

p16 immunostaining can avoid overdiagnosis in postmenopausal cervical cytology

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ABSTRACT

Senile (atrophic) colpitis is microscopically characterized by the predominance of parabasal squamous cells and the paucity of superficial cells. The activated parabasal cells or dyskeratotic superficial cells may be confused with squamous intraepithelial lesion (SIL) in the routine cytology practice. A total of 29 cervical cytology specimens diagnosed as atypical squamous cells (ASC) or SIL were retrospectively sampled from 24 postmenopausal women (age range: 56–84 years, mean: 65.5, median: 65). Cytological diagnoses in the routine services included ASC-US 20, ASC-H 2, LSIL 4 and HSIL 3. All the specimens showed an atrophic background (senile colpitis). There were two microscopic patterns of senile colpitis with atypia: 1) parabasal cells were clustered (n = 23) and 2) dyskeratotic superficial cells were seen in a highly inflamed background (n = 6). Immunostaining for p16-INK4a (p16 in short) was performed, after the cells were transferred to trimethoxy[3-(phenylamino)propyl]silane-coated glass slides. Only three of the 29 cytology specimens (two of the 24 cases) judged as HSIL cytologically revealed p16 positivity in clustered atypical parabasal cells. Biopsy was performed in 11 cases, and chronic cervicitis without p16 expression was seen in nine. Two lesions showed p16-positive dysplasia (one mild dysplasia and another moderate dysplasia). In one lesion in an 84 year-old female, both cytology and histology specimens showed p16 positivity (HSIL). Discrepancy of p16 expression between the cytology and histology specimens was encountered in two lesions, representing sampling errors. It is of note that the overcytodiagnosis is avoidable with the aid of p16 immunostaining.

Keywords: Atypical squamous cells (ASC), cell transfer technique, p16-INK4a, senile colpitis, squamous intraepithelial lesion (SIL).

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Abbreviations: ASC, Atypical squamous cells; ASC-H, atypical squamous cells, cannot exclude HSIL; ASC-US, atypical squamous cells with uncertain significance; HPV, human papillomavirus; HSIL, high-grade squamous intraepithelial lesion; LBC, liquid-based cytology; LSIL, low-grade squamous intraepithelial lesion; p16, p16-INK4a; SIL, squamous intraepithelial lesion.

INTRODUCTION

Senile colpitis (atrophic vaginitis) represents a status of postmenopausal atrophy of the vaginal squamous mucosa. Loss of secretion of ovary-derived estrogen induces thinning and dryness of the vaginal mucosa. Half of the females are symptomatic (Bachmann and Nevadunsky, 2000; Mac Bride et al., 2010). Senile colpitis is microscopically characterized by 1) decrease or loss of superficial-type keratinocytes, 2) relative predominance

of parabasal keratinocytes, 3) pyknosis seen in the superficial keratinocytes, 4) Loss of vaginal flora such as Döderlein bacilli and *Gardnerella vaginalis*, and 5) Neutrophilic infiltration seen after secondary infection of non-resident microbes. Dryness and inflammation in the vagina may provoke nuclear enlargement in the parabasal keratinocytes (Crothers et al., 2012). Particularly with brushing cytology, parabasal

keratinocytes are often clustered. In these cases, cytological diagnosis of atypical squamous cells (ASC) may be made, according to the Bethesda system for reporting cervical cytology (Nayar and Wilbur, 2015). Accelerated nuclear atypia in the parabasal keratinocytes may be categorized in ASC-H (ASC, cannot exclude high-grade squamous intraepithelial lesion [HSIL]). The diagnostic term ASC-US (ASC of uncertain significance) is used when the possibility of low-grade squamous intraepithelial lesion (LSIL) caused by infection of human papillomavirus (HPV) (Burd, 2003) should be excluded. When reactive atypia associated with HPV-unrelated senile colpitis is suspected cytologically, the diagnosis of ASC-US should thus be avoided as much as possible, we believe.

