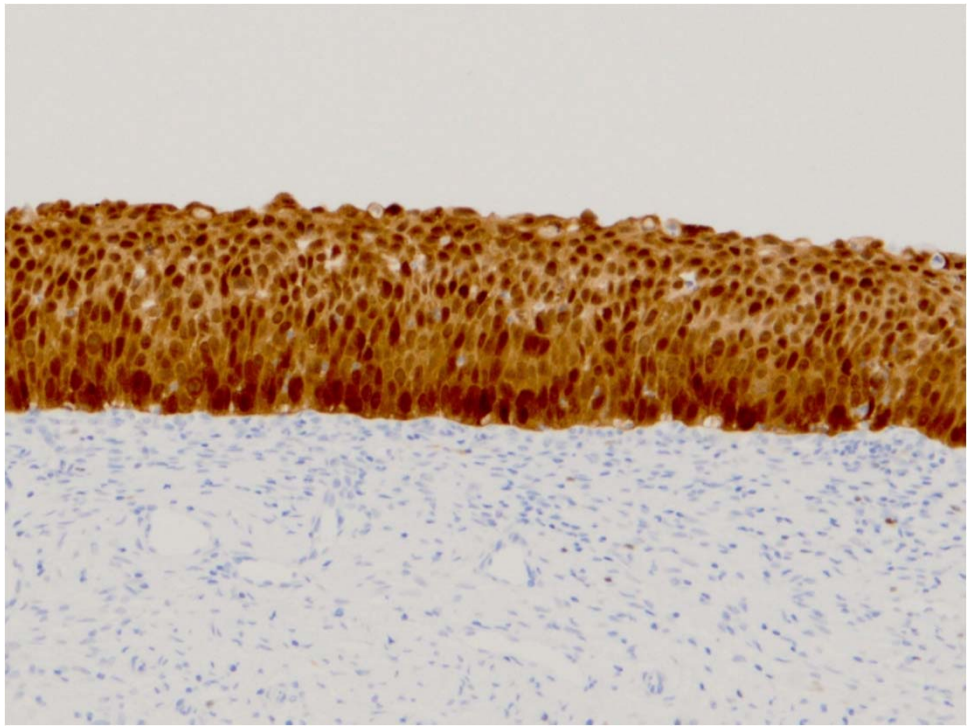

Figure 1

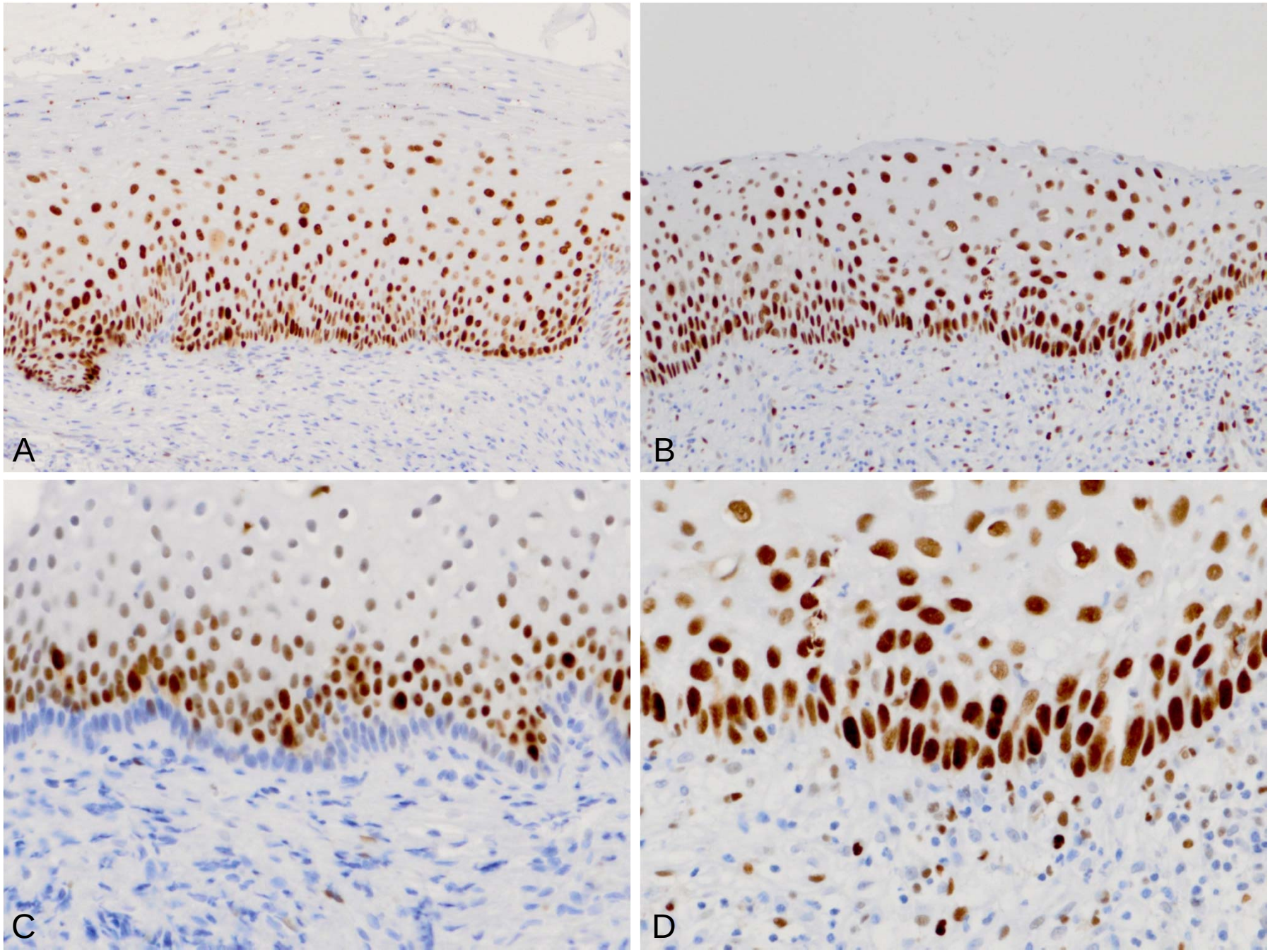

Figure 2