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# Investigation of the Possible Cytopathological Effect of Human Papillomavirus Infection on p-16$^{INK4a}$ Overexpressed Urothelial Carcinomas of the Bladder in the Urine

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**Abstract**

**Background and objectives:** Human papillomavirus (HPV) is well-known as the etiological agent for uterine cervical cancer or carcinoma of the oropharynx, anus and vulva. However, the cytopathologic effect of the HPV infection in urinary bladder tumors has rarely been reported. The present study has been carried out to search for cytopathologic differences between HPV positive and negative cases with urothelial carcinoma.

**Methods:** We examined 91 specimens from 63 patients who underwent transurethral resection or biopsy for papillary urothelial carcinomas between May 2010 and September 2012. p-16$^{INK4a}$ expression was evaluated by immunohistochemistry. Detection of HPV DNA was carried out by in situ hybridization on formalin-fixed, paraffin-embedded tissue sections. Urine smears were compared cytomorphologically between HPV-positive and HPV-negative cases.

**Results:** p-16$^{INK4a}$ overexpression was detected in 29 cases (31.9%, 29/91). Of them, HPV DNA was detected in 11 cases. No significant cytopathologic differences were found in tumor cells when HPV-positive and HPV-negative cases were compared.

**Conclusion:** HPV could be detected in urothelial carcinomas of the urinary bladder. There were no significant differences in cytopathologic features of urine smears between HPV-positive and HPV-negative cases with urothelial carcinomas. These results suggest that HPV infection in urinary tract does not add substantial clinically relevant importance to the carcinogenesis and cytomorphology of urothelial carcinomas.

**Keywords**: Human papillomavirus; Urinary bladder; p16$^{INK4}$; In situ hybridization; Urine; Cytology

## Introduction

Human papillomavirus (HPV) is well-known as the major etiological agent for uterine cervical cancer. Its possible relationship with cancers of the oropharynx, anus and vulva has also been investigated [1-7]. Over the past few decades, a considerable number of studies have been conducted to determine the role of HPV in urinary bladder tumors [8-17]. However, the most of their studies have focused on the detection of HPV infection, and little attention has been paid to the cytologic morphology correlated with HPV infection in urinary bladder tumor. On the other hand, detection of HPV-DNA and overexpression of p16 INK4a has been reported at a high rate in cervical squamous cell carcinoma, but there are only occasional reports of HPV-DNA and p16 INK4a expression in urothelial carcinoma of the bladder. The present study has been carried out to search for cytopathologic differences between HPV positive and negative cases with urothelial carcinoma and the possible role of p16 expression in the carcinogenesis of urothelial carcinoma or association with the HPV infection.

## Materials and Methods

### Cases

We examined 63 patients who underwent transurethral resection for papillary urothelial carcinomas, based on the 2004 WHO classification from May 2010 to September 2012 in the Department of Urology, Saiseikai Noe Hospital, Osaka, Japan. The present study included 91 tissue samples and 76 urine specimens (20 voided urines and 56 bladder washings), and was approved by the institutional "Ethical Review Board" of Saiseikai Noe Hospital.

### Immunohistochemistry for p16$^{INK4a}$

Immunohistochemical staining was carried out on 91 formalin-fixed, paraffin-embedded blocks. From each block, 4-μm-thick paraffin sections were cut and mounted on coated slides. The Ventana automated system (Ventana Automated Systems, Inc., Tucson, AZ) was used with an antibody p16$^{INK4a}$ (clone E6H4, 1: 200 dilution, Becton-Dickinson Company, Ltd), and a paraffin-embedded section of uterine cervical intraepithelial neoplasia was included as a positive control for each run. The expression of p16$^{INK4a}$ was evaluated in a semi-quantitative fashion as follows: score 0, no staining; score 1, <20%; score

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2, 21%–70%; score 3, >71% (Figures 1-3). The samples displaying score 3 immunoreactivity were considered to have overexpression of $p16^{INK4a}$ following the Nakazawa et al. criteria [18].