p16-INK4a (p16 in short) is a product of the tumor suppressor gene encoded by cyclin-dependent kinase inhibitor 2A (CDKN2A). p16 regulates the cell cycle to induce cell senescence: activation of p16 maintains a dephosphorylated state of retinoblastoma (Rb) gene to suspend the cell cycle (Romagosa et al., 2011). The E7 gene of carcinogenic HPV inactivates Rb gene in the infected cell. In the high-grade (carcinogenic) HPV-infected dysplasia and squamous cell carcinoma of the uterine cervix, the cells proliferate, with p16 overexpressed (Romagosa et al., 2011). Namely, p16 overexpression represents E7 activation: strong immunostaining of p16 indicates infection of high-grade HPV (Mulvany et al., 2008; Tsoumpou et al., 2009).

p16 immunostaining has been applied to cytology specimens (Monsonogo et al., 2007; Tsoumpou et al., 2009; Reuschenbach et al., 2010; Gustinucci et al., 2012; Gustinucci et al., 2016). Both the nuclei and cytoplasm are clearly p16-positive in the squamous intraepithelial lesion (SIL)-derived cells. So far, the immunostaining has mostly been applied to liquid-based cytology (LBC) specimens. In most city hospitals in Japan, however, LBC is not yet popular, simply because of low cost performance. In the present study analyzing the cytology specimens sampled from postmenopausal women and routinely diagnosed as ASC or SIL, conventional smear cytology preparations were utilized for immunostaining for p16. Because only one Papanicolaou (Pap)-stained glass slide was in hand in the respective cases, the cell transfer technique was applied to yielding plural specimens on trimethoxy[3-(phenylamino)propyl]silane-coated glass slides (Itoh et al., 2002; Marshall et al., 2014). The aim of the present study is to evaluate the usefulness of p16 immunostaining for distinguishing reactive atypia in senile colpitis and true carcinogenic atypia in SIL.

MATERIALS AND METHODS

Patients

From the computer-filed data, we searched for cytology specimens sampled from women aged 55 years or more and diagnosed as

ASC-US, ASC-H, LSIL or HSIL in the period 2014 through 2017 in Keiyu hospital, Yokohama, Kanagawa, Japan. We recruited a total of 29 Pap-stained specimens from 24 female patients. The age ranged from 56 to 84 years, with the mean of 65.5 and the median of 65. Cytological diagnoses in routine services included ASC-US 20, ASC-H 2, LSIL 4 and HSIL 3. All the specimens microscopically showed an atrophic background (senile colpitis). Hematoxylin and eosin (H&E)-stained cervical biopsy specimens were evaluated in 11 patients.

Cell transfer

We had only one Pap-stained cervical smear preparation on an uncoated glass slide in hand in respective cases. For evaluating p16 immunostaining, the cells smeared on the glass slide were transferred to silane-coated glass slides, according to the cell transfer technique (Marshall et al., 2014). In the present study, Itoh's quick modification (Itoh et al., 2002) was employed (Figure 1). The resin membrane (solidified mounting media, Malinol®, Muto Chemicals, Tokyo, Japan) was cut into two pieces to preserve one piece as Pap-stained preparation in a hospital file. After removing the resin membrane in xylene, the stained dyes were breached in acid alcohol solution for three hours. Then, another piece of the cytology specimen was ready for p16 immunostaining.

Immunostaining

For the heat-induced epitope retrieval of the p16 antigen, the transferred cytology specimens were heated with an electric water boiler (electric pot) in 10 mM ethylenediamine tetraacetic acid (EDTA), pH 8.0, for 30 min. For immunostaining in biopsy specimens, heating in a pressure cooker was employed: The hydrated heating at 121°C was applied for 10 minutes in the EDTA solution. An anti-p16 mouse monoclonal antibody E6H4 available from Ventana Medical Systems, Oro Valley, AZ, USA, was used at a 1:10 dilution, and incubated overnight at room temperature. As the secondary reagent, the Simple Stain-MAX-PO (MULTI) available from Nichirei Bioscience, Tokyo, Japan, was applied for 30 min. The diaminobenzidine color reaction and hematoxylin nuclear counterstaining followed.

