RESEARCH ARTICLE

# Significance of Compression in Binucleation while Differentiating Reactive Cellular Changes Between Human Papillomavirus and *Candida* Infections

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## Abstract

**Purpose:** Binucleation is a reactive cellular change (RCC) in Pap smears due to *Candida* infection. However, the origin of these binucleated cells as RCCs remains unclear. The purpose of this study was to examine binucleation in patients negative for intraepithelial lesion or malignancy (NILM) and infected with *Candida* and those infected with high-risk human papillomavirus (hr-HPV) and to clarify the origin of the binucleated cells. **Methods:** A total of 115 endocervical swab specimens with a combined diagnosis of NILM, *Candida* infection, and RCCs were used for this study. Pap smears were used to identify binucleated cells and then separate them into two groups, compression-positive and compression-negative. In addition, hr-HPV was detected using polymerase chain reaction (PCR) with a specific primer on the DNA extracted from the remaining residual cytology specimens. To make the hr-HPV-infected binucleated cells visible, an *in situ* PCR assay was performed on the Pap smear. **Result:** Of the 115 specimens, 69.6% contained binucleated cells, 26 (32.5%) showed only the compressed form, 35 (43.8%) showed only the non-compressed form, and 19 showed both the compressed and non-compressed forms of binucleated cells. Also, 34 specimens (29.6%) were positive for hr-HPV. The sensitivity and specificity of compression-positive binucleated cells were 91.2% and 82.7% ($p < 0.001$), but they were not significant in the compression-negative group ($p = 0.156$). Also, 34 cases with hr-HPV contained 99 compression-positive and 24 compression-negative cells. The hr-HPV-positive cells accounted for 68 (68.7%) of the 99 compression-positive and 2 (8.3%) of the 24 compression-negative binucleated cells as determined by an *in situ* PCR assay for hr-HPV. The relationship between compression and hr-HPV was statistically significant ($p < 0.001$). **Conclusion:** Compression-positive binucleated cells may be present as a result of hr-HPV infection and not RCC, which is caused due to inflammation in NILM cases infected with *Candida*.

**Keywords:** *Candida* infection- binucleated cell- compression- HPV

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## Introduction

In Pap smears, STD pathogens, such as *Candida*, can cause morphological epithelial changes, such as slight nuclear enlargement, mild hyperchromasia, binucleation, multinucleation, perinuclear halo, and eosinophilic changes of the cytoplasm (Solomon et al., 2004). These changes are defined as reactive cellular changes (RCCs) under the negative for intraepithelial lesion or malignancy (NILM) category in the Bethesda system. These morphological changes can mimic epithelial cellular changes observed in atypical squamous cells of undetermined significance (ASC-US) or other intraepithelial changes, which can cause diagnostic dilemmas when diagnosing Pap smears in the presence of STD pathogens (Migue et al., 1997, Soofer et al., 1997, Barcelos et al., 2006, Wong et al., 2008, Hall et al., 2009, Nasser et al., 2011). However, the possibility that the inflammatory cellular effects due to *Candida* infection do not affect ASC-US diagnoses was suggested by Hall (2009) and Nasser (2011). In contrast, 12%–45% of Pap smears with *Candida* infections have been reported to present with human papillomavirus (HPV) infections (Voog et al., 1995, Murta et al., 2000, Mendoza et al., 2013), and intraepithelial abnormality rates are higher in RCC-positive NILM cases than in RCC-negative ones (Singh et al., 1999, Choi et al., 2014). Thus, some RCCs due to infection with *Candida* or other STD pathogens may be associated with the presence of ASC-US or other intraepithelial abnormalities. Thus, some new criteria for RCC under NILM, which have not been defined in the Bethesda system, may be needed to account for possible underlying HPV infections or other intraepithelial changes.

Historically, nonclassic morphological signs of HPV

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infections in the NILM category included dyskeratosis, abortive keratosis, and bi/multinucleation (Schneider et al., 1987, Cramer et al., 1997, Bollmann et al., 2005, Nijhawan et al., 2010). Notable, the sensitivity and specificity for HPV detection in bi/multinucleated cells are high (Nijhawan et al., 2010). Moreover, recent studies exploring HPV infections and compression-positive binucleated cells reported that these cells can be associated with HPV infection and were useful in discriminating ASC-US diagnoses on Pap smears (Okayama et al., 2010a, Washiya et al., 2013).

The purpose of this study was to define the significance of binucleated reactive cells on Pap smears in the presence of inflammatory changes due to *Candida* infection. We reviewed Pap smears with a diagnosis of NILM with *Candida* infection to establish the presence of binucleated cells and to determine their relationship with HPV infection using *in situ* polymerase chain reaction (PCR).