## HPV DNA in situ hybridization

ISH was carried out on sections showing $p16^{INK4a}$ overexpression on formalin-fixed, paraffin embedded tissue sections cut at a thickness of 4 μm. Following deparaffinization, the tissue was digested in protease solution for 7.5 minutes at 37°C. Slides were dehydrated in ascending order of ethanol concentration (70%, 95%, and 100%) for 1 minute each and air-dried. The probe used was Wide Spectrum HPV Biotinylated DNA probe, which detects HPVs 6, 11, 16, 18, 31, 33, 35, 45, 51, and 52 (Y1404, Dako North America Inc., Carpinteria, CA). It was applied to slides and coverslips were immediately placed over the probe solution and sealed with rubber cement. The slides were placed in a dry oven and denatured at 95°C for 5 minutes. Hybridization was carried out at 37°C overnight, and afterwards, the rubber cement and coverslip were removed and the slides immersed in the warmed stringent wash solution to incubate at 48°C for 30 minutes. Primary streptavidin-AP reagent was applied to the slides at room temperature for 20 minutes. BCIP/NBT substrate solution applied to the slides at room temperature for 2 hours. Tissues have been counterstained by Nuclear Fast Red solution. Positive ISH signal patterns were identified and classified by Cooper et al. [18,19] as follows: (1) punctate, when distinct dot-like intra-nuclear signals were stained (indicative of integrated HPV); (2) diffuse, when nuclei were completely stained (indicative of episomal HPV).

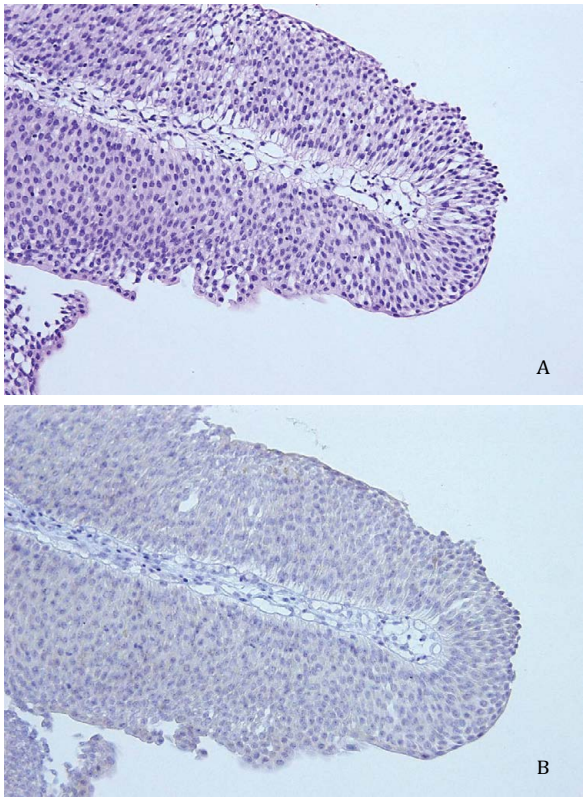


**Figure 1:** (A) Papillary urothelial carcinoma, low grade; (B) Score 0: no staining Expression of p16 is not detected at all. ( a. H.E. stain 100X; b. $p16^{INK4a}$ immunostain, 100X)..