Ethical issues

Informed consent of the patients was guaranteed based on the opt-out method. The purpose of the study and how to protect private information were presented in the home page of both the Department of Diagnostic Pathology, Keiyu Hospital, Yokohama and Diagnostic Pathology Clinic, Pathos Tsutsumi, Nagoya, Japan. The present retrospective study was approved by the Ethics Committee for the Clinical and Epidemiological Study, Keiyu Hospital, Yokohama in 2020 (approval number: R2-63).

RESULTS

Two microscopic patterns of senile colpitis

All the 29 cytology specimens showed an atrophic background (senile colpitis). There were two predominant microscopic patterns of senile colpitis with atypia, as illustrated in Figure 2.

1) Parabasal cells with atypia were clustered (n = 23).

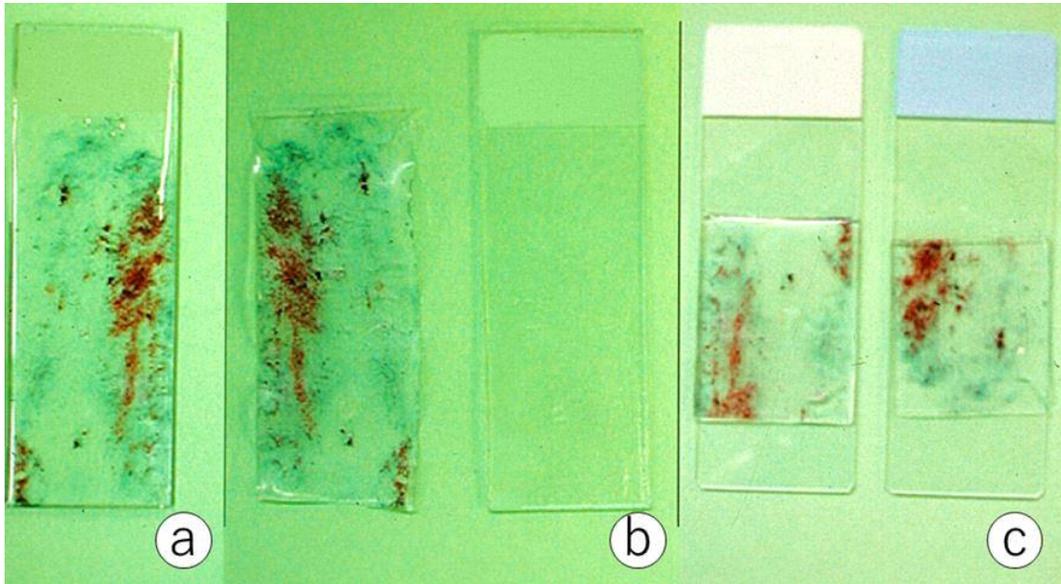


Figure 1. The cell transfer technique. Liquid resin (mounting media) is covered on the Pap-stained cells after removal of the cover slip (a). All the cells on the glass slide can be transferred to the solidified resin membrane (b). Then, the membrane is cut into two pieces (c). One piece is employed for p16 immunostaining after dye breaching, while another should be kept as Pap-stained preparation.