## Materials and Methods

*Clinical samples*

The study protocol was approved by the ethics committee of the Faculty of Health Science, Kyorin University. The study subjects were patients who visited Fukui Maternity Clinic between January 2010 and September 2012. All liquid-based Pap smears of the cervix uteri obtained by cotton swab scraping were screened by a cytotechnologist. Among cases diagnosed as NILM having hyphae and/or blastospores of *Candida* spp., 115 showing RCCs, such as nuclear enlargement with slight focal hyperchromasia, binucleation, changes of orangeophilic cytoplasm, and perinuclear halos, were confirmed and used as specimens.

*High-risk HPV (hr-HPV) test*

DNA was extracted from residual liquid cervical cytology specimens (100 µl) using the High Pure PCR Template Preparation Kit (Roche Diagnostics, Mannheim, Germany). DNA from hr-HPV was amplified using PCR with specific primers for the HPV E6 region (Okayama et al., 2013). The PCR reaction mixture included 1× AmpliTaq Gold® 360 buffer, 2 mM $MgCl_2$, 0.025 U/µl AmpliTaq Gold 360 DNA Polymerase (Applied Biosystems, CA, USA), 1 µl DNA, and 0.5 pM primers (HPV 16, 18, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 68) in a total volume of 25 µl. PCR amplification was performed using a thermal cycler with 35 cycles of denaturation at 95°C for 30 s, annealing at 60°C for 30 s, and extension at 72°C for 30 s, including an initial denaturation step of 10 min and a final extension step of 5 min. Human β-actin expression, determined using an additional PCR, was used as an internal standard; the resulting amplicon was 262-bp long.

*Morphological classification and detection of binuclear squamous epithelial cells*

The smear preparations were rescreened and the cases showing binucleated squamous epithelial cells with positive and negative compressions in NILM with *Candida* infections were counted. Binucleated cells with their nuclei pressing against each other or with an adhesion distance of approximately >5 µm (Okayama et al., 2010a) were defined as compression-positive, whereas those with separate or overlapping nuclei or with an adhesion distance between the two nuclei of <5 µm were defined as compression-negative.

*HPV detection by in situ PCR using hr-HPV-specific primers*

The protocol for *in situ* PCR was based on a method devised by our team (Okayama et al., 2010b). After decolorizing a Pap smear specimen, endogenous peroxides were removed and proteolyzed using 0.01% trypsin (Sigma-Aldrich, Tokyo, Japan). The *in situ* PCR reaction mixture included 10 mM Tris-HCl (pH 8.0), 50 mM KCl, 3.75 mM $MgCl_2$, 0.045 U/ul Ex Taq DNA polymerase (Takara Bio, Shiga, Japan), 0.2 mM digoxigenin (DIG) labeling mix (Roche Diagnostics), and 1 pM HPV primers (Okayama et al., 2013). Specimens were enclosed within gaskets, reaction mixture was placed into the area surrounded by the gasket, and a plastic film was placed over the gasket to prevent evaporation. Slides were initially denatured for 10 minutes at 93°C, then subjected to 30 rounds of thermal cycling, which consisted of denaturation at 93°C for 1 min, annealing at 60°C for 1 min, and extension at 72°C for 1 min. DIG incorporated into PCR amplicons was detected using an immunoperoxidase assay with an anti-DIG antibody (Roche Diagnostics) for 1 h at room temperature. HPV-16-positive human cervical cancer cell line SiHa (ATCC® HTB-35) was use as a positive control and human promyelocytic leukemia cell line HL60 as a negative control.

*Statistical analysis*

Statistical analysis was performed using SPSS version 21.0 (SPSS, Chicago, IL, USA). Differences between groups were examined using the $X^2$ or Fisher's exact probability test according to the characteristics of data distribution. A p-value of <0.05 was considered statistically significant. The sensitivity and specificity of binary tests for detecting hr-HPV on cervical Pap smears based on the presence or absence of binucleated cells and whether they were compression-negative or compression-positive were calculated.