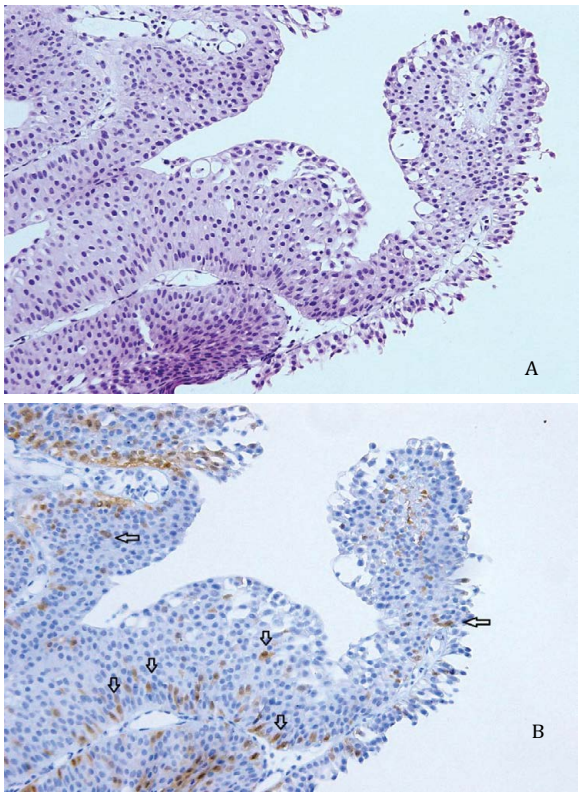


**Figure 2:** (A) Papillary urothelial carcinoma, low grade; (B) Score1: expression of p16 is detected in the nuclei and/or cytoplasm in less than 20%. (a. H.E. stain 100X; b. $p16^{INK4a}$ immunostain, 100X).

## Comparison of cytopathological finding

Urine cytologic specimens consisted of 20 voided urines and 56 bladder washings. They were prepared by conventional methods, such as Cytospin (Thermo Shandon, Pittsburgh, PA) or smear preparations following centrifugation, and fixed in 95% ethanol or air-dried for Papanicolaou staining or May-Grünwald-Giemsa staining. We compared the cytomorphological difference in urinary cytology between HPV-positive and HPV-negative cases. The cytopathological features of tumor cells were reviewed, including their background, arrangement, and cellular pleomorphism (variation of cell diameter occurring more than twice), and nuclear and cytoplasmic features. Furthermore, the presence of cytomorphological parameters associated with HPV infection considered previously by Bollmann et al. [20], such like koilocytosis, dyskeratocytes, abortive koilocytes, mild dyskeratosis, parakeratosis, mild nuclear hyperchromasia, mild nuclear variations, binucleation or multinucleation, measles cells, keratohyalin and keratohyalin-like granules, macrocytes, and cytoplasmic folding were reviewed.

## Statistical analysis

Data were entered into JMP8.0 (SAS Institute, Inc. North Carolina, USA) software and analyzed. Significant differences between groups were found using the Chi-square test and Fisher's exact test. *P*-values<0.05 were considered significant.

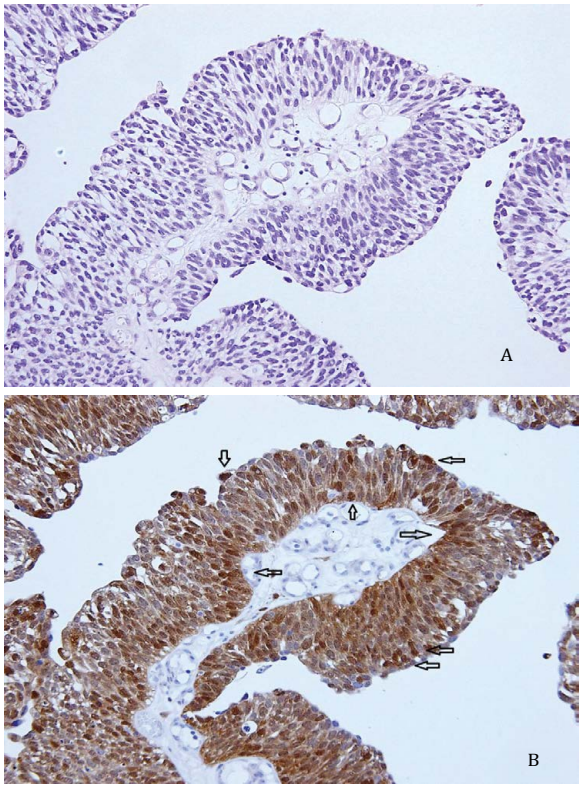


**Figure 3:** (A) Papillary urothelial carcinoma, high grade (H.E. stain 100X); (B) Score3: Over expression of p16 is detected in the nuclei and/or cytoplasm in more than 70%. (p16$^{INK4a}$ immunostain, 100X)

## Results

### Patient characteristics

Clinicopathological characteristics of our samples are shown in Table 1. The mean age at the time of urothelial carcinoma diagnosis was 75.3 years (range, 46-91 years). The patients included 72 males and 19 females. Fifty-four patients had low grade urothelial carcinomas (LGUC), and 37 patients had high grade urothelial carcinomas (HGUC). Seventy-four patients had non-invasive tumors, and 17 patients had invasive disease. Sixty-one patients had original tumors and 30 patients had recurrent disease.