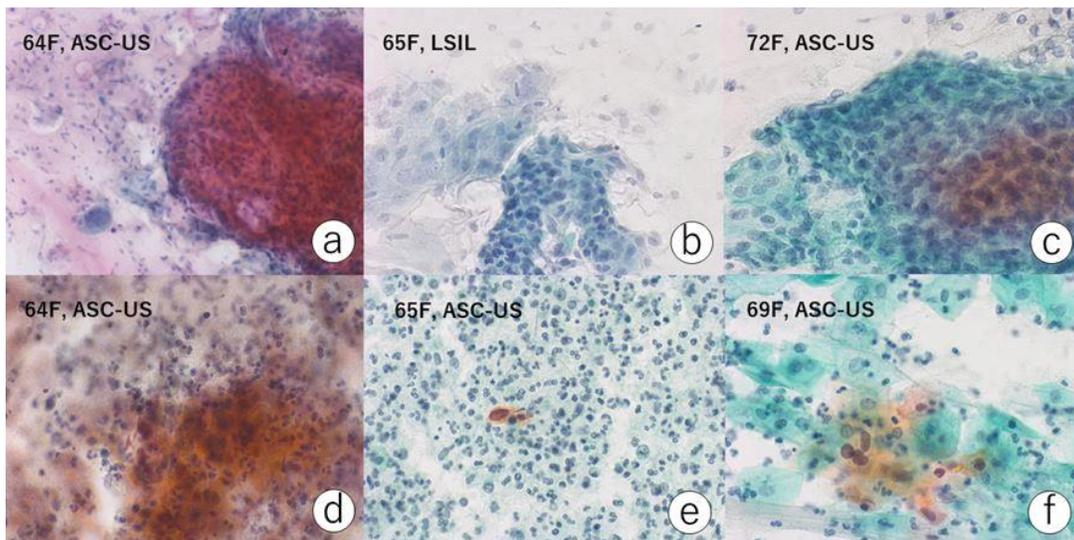


Figure 2. Two microscopic patterns of senile colpitosis. 1) Parabasal cells with atypia are clustered, as seen in the upper three panels (a-c: cases 9, 11 and 23, respectively). Case 11 was cytologically diagnosed as LSIL, but with negativity of p16. 2) Dyskeratotic superficial cells are seen in an inflammatory background, as demonstrated in the lower three panels (d-f: cases 10, 12 and 21, respectively). Case 21 was histologically diagnosed as p16-positive LSIL, but cytology specimen was p16-negative.

2) Dyskeratotic superficial cells were seen in an inflammatory background (n = 6).

Results of p16 immunostaining

The results are summarized in Table 1. Only three of 29

cytology specimens (two of 24 cases) revealed p16 positivity in clustered atypical parabasal cells, and all the three specimens were cytologically judged as HSIL. The diagnoses of ASC-US, ASC-H and LSIL were made in 20 (69%), 2 (7%) and 4 (14%) of 29 specimens, respectively, and all of them were p16-negative, as represented in Figure 3. All the lesions with dyskeratotic superficial

Table 1. Summary of 29 lesions (24 cases) analyzed in the present study.

Case	Age	Cytology Dx	SC type	p16 cytology	Biopsy Dx	p16 biopsy
1	56	LSIL	1	-	CC	-
2	57	ASC-US	1	-	CC	-
3	61	ASC-US	1	-	CC	-
4	61	ASC-H	1	-		
5	62	ASC-US	1	-	CC	-
6	62	HSIL	1	+	CC	-
7	64	LSIL	1	-	CC	-
8	64	ASC-US	1	-		
9	64	ASC-US	1	-	CC	-
10	64	ASC-US	2	-	CC	-
11	65	LSIL	1	-		
12	65	ASC-US	2	-		
13	65	ASC-US	1	-		
14	66	ASC-US	1	-		
15	66	ASC-US	1	-		
16	66	ASC-US	2	-		
17	66	ASC-US	1	-		
18	66	ASC-US	1	-		
19	67	ASC-US	1	-		
20	68	ASC-US	2	-		
21	69	ASC-US	2	-	LSIL	+
22	72	ASC-US	1	-		
23	72	ASC-US	1	-	CC	-
24	82	HSIL	1	+		
	82	ASC-H	2	-		
	84	HSIL	1	+	HSIL	+

SC: senile colpitis, **CC:** chronic cervicitis

SC type 1: SC with clustered parabasal cells

SC type 2: SC with dyskeratotic superficial cells

keratinocytes (n=6) were negative for p16. Biopsy was performed in 11 cases, and chronic cervicitis without p16 expression was observed in nine. Two lesions showed p16-positive dysplasia (one mild dysplasia/LSIL and another moderate dysplasia/HSIL). In one lesion in an 84 year-old female (case 24), both cytology and histology specimens showed p16 positivity and diagnosed as HSIL (Figure 4). Two of three cytology specimens in case 24 were diagnosed as HSIL. Discrepancy of p16 expression between the cytology and histology specimens was encountered in two lesions. One of the three p16-positive cytology specimens (case 6) was negative in the biopsy sample (Figure 5). This should be regarded as a sampling error of the biopsy procedure. Another false-negative (cytology-negative and biopsy-positive) case (case 21) was experienced: the biopsy diagnosis was mild dysplasia/LSIL as displayed in Figure 6.