## Results

Of the 115 NILM cases with *Candida* infection, 80 (69.6%) contained binucleated cells. Among these cases, 26 (32.5%) showed only the compressed form, 35 (43.8%) showed only the non-compressed form, and 19 showed both the compressed and non-compressed forms of binucleated cells. In addition, 34 cases (29.6%) were positive for hr-HPV. Table 1 shows the relationship between binucleated cells and hr-HPV in Pap cervical smears as well as their sensitivity and specificity. The sensitivity and specificity for detecting hr-HPV were 94.1% (32/34) and 40.7% (33/81) in binucleated cells and 91.2% (31/34) and 82.7% (67/81) in compression-positive binucleated cells on cervical Pap smears, respectively; however, the sensitivity and specificity were not statistically significant

Table 1. Relationship between Binucleated Cells and High-Risk HPV in Cervical Pap Smear

| | High-risk HPV | | | | |
|---|---|---|---|---|---|
| | Positive N=34 | Negative N=81 | P-value | Sensitivity | Specificity |
| Binucleated cells + | 32 | 48 | 0.000 | 94.1% | 40.7% |
| Positive-compression + | 31 | 14 | 0.000 | 91.2% | 82.7% |
| Negative-compression + | 13 | 41 | 0.156 | 38.2% | 49.4% |

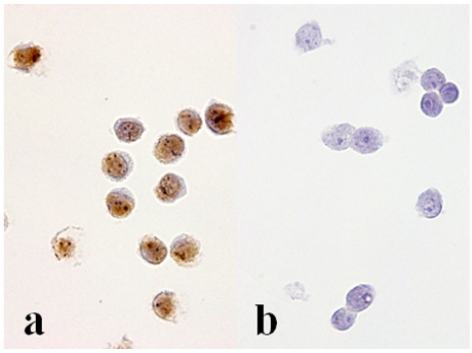


Figure 1. (a) SiHa cells as positive controls diffusely stained by *in situ* PCR with high-risk-HPV-specific primers. (b) HL60 cells as negative controls not stained by *in situ* PCR with high-risk-HPV-specific primers.

Table 2. Positive high-risk HPV by *in situ* PCR in binucleated cells

| | Number of cells | Number of high-risk HPV positive by in situ PCR | Positive rate | P-value |
|---|---|---|---|---|
| Positive-compression | 99 | 68 | 68.7% | 0.000 |
| Negative-compression | 24 | 2 | 8.3% | |

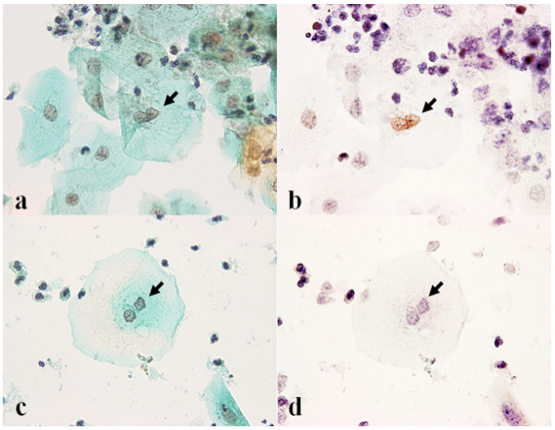


Figure 2. Compression-Positive Binucleated Cells: (a) Pap staining (40×), (b) positive nuclear staining by in situ PCR with high-risk-HPV-specific primers (40×). Compression-negative binucleated cells: (c) Pap staining (40×), d) negative nuclear staining by *in situ* PCR with high-risk-HPV-specific primers (40×).

for compression-negative binucleated cells ($p = 0.156$). In the 34 hr-HPV-positive cases, 99 compression-positive and 24 compression-negative cells were observed. The results of HPV detection using *in situ* PCR with hr-HPV-specific primers are shown in Figures 1 and 2. The nuclei stained positive in SiHa cells but negative in HL60 cells. Hr-HPV-positive cells accounted for 68 (68.7%) of the 99 compression-positive binucleated cells (Figure 2) and 2 (8.3%) of the 24 compression-negative binucleated cells (Figure 2) as determined by *in situ* PCR for hr-HPV. The relationship between compression and hr-HPV was statistically significant ($p < 0.001$).

## Discussion

Binucleated cells have always been recognized as one of the cytological changes of inflammation due to *Candida* infection. However, these cytological changes have not been clearly defined or shown in the fields of biochemistry and molecular biology (Kiviat et al., 1985). Also, because an association between *Candida* infection and intraepithelial lesions is speculated (Singh et al., 1999, Choi et al., 2014), it is worthwhile to see if binucleated cells are associated with HPV infection.