### Immunohistochemistry for p16$^{INK4a}$

Twenty-nine of 91 (31.9%) patients showed overexpression (Score 3) of p16$^{INK4a}$ (Table 1). With regard to tumor grade, stage, and original or recurrent disease, overexpression of p16$^{INK4a}$ was detected in 14 (25.9%) of 54 LGUC, 15 (40.5%) of 37 HGUC, 22(29.7%) of 74 non-invasive tumors, 7 (41.2%) of 17 invasive disease, 25 (41.0%) of 61 original tumors, and 4 (13.3%) of 30 recurrent disease.

### Detection of HPV-DNA

HPV-DNA was detected in 11 (37.9%) out of 29 cases of p16$^{INK4A}$ overexpression (Table 2). As for tumor grade, stage, and original or recurrent, HPV-DNA was detected in 4 (28.6%) of 14 LGUC, 7 (46.7%) of 15 HGUC, 9 (40.9%) of 22 non-invasive tumors, 2 (28.6%) of 7 invasive cancer, 9 (36%) of 25 original tumors, and 1 (25%) of 4 recurrent disease. All of the HPV DNA-positive specimens showed punctate signals in the nuclei (Figure 4).

### Comparison of cytologic findings of HPV positive UC and HPV negative UC

Cytopathologic findings could be evaluated in 29 samples (8 voided urine, 21 bladder washings) consisting of 11 HPV-positive cases and 18 HPV-negative cases. The cytomorphological findings are summarized in Table 3. No significant differences were found in the cytology of tumor cells when HPV-positive case and HPV-negative cases were compared (Figure 5 and Table 3 with corresponding p-values). The cytomorphological parameters associated with HPV infection were observed in 2 (18.2%) cases of HPV-positive cases, and in three (16.7%) HPV-negative cases of benign squamous cells. The cytopathologic parameters observed were bi- or multinucleation, abortive koilocytes, and keratohyalin-like granules (Figure 6). No significant differences were present in cytomorphological findings among HPV-positive and HPV-negative cases.

## Discussion

In this study, detection of HPV-DNA by ISH was performed only on cases which had p16$^{INK4a}$ protein as a surrogate marker of HPV infection in cervical tissue with overexpression. p16$^{INK4a}$ binds to cyclin-dependent kinase4 and inhibits its activity [21,22]. In the cervix, HPV infection inactivates RB protein by HPV E7 protein, and then the free transcription factor E2F increases. The cell cycle proceeds from G1 phase to S phase and cell proliferation is enhanced. At this time, since the E2F protein induces the expression of p16$^{INK4a}$, it is overexpressed. Detection of HPV-DNA and overexpression of p16$^{INK4a}$ has been reported at a high rate in cervical squamous cell carcinoma [23-25], but reports of HPV-DNA and p16$^{INK4a}$ expression of the bladder have been few. Steinestel et al. [26] found the expression of p16$^{INK4a}$ in 25 specimens of 27 (92.6%), but HPV-DNA was not detected. Moreover, Piaton et al. [27,28] showed that in three patients with p16$^{INK4a}$ immunoreactive tumor cells and high risk-HPV in the urine, HPV genotyping and in situ hybridization for high risk-HPV were negative in tissue sections. However, Shigehara et al. [16] examined 106 cases of urothelial carcinoma, 4 cases of squamous cell carcinoma, 6 cases of adenocarcinoma, and one case of another bladder cancer and found HPV-DNA in 18 out of 117samples (15%). Among them, they reported score3 (>50% of the cells were positive) staining of p16 $^{INK4a}$ in10 samples [16]. In this study, we admitted overexpression in 29 of 91 papillary urothelial carcinoma samples. We conducted ISH, and HPV-DNA was detected in 4 of

