

## RESEARCH ARTICLE

# Significance of the Cytological Signs of Human Papillomavirus Infection in Anal Pap Smears of Human Immunodeficiency Virus-Infected Japanese Men Who Have Sex with Men

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### Abstract

**Purpose:** The incidence of invasive anal cancer (IAC) has been increasing among human immunodeficiency virus (HIV)-positive men who have sex with men (MSM). Although cytological diagnosis is the modality of choice for screening cases of IAC, it is associated with lower sensitivity and specificity. Therefore, the present study aimed to evaluate new cytological signs of human papillomavirus (HPV) infection that may contribute to improving anal cytology. **Methods:** Anal cytology and HPV testing were performed using SurePath liquid-based cytology on samples obtained from 37 HIV-positive Japanese MSM. Subsequently, a histological biopsy based on high-resolution anoscopy was performed in MSM with abnormal cytological findings indicative of atypical squamous cells of undetermined significance (ASC-US) +. Also, anal Papanicolaou (Pap) smears were performed to determine cellularity, presence of dysplastic squamous cells, and other cytological signs of HPV infection. **Results:** Of the 37 MSM who underwent anal cytology, six tested negative for intraepithelial lesion or malignancy, three cases exhibited ASC-US, 17 exhibited low-grade squamous intraepithelial lesion (LSIL), nine exhibited high-grade squamous intraepithelial lesion (HSIL), and two remained undiagnosed. The anal Pap smears of 28 (96.6%) of the 29 MSM with abnormal cytological findings of ASC-US+ exhibited anal intraepithelial neoplasia (AIN), as revealed by histological biopsy. The median value (minimum–maximum) of the cellularity of anal Pap smears was 12 (0–70.5) nsc/hpf. In 26 MSM with LSIL and HSIL, the median dysplastic squamous cells count was 14 (2–152) dsc/smear and the cytological sign of HPV infection was 11 (2–71) hpv/smear. Of all anal Pap smears that revealed ASC-US+, 96.6% exhibited cytological signs of HPV infection. Compression-positive binucleated cells were the most prevalent among all cytological signs of HPV infection. **Conclusion:** For anal cytology, instead of considering a small number of dysplastic squamous cells, screening based on cytological signs of HPV infection may be beneficial for improving the diagnosis of AIN.

**Keywords:** Human immunodeficiency virus (HIV)- human papillomavirus (HPV)- MSM- anal cytology- HRA

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### Introduction

The incidence of invasive anal cancer (IAC) is highest among human immunodeficiency virus (HIV)-infected men who have sex with men (MSM), followed by HIV-infected heterosexual men and women (del Amo et al., 2013). Notably, a history of receptive anal intercourse is significantly associated with the development of anal cancer, thus representing a primary risk factor for high-risk human papillomavirus (HR-HPV) infection (Abbas et al., 2010).

The biological behavior of IAC is similar to that of cervical cancer; therefore, an anal cancer screening program for this high-risk population has been proposed by researchers (Park et al., 2010). Accordingly, the screening program is based on the cytological detection of HPV-related abnormalities or by the direct detection

of HPV-related biomarkers. Screening test for cytological changes can be performed using methods similar to those used for cervical screening using Papanicolaou (Pap) stain. Cervical and anal cytology share histopathological features because of some similarities in the genital areas, such as the transformation zone in both cervix and rectum.

Furthermore, it has been recommended that anal cytology must be evaluated according to the 2001 Bethesda System, which is used to evaluate cervical cytology. However, a standard screening strategy for anal cancer among MSM remains controversial (Darragh et al., 2011). Although the sensitivity of anal cytology for high-grade anal intraepithelial neoplasia (AIN) using atypical squamous cells of undetermined significance (ASC-US) as the threshold for triage to high-resolution anoscopy (HRA, colposcopy modified for anus) is considered high, its specificity is low (Salit et al., 2010).

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Nevertheless, different sensitivity and specificity rates have been reported for anal cytology (Goldie et al., 1999). Therefore, the accuracy of anal cytology needs further improvement for the early detection of AIN. The detection of cytological signs of HPV infection using anal cytology remains poor because anal cytology is altered in only a small number of cases. Concerns have been raised on the low accuracy of anal cytology due to the variation in the sensitivity rates of atypical cells, including cells associated with HPV infection.

The present study aimed to evaluate the cytological signs of HPV infection that may contribute to the improvement of anal cytology. We reviewed anal Pap smears obtained from HIV-infected Japanese MSM to confirm the cellularity, presence of dysplastic squamous cells, and cytological signs of HPV infection.