DISCUSSION

Senile colpitis seen in postmenopausal women is cytologically featured by atrophic smears (Crothers et al., 2012). The atrophic smears may be misinterpreted as positive or equivocal (Abati et al., 2000; Qiao et al., 2005; Tabrizi, 2018). This happens when condensed nuclear chromatin resembling nuclear hyperchromasia is seen in parabasal squamous cells. It may be confused with SIL, as was so in the present series. Microscopically, a hypocellular background and the lack of chromatin clumping, nucleoli and mitotic activity are the hallmark of atrophic smears (Crothers et al., 2012). Superficial keratinocytes significantly decrease or disappear in many occasions. When pyknotic and parakeratotic change is seen in superficial squamous cells in senile colpitis in association with accelerated inflammation, distinction

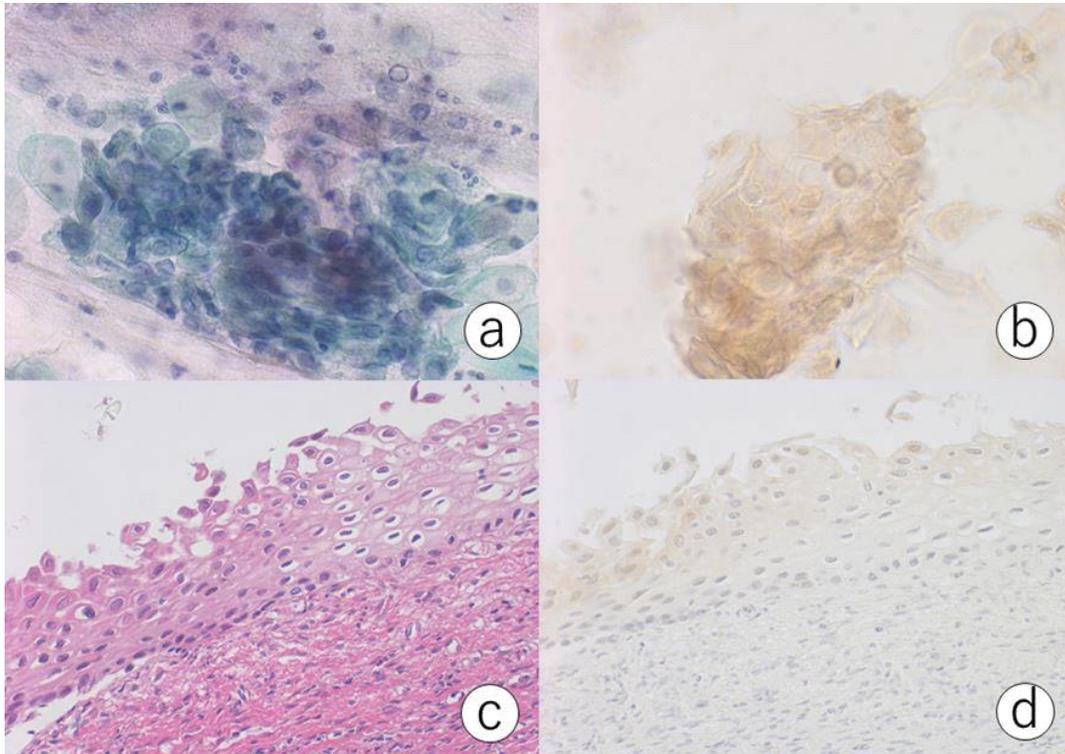


Figure 3. Senile colpitis diagnosed as ASC-US without p16 expression (case 5: a&b: cytology, c&d: biopsy, a: Pap, c: H&E, b&d: p16). Both the parbasal cell clusters in the cytology specimen and atrophic mucosa in the biopsy specimen are negative for p16.