| | | p16$^{INK4a}$ status | | | |
|---|---|---|---|---|---|
| | | Score 0 | Score 1 | Score 2 | Score 3 |
| **n=91** | | **42** | **12** | **8** | **29** |
| Age (mean ± S.D) | 75.3 ± 8.7 | | | | |
| Gender | | | | | |
| Male | 72 | 29 | 11 | 6 | 26 |
| Female | 19 | 13 | 1 | 2 | 3 |
| Tumor grade | | | | | |
| Low | 54 | 32 | 5 | 3 | 14 |
| High | 37 | 10 | 7 | 5 | 15 |
| Tumor invasion | | | | | |
| noninvasive | 74 | 38 | 7 | 7 | 22 |
| invasive | 17 | 4 | 5 | 1 | 7 |
| | | | | | |
| original | 61 | 24 | 7 | 5 | 25 |
| recurrence | 30 | 18 | 5 | 3 | 4 |

Score 0, no staining; Score1, <20%; Score2, 21%–70%; Score3, >71%

**Table 1:** Clinicopathological characteristics of the patients and correlation with p16$^{INK4a}$ immunophenotype.

| Case no. | Age | Sex | Tumor grade | Tumor invasion | Original/Recurrence | ISH signal pattern |
|---|---|---|---|---|---|---|
| 1 | 64 | M | low | noninvasive | recurrence | punctate |
| 2 | 81 | M | low | noninvasive | original | punctate |
| 3 | 65 | M | low | noninvasive | original | punctate |
| 4 | 79 | M | low | noninvasive | original | punctate |
| 5 | 79 | F | high | noninvasive | original | punctate |
| 6 | 46 | M | high | noninvasive | original | punctate |
| 7 | 60 | M | high | noninvasive | original | punctate |
| 8 | 68 | M | high | noninvasive | original | punctate |
| 9 | 77 | M | high | noninvasive | original | punctate |
| 10 | 72 | M | high | invasive | original | punctate |
| 11 | 74 | M | high | invasive | original | punctate |

M: Male; F: Female

**Table 2:** Clinicopathological features of the eleven cases of UC with presence of HPV-DNA.

| | Voided urine n=5 | | Bladder washings n=12 | | p-value |
|---|---|---|---|---|---|
| | HPV (+) n=1 | HPV (-) n=4 | HPV (+) n=4 | HPV (-) n=8 | |
| **General features** | | | | | |
| **Background** | | | | | |
| clean | 0 | 2 ( 50% ) | 1 ( 25% ) | 4 ( 50% ) | 0.338 |
| Inflammation | 0 | 0 | 1 ( 25% ) | 0 | 0.294 |
| Bloody | 1 ( 100% ) | 2 ( 50% ) | 3 ( 75% ) | 3 ( 37.5% ) | 0.294 |
| Tumor diathesis | 1 ( 100% ) | 0 | 1 ( 25% ) | 1 ( 12.5% ) | 0.191 |
| **Arrangement** | | | | | 1.000 |
| Mostly clusters | 0 | 2 ( 50% ) | 2 ( 50% ) | 4 ( 50% ) | |
| Single cell and clusters | 0 | 1 ( 25% ) | 1 ( 25% ) | 2 ( 25% ) | |
| Mostly single cells | 1 ( 100% ) | 1 ( 25% ) | 1 ( 25% ) | 2 ( 25% ) | |
| **Cell uniformity** | | | | | 0.117 |
| Monomorphic | 0 | 3 ( 75% ) | 2 ( 50% ) | 7 ( 87.5% ) | |
| Pleomorphic | 1 ( 100% ) | 1 ( 25% ) | 2 ( 50% ) | 1 ( 12.5% ) | |
| ***Nuclear features*** | | | | | |
| **Chromatin pattern** | | | | | 1.000 |
| Fine granular | 0 | 3 ( 75% ) | 2 ( 50% ) | 2 ( 25% ) | |
| Granular | 1 ( 100% ) | 1 ( 25% ) | 2 ( 50% ) | 6 ( 75% ) | |
| **Conspicuous nucleoli** | | | | | 0.538 |
| Absent | 1 ( 100% ) | 3 ( 75% ) | 2 ( 50% ) | 7 ( 87.5% ) | |
| Indistinct/small | 0 | 1 ( 25% ) | 1 ( 25% ) | 1 ( 12.5% ) | |
| Present/occasional | 0 | 0 | 1 ( 25% ) | 0 | |
| ***Cytoplasmic features*** | | | | | |
| **Border** | | | | | 0.620 |
| Indistinct | 0 | 1 ( 25% ) | 3 ( 75% ) | 4 ( 50% ) | |
| Distinct | 1 ( 100% ) | 3 ( 75% ) | 1 ( 25% ) | 4 ( 50% ) | |
| **Texture** | | | | | 0.319 |
| Homogenous | 1 ( 100% ) | 2 ( 50% ) | 0 | 6 ( 75% ) | |
| Variable | 0 | 2 ( 50% ) | 4 ( 100% ) | 2 ( 25% ) | |