## Materials and Methods

### *Clinical samples*

The study protocol was approved by the Ethics Committee of the Faculty of Health Sciences, Kyorin University. SurePath™ (BD Diagnostics, Franklin Lakes, NJ, USA) liquid-based cytology samples were obtained from 37 HIV-infected Japanese MSM treated with antiretroviral therapy at Kyorin University between April 2014 and September 2016. The samples were collected from the anal canal using a brush from the SurePath™ sample collection kit. After digital rectal examination with lubricant jelly, the brush was inserted into the anal canal and rotated approximately 20 times clockwise and counter-clockwise with gentle pressure.

### *Cytology*

Thin-layer slides were prepared using the SurePath system as described elsewhere (Kirschner et al., 2006). The slides were fixed in 95% ethanol and stained with Pap stain. The anal Pap smears were classified by two cytotechnologists according to the modified Bethesda System 2001 (Solomon et al., 2002): negative for intraepithelial lesion or malignancy (NILM); ASC-US; low-grade squamous intraepithelial lesion (LSIL); high-grade squamous intraepithelial lesion (HSIL); atypical squamous cells, cannot exclude an HSIL (ASC-H), or invasive carcinoma. In the case of ASC-US+ findings, an HRA-directed biopsy was performed. For NILM cases, a follow-up with cytology and HPV test for >1 year was conducted. Six cytological signs of HPV infection were selected in the present study: koilocytes, koilocytoids, atypical parakeratotic (APK) cells, compression-positive binucleated cells (Okodo et al., 2017), compression-negative binucleated cells, and multinucleated cells. Koilocytoids were defined as “abortive koilocytes” exhibiting some perinuclear cytoplasmic clearing around a small nucleus (Wong et al., 2008). APK cells were characterized by atypical, small, densely keratinized squamous cells with dense orangeophilic cytoplasm and a small pyknotic nucleus (Bollmann et al., 2005). Furthermore, multinucleated cells were identified as large cells showing  $\geq 3$  nuclei. Binucleated cells with their nuclei pressing against each other or with an adhesion distance

of approximately  $>5 \mu\text{m}$  were identified as compression-positive binucleated cells, whereas those with separate or overlapping nuclei or with an adhesion distance of  $<5 \mu\text{m}$  between two nuclei were defined as compression-negative binucleated cells (Okodo et al., 2017). In addition, the ASC-US criterion does not reflect the presence or absence of cytological signs of HPV infection. Cells exhibiting each morphological feature were recorded as absent or present. Adequate cellularity was defined as the median number of nucleated squamous cells per  $40 \times$  high-power field (nsc/hpf), which was calculated by counting 10 hpf. The presence of dysplastic squamous cells and cytological signs of HPV infection was examined in case of abnormal cytology of LSIL and HSIL; these were defined as the median number of cells counted in the complete smear by observing at  $10 \times$  low power (dsc/smear and hpv/smear for the presence of dysplastic squamous cells and cytological signs of HPV infection, respectively).

### *HR-HPV test*

DNA was extracted from liquid cervical cytology specimens (100  $\mu\text{l}$ ) using the High Pure PCR Template Preparation Kit (Roche Diagnostics, Mannheim, Germany). DNA was amplified using PCR with specific primers for the E6 region of HPV (Okayama et al., 2013). The final 25  $\mu\text{l}$  of PCR reaction mixture contained  $1 \times$  AmpliTaq Gold 360 buffer, 2 mM  $\text{MgCl}_2$ , 0.025 U/ $\mu\text{l}$  AmpliTaq Gold 360 DNA Polymerase (Applied Biosystems, CA, USA), 1  $\mu\text{l}$  DNA, and 0.5 pM primers (HPV 16, 18, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, and 68). PCR amplification was performed using a thermal cycler with the following cycling: initial denaturation at  $94^\circ\text{C}$  for 10 min; 35 cycles of denaturation at  $95^\circ\text{C}$  for 30 s, annealing at  $60^\circ\text{C}$  for 30 s, extension at  $72^\circ\text{C}$  for 30 s, and a final extension at  $72^\circ\text{C}$  for 5 min. Human  $\beta$ -actin expression, amplified using an additional PCR, was used as an internal standard; the resulting amplicon was 262-bp long (Okodo et al., 2016).