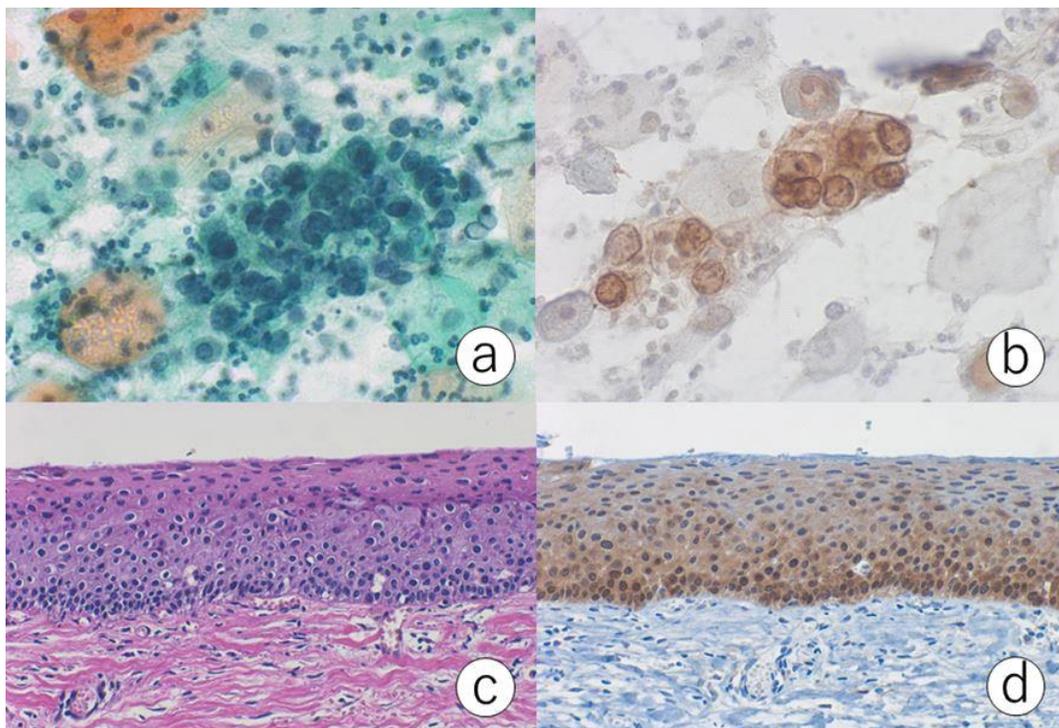


Figure 4. p16-positive HSIL seen in an 84 year-old female patient (case 24: a&b: cytology, c&d: biopsy, a: Pap, c: H&E, b&d: p16). Truly dysplastic cells are p16-immunoreactive in both cytology and histology. In Pap-stained preparation, hyperchromasia is discernible in the atypical parbasal cells.