**Table 3:** Comparison of cytopathological findings between HPV-positive UC and HPV-negative UC.

14 LGUC cases (28.6%) and 7 of 15 HGUC cases (46.7%). Previous reports showed either a high rate in Grade 3 [10,17] or a higher rate in Grade 1 [16]. In this study, HGUC showed a higher detection rate of HPV-DNA. This controversy is probably related to our selected cases having IHC overexpression of $p16^{INK4a}$. Criteria of overexpression of $p16^{INK4a}$ in tissue specimens varied in the literature [3,16,25,27,29]. In our study, overexpressed positive findings meant that there was diffused distribution in more than 70% of the nucleus and cytoplasm of tumor cells. In Shigehara's report [16], among 18 HPV-DNA positive cases there were 6 cases of $p16^{INK4a}$ from 20% to 50%. They were 3 cases of urothelial carcinoma, Grade 1>2, and 3 cases of urothelial carcinoma, Grade 1. It is therefore expected when the threshold of overexpression of $p16^{INK4a}$ is lowered, HPV-DNA detection by LGUC will rise. Differences in cellular findings caused by the presence or absence of HPV infection was not observed in papillary urothelial carcinoma in this study. Having an HPV infection suggests the presence of atypical squamous cells, koilocytes, dyskeratotic cells and multinucleated cells in cervical smears [20]. These cells appear mainly at the stages of CIN1 and CIN2 but are rare in the stage of invasive cancer, except when associated with CIN1 and CIN2 lesions. HPV infection is not only involved in squamous cell carcinoma, but also adenocarcinoma in the uterine cervix [30,31]. However, the cellular changes of HPV infection in glandular cells are not well established. In urothelial cells also, cellular changes due to HPV infection is not well understood. HPV-DNA detected by ISH method in this study showed the all integrate pattern; no episomal pattern was observed. Because of these results, finding

the cytologic differences between HPV-DNA positive and negative UC cases on urine was difficult. Also, in the urine cytology specimens, because HPV-DNA positive cases can focus on non-tumor cells unlike HPV-DNA negative cases, significant differences in the incidence of abortive koilocytes or bi-nucleation were observed. Atypical squamous cells that appeared in urine cytology specimens of HPV-DNA positive cases in this study were of the "Non-classic type" rather than "Classic type" which includes koilocytes and dyskeratotic cells. There are many established reports of the appearance of atypical squamous cells of non-classic type in uterine cervical smears, and there are types of observed