## Results

### *Cytology, HR-HPV infection, and biopsy*

Of the 37 MSM who underwent anal cytological evaluation with Pap smear, six exhibited NILM, three exhibited ASC-US, 17 exhibited LSIL, and nine exhibited HSIL; however, two cases remained undiagnosed. Besides, 29 MSM (78.4%) displayed abnormal cytological findings of ASC-US+. Of the 37 MSM, 31 (83.8%) tested positive for HR-HPV. According to cytological results, 33.3% (2/6), 100% (3/3), 94.1% (16/17), and 100% (9/9) of MSM with NILM, ASC-US, LSIL, and HSIL, respectively, tested positive for HR-HPV. Of the 29 MSM with abnormal cytological findings of ASC-US+, 28 exhibited AIN (96.6%), as revealed by histological biopsy. All NILM cases were interpreted as no lesions because they were NILM and HPV negative for >1 year of follow-up.

### *Cellularity in anal Pap smears*

Table 1 shows the cellularity values of the anal Pap smears of all 37 MSM. The median (minimum–maximum)

Table 1. The Cellularity Values, Number of Dysplastic Squamous Cells and Cytological Signs of Human Papillomavirus (HPV) Infection in the Anal Pap Smears of All 37 Men Who Have Sex with Men (MSM)

Case No.	High-risk HPV infection	Cytology	Cellularity (nsc/hpf)	Dysplastic squamous cells (dsc/smear)	Cells (hpv/smear)	Cytological signs of HPV infection					
						Koilo	Koiloid	APK cells	bin (+)	bin (-)	Multi
1	+	UN	0.3	ND	ND						
2	-	UN	1.3	ND	ND						
3	-	NILM	11	ND	2					+	
4	-	NILM	11.5	ND	0						
5	+	NILM	7.5	ND	0						
6	+	NILM	11.5	ND	2					+	
7	-	NILM	14	ND	0						
8	-	NILM	17.5	ND	0						
9	+	ASC-US	52	ND	0						
10	+	ASC-US	12	ND	2				+		+
11	+	ASC-US	18	ND	4			+	+		
12	+	LSIL	11	3	5				+		
13	+	LSIL	33	18	6			+	+	+	
14	+	LSIL	12	5	2				+		
15	+	LSIL	10.5	18	19	+		+	+	+	
16	+	LSIL	70.5	18	62	+	+	+	+	+	
17	+	LSIL	12	31	24	+		+	+		
18	-	LSIL	40.5	20	2			+			
19	+	LSIL	10	5	9			+	+		
20	+	LSIL	15	10	16	+	+	+	+		
21	+	LSIL	31.5	7	18			+	+		
22	+	LSIL	57.5	9	9	+		+	+		
23	+	LSIL	27.5	8	8			+	+		+
24	+	LSIL	3	29	43	+	+	+	+		
25	+	LSIL	31.5	21	19	+		+	+		
26	+	LSIL	10	31	16	+	+	+	+		
27	+	LSIL	1.5	3	3		+				
28	+	LSIL	2	2	2				+		
29	+	HSIL	24.5	13	2				+		
30	+	HSIL	8.5	17	25			+	+	+	+
31	+	HSIL	25.5	10	10	+	+		+	+	+
32	+	HSIL	23.5	24	71	+	+	+	+	+	+
33	+	HSIL	48	34	9			+	+	+	+
34	+	HSIL	19.5	2	25	+			+		
35	+	HSIL	2.5	15	8			+	+	+	
36	+	HSIL	2	12	18	+		+	+	+	+
37	+	HSIL	20	152	12			+	+	+	

HPV, human papillomavirus; UN, undiagnosable; ND, not detected; NILM, negative for intraepithelial lesion or malignancy; SIL, squamous intraepithelial lesion; ASC-US, atypical squamous cells of undetermined significance; LSIL, low-grade squamous intraepithelial lesion; HSIL, high-grade squamous intraepithelial lesion; Koilo, Koilocytes; Koiloid, Koilocytoids; APK cells, atypical parakeratotic cells; bin (+), compression-positive binucleated cells; bin (-), compression-negative binucleated cells; Multi, Multinucleated cells

of the cellularity values was 12 (0–70.5) nsc/hpf. Of all 37 MSM, seven showed a cellularity value of  $\leq 6$  nsc/hpf; of these, two cases with cellularity values of 0.3 nsc/hpf and 1.3 nsc/hpf were excluded considering them to be undiagnosable. The remaining four MSM were considered as diagnosable due to the presence of a small number of

dysplastic cells. Although many anucleated squamous epithelial cells were detected in all cases, the number was particularly high in undiagnosable cases.