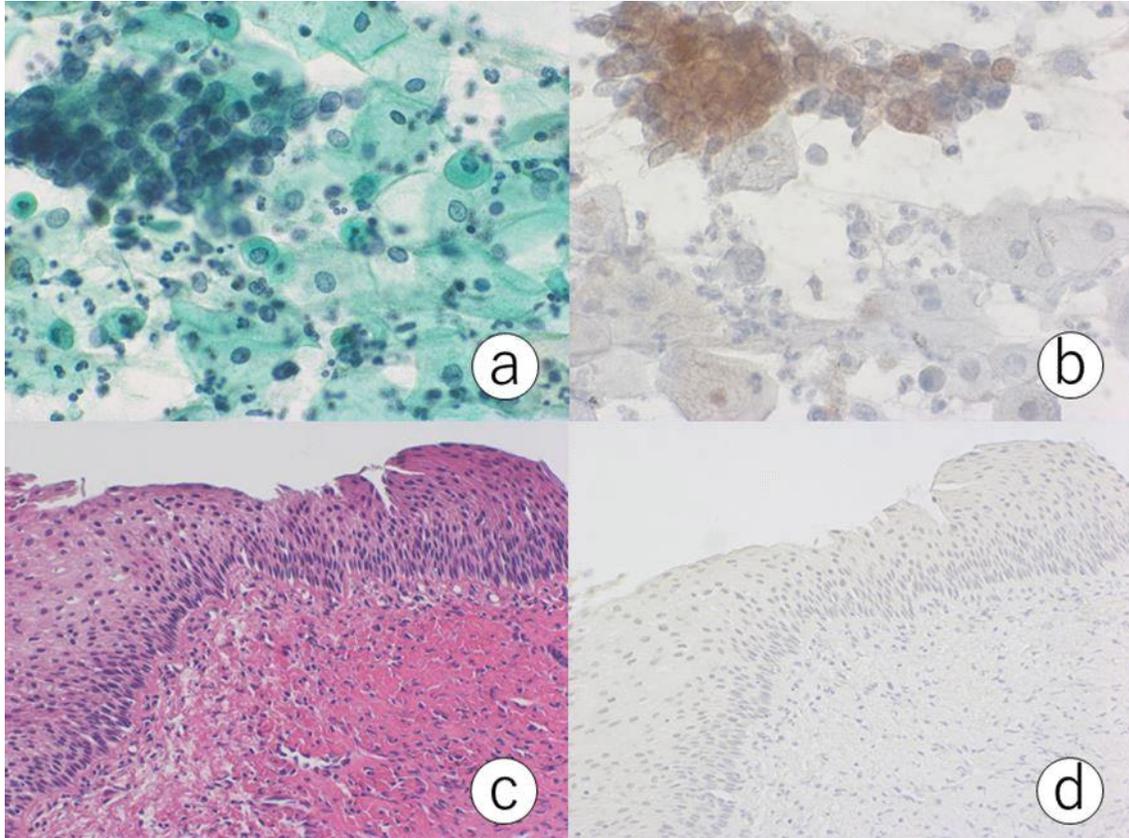


Figure 5. Discrepancy between cytology and biopsy specimens (1) (case 6: a&b: cytology, c&d: biopsy, a: Pap, c: H&E, b&d: p16). In the cytology specimen, the atypical parabasal cells express p16, but no p16 immunoreactivity is noted in the biopsy sample. Sampling error of the biopsy procedure is indicated.

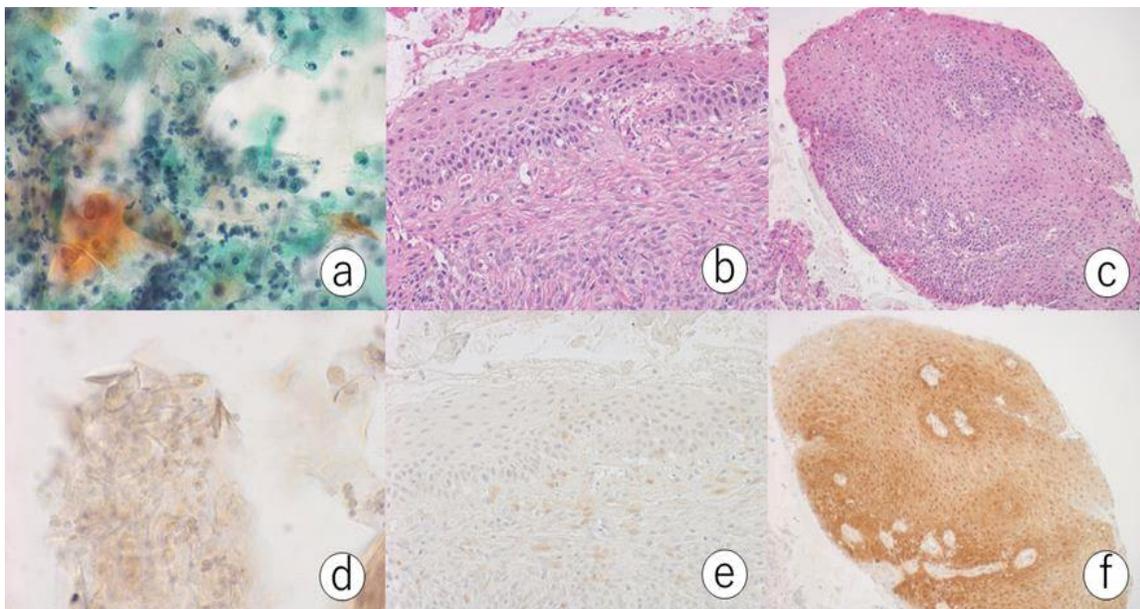


Figure 6. Discrepancy between cytology and biopsy specimens (2) (case 21: a&d: cytology, b,c,e&f: biopsy, a: Pap, b&c: H&E, d-f: p16). The cytology specimen with dyskeratotic superficial cells in the inflamed background is negative for p16. One of the biopsy specimens (c&f) shows p16 expression, while the other piece accompanying features of senile colpitis (b&e) is p16-negative.

from LSIL is needed.