**Figure 4:** Detection of HPV DNA with in situ hybridization method showing nuclear punctate signals (HPV ISH 1000X).

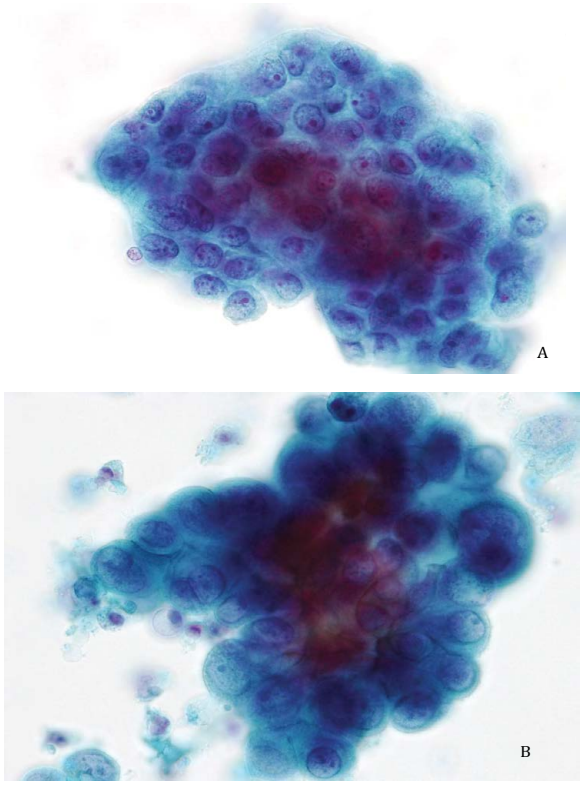


**Figure 5:** Cytopathological findings of tumor cells. The difference was not observed in the cell findings of tumor cells with HPV-negative and positive cases. (A) HPV negative of LGUC; (B) HPV positive of HGUC; (C) HPV negative of HGUC, Papanicolaou stain 1000X).

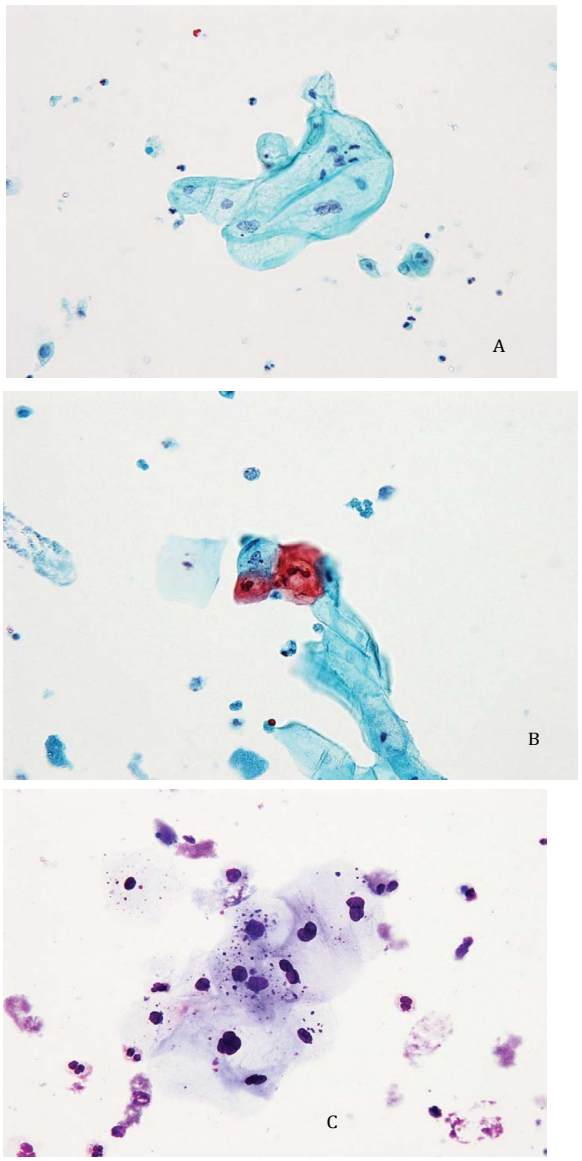


**Figure 6:** Cytopathological features of squamous cells. "Non-classical type" observed in voided urine cytology with HPV-positive cases (A and B: binucleate cell, papanicolaou stain 400X) and washing fluid cytology with HPV-negative cases (C: keratohyalin-like granules, May-Grunwald-Giemsa stain 400X).

parameters or combination of parameters indicative of HPV infection [20,31]. However, atypical squamous cells that appeared in HPV-DNA positive cases were observed in both cases of the voided urine and it was not possible to limit them from the urinary bladder epithelium.

Cancer generated from uterine cervix, oral cavity, tonsil, pharynx, anus, vulva, vagina and penis are carcinogenic by HPV infection as observed by IARC. Although attempts have been made to study urinary bladder cancer associated with HPV infection, carcinogenic HPV in bladder cancer is still controversial. Our results support the theory that HPV infection in urinary tract does not add clinically relevant importance to the carcinogenesis and cytomorphology of urothelial carcinomas. Further investigations are needed to clarify the role and correlation between bladder cancer and HPV infection.

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