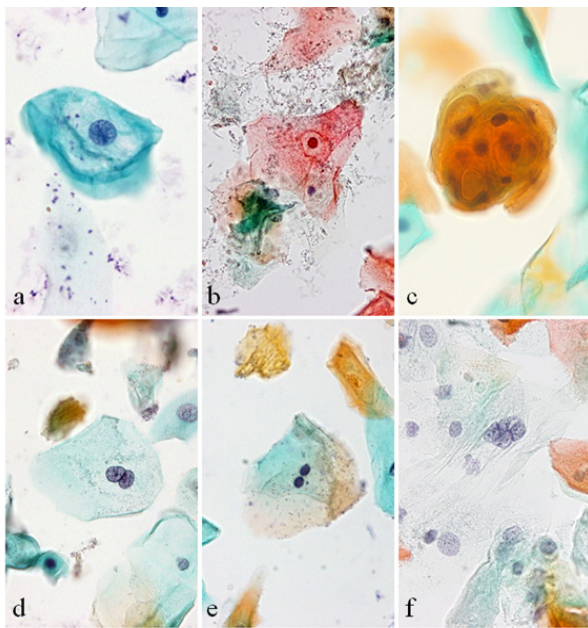


Figure 1. Six Cytological Signs of Human Papillomavirus (HPV) Infection. Pap staining (40 $\times$ ) : (A) koilocite, (B) koilocytoid, (C) atypical parakeratotic cells (APK), (D) compression-positive binucleated cell, (E) compression-negative binucleated cell, and (F) multinucleated cell.

#### *Dysplastic squamous cells and cytological signs of HPV infection in anal Pap smears*

Table 1 shows the number of dysplastic squamous cells and cytological signs of HPV infection in the anal Pap smears of all 37 MSM. In the anal smears of 26 MSM with LSIL or HSIL, the median dysplastic cell count was 14 (2–152) dsc/smear and the median number of cytological signs of HPV infection was 11 (2–71) hpv/smear. Figure 1 shows the six cytological signs of HPV infection and the anal cytology. The cytological signs of HPV infection were detected in anal Pap smears of 28 (96.6%) of the 29 MSM with abnormal cytological findings of ASC-US+, with koilocytes and compression-negative binucleated cells detected in 42.9% (12/28) of the MSM, koilocytoids and multinucleated cells in 25.0% (7/28), APK cells in 71.4% (20/28), and compression-positive binucleated cells in 92.9% (26/28). However, of the six MSM with NILM, two (33.3%) exhibited cytological signs of HPV infection (Table 1), all of which were compression-negative binucleated cells.

## Discussion

Anal cytology has been suggested to be a potential screening modality for the early detection of AIN (Gingelmaier et al., 2010). However, the reported sensitivity and specificity of anal cytology for AIN are 69%–93% and 32%–59%, respectively (Chiao et al., 2006). Therefore, it is possible that the true prevalence of AIN is underestimated. Nevertheless, three reasons were attributed to the low sensitivity and specificity of anal cytology. First, insufficient, incomplete, or inappropriate swabbing of the anal epithelium, which is particularly challenging compared with the vaginal canal because of the difference between the creased lining of

the anus and the smooth mucous membrane of the uterine cervix, resulting in inadequate diagnoses of cytological abnormalities (Ruanpeng et al., 2016). Second, the abundance of anucleated squamous epithelial cells (Arain et al., 2005, Patarapadungkit et al., 2012), in addition to the inadequacy of dysplastic cells in specimens, hinders the detection of abnormal cells. Third, anal cytology is a relatively new concept and thus subjected to high rates of interobserver variation regarding of the interpretation of morphological characteristics of dysplastic cells (Arora et al., 2014, Johnson et al., 2016).

We evaluated the cellularity values of anal Pap smears in HIV-positive MSM and thus found that the median value of cellularity was 12 nsc/hpf. As the Bethesda System recommends that the mean cellularity of satisfactory samples (SurePath samples) to be at least 6 nsc/hpf for the cytological diagnosis of the uterine cervix (Solomon et al., 2002), the samples prepared in the present study were mostly appropriate for diagnosis with regard to cellularity. Anal Pap smears are likely to yield unsatisfactory samples because of small numbers of nucleated cells and large numbers of anucleated squamous epithelial cells; 13.6% unsatisfactory samples have been previously reported (Donà et al., 2012). The percentage of unsatisfactory anal Pap smear samples in the present study was approximately 5%, which was lower than the reported percentage. The anal cell samples were collected using SurePath brushes, which are routinely used for the uterine cervical canal by inserting the brush into the anus and rotating it 20 times clockwise and counter clockwise to scrape the anal transitional zone (anTz). This result may indicate that the number of unsatisfactory samples can be reduced if the collection procedure is correctly performed. However, it is necessary to evaluate the differences among other sampling instruments and scraping methods. In addition, the ASC-US criterion does not reflect the presence or absence of cytological signs of HPV infection.