In the present study, we found binucleated cells in 70% of the cases infected with *Candida*; there were two types of binucleated cells, compression-positive and compression-negative. Before examining the essential cytological significance of these two types of binucleated cells, we tested NILM cases with *Candida* infection for hr-HPV as a surrogate marker and found that 29.6% of the cases were positive, and this was within the limits set by previous studies (Voog et al., 1995, Murta et al., 2000, Mendoza et al., 2013). Because the HPV positive rate of *Candida*-infected NILM cases was higher than that (approximately 20%) of the general Japanese female population (Sasagawa et al., 2016), inflammatory changes due to *Candida* infection could represent an increased possibility of an underlying HPV infection. inflammatory changes due to *Candida* infection could represent an increased possibility of an underlying HPV infection. *Candida albicans* secretes several proteolytic enzymes that support penetration and invasion, and by generating oxygen radicals, it can increase inflammation and tissue destruction (Danley et al., 1983, Schröter et al., 2000). The disruption of the inflamed cervical epithelium by STD pathogens has been hypothesized to increase the risk of HPV infection by allowing the virus to penetrate the basal cells of the epithelium (Roeters et al., 2010). In contrast, Ghosh (2017) reported that co-infection with *Candida* spp. did not increase the carcinogenic effect of HPV on the cervix. Thus, whether a relationship exists between the presence of *Candida* infection and endocervical lesions remains controversial.

We have examined binucleated cells using hr-HPV test, and only non-distinguished binucleated cells and compression-positive binucleated cells showed statistical significance ($p < 0.001$). In contrast, compression-negative binucleated cells showed no relationship with hr-HPV, and these cells may be associated with RCC due to inflammation. The sensitivities of both non-distinguished and compression-positive binucleated cells were >90%,

but their specificities were significantly different at 40.7% and 82.7%, respectively. In previous studies of HPV infection and its non-classical cytological morphology, an association of bi/multinucleated cells with HPV infection was reported, but the results of Bollmann (2005) and Nijhawan (2010) were significantly contrasting. Bollmann's study showed a sensitivity of 91% and a specificity of 40%, while Nijhawan's study showed the same as 40% and 86%, respectively. We believe that these disparities occurred due to different cell groups being targeted. We have only targeted *Candida*-infected cases, whereas others have targeted unsatisfactory ASC-US and RCCs, such as those associated with inflammation, cellular repair, and intrauterine contraceptive device or atrophy, as well as intraepithelial lesions. Interestingly, the findings of Bollmann (2005) and our study of binucleated cells with non-distinguished compression were very similar. It is unclear whether compression-positive binucleated cells were evaluated in Bollmann's non-classical cytological findings of binucleated cells with HPV infection; if these cells were not distinguished using the same compression-positive binucleated cell criteria, it would explain why our specificity value has increased.

Other studies have used molecular biology tests to determine HPV infection. These studies did not apply Pap smears, so it is unknown if their specimens contained binucleated cells. Thus, further studies are needed to determine if HPV infection causes binucleation. In our study, we implemented *in situ* PCR to visualize the HPV-infected binucleated cells. The hr-HPV-positive rate of compression-positive binucleated cells was eight times higher than that of compression-negative binucleated cells, with statistical significance. In cases that contained both compression-positive and compression-negative binucleated cells, the results were mostly hr-HPV-negative; therefore, we encourage investigators to distinguish the compression state of binucleated cells when evaluating the existence of hr-HPV. A previous study of compression-positive binucleated cells in abnormal cytology cases reported these cells as HPV-infected (Okayama et al., 2010a, Okodo et al., 2016); this result was also confirmed by other researchers (Washiya et al., 2013). It remains unclear why binucleated cells are compressed, but Washiya (2013) presumed that mitosis is completed with incomplete chromosomal segregation due to HPV infection, thus regenerating a nuclear envelope around the scattered chromosomes, which results in multinucleation.

These findings of compression-positive binucleated cells may help cytotechnologists to better understand and confidently diagnose RCCs due to *Candida* infection. However, with the rapid developments in molecular biology and with its highly sensitive and specific diagnostics becoming more popular, the use of cytological findings for HPV infection may seem as a step backward. We believe that this retrogressive step may still be effective if we can determine HPV infections on a Pap smear in a triage stage, potentially avoiding costly molecular biological HPV testing. Also, by pursuing more definitive cytological findings, these studies could lead to new cytological findings associated with the specific morphological changes of a tumor or lesion.

In conclusion, our study showed that binucleated cells present in NILM cases infected with *Candida* can be separated into compression-positive and compression-negative types, and the compression-positive cells are likely to be associated with hr-HPV infection and not with RCCs due to *Candida*-related inflammation. Future studies using larger samples should be conducted, and methods to rule out other residual confounding effects caused by non-HPV factors should be developed.

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