In routine cytodiagnostic services, we tend to use the term ASC for interpretation in equivocal (gray-zone) cases. When nuclear atypia is relatively weak, the term ASC-US may be chosen for the cytodiagnosis. However, the clinician will understand with the diagnostic term ASC-US that HPV genomic examination is recommended by the cytopathologists (Cuzick et al., 2006; Ndifon and Al-Eyd, 2020).

It has been reported that p16 overexpressed by high-grade (carcinogenic) HPV infection is an excellent immunocyto- and histochemical marker for distinguishing premalignant and malignant cervical lesions from reactive cervical lesions, including senile colpititis (Monsonogo et al., 2007; Tsoumpou et al., 2009; Reuschenbach et al., 2010; Gustinucci et al., 2012; Gustinucci et al., 2016). The Lower Anogenital Squamous Terminology Standardization Project for HPV-Associated Lesions substantiated the use of p16 immunostaining for biologically equivocal lesions falling between the microscopic changes of low-grade and high-grade HPV infection (Darragh et al., 2012). p16 positivity is expected to indicate viral integration to the host cell genome and thus could be used to determine which LSIL lesions will regress and which will progress (del Pino et al., 2009). In the present study, 26 (90%) of the 29 specimens or 22 (92%) of the 24 cases of senile colpititis with atypia were p16-negative, hence high-grade HPV-unrelated. All the three p16-positive specimens were diagnosed as HSIL in a routine cytology services, and the nuclei showed hyperchromasia and chromatin clumping. The p16-positive biopsy lesions (n = 2) were diagnosed as LSIL and HSIL. All the cytology specimens with dyskeratotic superficial keratinocytes (n = 6) were negative for p16 expression. The diagnosis of ASC-US was made in 20 (69%) of the 29 specimens, and all of them were p16-negative. When senile colpititis with reactive atypia was suspected cytologically, we should recommend to using the term NILM (negative for intraepithelial lesion or malignancy), instead of ASC-US. p16 immunostaining was shown as a sharp and excellent indicator to avoid such misleading interpretation (Abati et al., 2000; Qiao et al., 2005; Tabrizi, 2018).

The p16 expression in the cytology and biopsy specimens was compared in the respective cases. In the lesion of an 84 year-old female patient, both cytology and histology specimens showed p16 positivity and diagnosed as HSIL. One of two p16-positive cytology specimens was p16-negative in the biopsy sample. This should be regarded as a sampling error of the biopsy procedure. Another false-negative (cytology-negative and biopsy-positive) case was experienced. This tells us the need for repeated evaluations for cytological and histological analyses.

In the present study, we created a silane-coated glass slide by the cell transfer technique meticulously one by one (Itoh et al., 2002; Marshall et al., 2014). In most of the common general hospitals in Japan, the LBC system

is not yet introduced because of low cost performance, according to the cost reduction plan by the Japan Health Insurance Association. Usually, only one smeared glass slide is in hand, so that the cell transfer technique is indispensable for evaluating p16 expression. It is of no doubt that introduction of the LBC system will facilitate the application of p16 immunostaining for the differential diagnosis of senile colpititis and precancerous lesions (Monsonogo et al., 2007; Tsoumpou et al., 2009). We sincerely hope that the medical environment for the cytology services is improved for allowing us to introduce the LBC system in the cytology division of general hospitals in Japan.

Conclusion

Senile (atrophic) colpititis may show reactive atypia, often confusing with HPV-related atypia. Overcytodiagnosis can be avoided with the aid of p16 immunostaining. A cell transfer technique is useful, even if only one smeared cytopreparation is available.

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