In the present study, 78.4% of the MSM presented with abnormal cytological findings of ASC-US+; 8.1% were diagnosed with ASC-US. Goldstone et al., (2012) indicated that the percentage of ASC-US in HIV-positive patients was 12.5%, which was twofold than that of HIV-negative patients. Moreover, Johnson et al., (2016) reported that approximately 20% of HIV-positive MSM were diagnosed with ASC-US; however, the anal Pap smear screening of HIV-positive MSM showed a higher percentage of ASC-US than that of the general population. These findings suggested that a diagnosis of ASC-US should be considered as the threshold for triage to HRA; consequently, the sensitivity became higher than when LSIL was set as the threshold (Drragh et al., 2011). In the present study, out of the 29 MSM with abnormal findings of ASC-US+, 28 exhibited AIN, leading us to believe that patients with ASC-US+ should be subjected to histological diagnosis for further examination such as HRA. The anal Pap smears used in the present study demonstrated a lower diagnostic rate of ASC-US than those used in previous studies, with the majority of abnormal cytological findings being LSIL+. This result suggested that we could conclusively detect the presence of scarce dysplastic cells in the samples. When performing

the screening of anal Pap smears of HIV-positive MSM, it is critical to have an understanding of the noticeably high proportion of LSIL, particularly in MSM compared with the general population before reaching a definite diagnosis.

To the best of our knowledge, there is a lack of comprehensive studies on cell abnormalities in anal cytological specimens; moreover, cytopathologists have inadequate data on how to efficiently identify dysplastic squamous cells. Furthermore, in the present study, the median dysplastic cell count was 14 (2–152) dsc/smear in both LSIL and HSIL anal smears. Arain et al., (2005) used SurePath specimens and reported a dysplastic cell count of <3 dsc/smear in 16% of patients with LSIL. Although our analysis detected a relatively high number of dysplastic cells, the dysplastic cell count varied considerably from one case to another, being as low as 2 dsc/smear in one case. This variability might have contributed to the low sensitivity and specificity of the cytological diagnosis. Besides, for detecting small numbers of dysplastic cells, we focused on the cytological signs of HPV infection. The median (minimum–maximum) number of cytological signs in both LSIL and HSIL anal smears was 11 (2–71) hpv/smear. Of all cytological signs of HPV infection, compression-positive binucleated cells and APK cells were not detected in MSM with NILM, but were recognized at predominantly high frequencies in MSM with LSIL and HSIL. Arain et al., (2005) reported that bi/multinucleated squamous cells and APK cells are significantly more common in squamous intraepithelial lesion (SIL) cases than in NILM cases. In particular, as APK cells were present in 72% of all SIL cases, they were shown to be useful for detecting AIN and were the most prevalent in HSIL cases. In our previous study, we demonstrated that among multinucleated squamous cells in uterine cervix smears, compression-positive binucleated cells are cytological signs of HPV infection that may be associated with a cervical intraepithelial neoplasm (Okayama et al., 2013). Similarly, in anal Pap smears, compression-positive binucleated cells are also highly likely to be abnormal cells that indicate the presence of AIN. Interestingly, many cytological signs of HPV infection as the number of dysplastic cells were identified in the present study. Furthermore, it has already been reported that HPV testing is a valuable tool for improving the sensitivity of AIN diagnosis (Goldstone et al., 2012). However, the positivity rate of HPV testing is higher in HIV-positive MSM than in the general population. The positivity rate in the subjects in our study was also as high as 83.8%. Nevertheless, not all HPV-positive individuals presented dysplastic cells or cytological signs of HPV infection. Thus, the effectiveness of this modality as a stand-alone screening method is uncertain. Consequently, to accurately evaluate the true prevalence of AIN by detecting small numbers of dysplastic cells in anal Pap smears, it may be essential to identify associated morphological changes in the epithelium produced by HPV infection. Limitations of the present study include the small sample size and the lack of evidence of HRA histological diagnosis in NILM cases. Thus, to corroborate our findings, further investigation including a larger group of patients is necessary.

In conclusion, the presence of cytological signs

of HPV infection in anal cytology is not considered as a critical index that may influence the outcomes of diagnosis. Nevertheless, screening based not only on SIL cells, which are present in a small number, but also on compression-positive binucleated cells and APK cells, which are critical cytological signs of HPV infection, may contribute to improving the diagnosis of AIN.

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#### Conflict of interest disclosures

The authors made no disclosures.

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