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p16 expression is not associated with human papillomavirus in urinary bladder squamous cell carcinoma

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Squamous cell carcinoma of the urinary bladder is unusual and of unknown etiology. There is a well-established association between human papillomavirus (HPV) infection and the development of cervical and head/neck squamous cell carcinomas. However, the role of HPV in the pathogenesis of squamous cell carcinoma of the urinary bladder is uncertain. The purposes of this study were to investigate the possible role of HPV in the development of squamous cell carcinoma of the urinary bladder and to determine if p16 expression could serve as a surrogate marker for HPV in this malignancy. In all, 42 cases of squamous cell carcinoma of the urinary bladder and 27 cases of urothelial carcinoma with squamous differentiation were investigated. HPV infection was analyzed by both *in situ* hybridization at the DNA level and immunohistochemistry at the protein level. p16 protein expression was analyzed by immunohistochemistry. HPV DNA and protein were not detected in 42 cases of squamous cell carcinoma (0%, 0/42) or 27 cases of urothelial carcinoma with squamous differentiation (0%, 0/15). p16 expression was detected in 13 cases (31%, 13/42) of squamous cell carcinoma and 9 cases (33%, 9/27) of urothelial carcinoma with squamous differentiation. There was no correlation between p16 expression and the presence of HPV infection in squamous cell carcinoma of the bladder or urothelial carcinoma with squamous differentiation. Our data suggest that HPV does not play a role in the development of squamous cell carcinoma of the urinary bladder or urothelial carcinoma with squamous differentiation. p16 expression should not be used as a surrogate marker for evidence of HVP infection in either squamous cell carcinoma of the urinary bladder or urothelial carcinoma with squamous differentiation as neither HVP DNA nor protein is detectable in these neoplasms.
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Primary squamous cell carcinoma of the urinary bladder is an uncommon malignant neoplasm, the etiology of which is not well understood. It accounts for 3–5% of bladder cancer in Western populations.[1–4] In areas of endemic *Schistosoma haematobium* (bilharziasis) infections, primarily in Middle Eastern countries, the contribution of squamous cell carcinoma to overall bladder cancer incidence is reportedly as high as 75%.[5,6] In Western populations, the development of urinary bladder squamous cell carcinoma has been attributed to chronic irritation from such things as urinary calculi, catheters, diverticula, or chronic nonbilharzial infections. In comparing reported cases of muscle-invasive bladder

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cancer, urinary bladder squamous cell carcinoma consistently presents at a higher stage and carries a worse-stage matched survival than urothelial carcinoma of the bladder.

While the association between human papillomavirus (HPV) and uterine cervical and head/neck squamous cell carcinoma is well established, the role of HPV in the pathogenesis of urinary bladder squamous cell carcinoma is not defined clearly. In urothelial carcinoma of the bladder, HPV has been suggested as a causative agent by some,[7–9] while others disagree with this concept.[10–12] p16 expression is often used as a surrogate marker for HPV infection in cervical neoplasia and squamous cell carcinoma at other organ sites.[13–33] However, whether p16 expression can be used as a surrogate marker for HPV infection in urinary bladder squamous cell carcinoma is also poorly understood. The purposes of this study were to investigate the possible role of HPV in the development of squamous cell carcinoma of the urinary bladder and to determine if p16 expression could serve as a surrogate marker for HPV in this malignancy.

Materials and methods

A total of 69 cases of interest were retrieved from the surgical pathology files of participating institutions, between the years of 1992 and 2011. Of these cases, 52 were from cystectomy specimens, 14 from transurethral resections, and the remaining 3 cases were from biopsy material. The study included 42 cases of pure squamous cell carcinoma urinary bladder and 27 cases of urinary bladder urothelial carcinoma with squamous differentiation. The latter subset cases were defined by tumors that exhibited keratin pearls, intercellular bridging, or intercellular keratin in the areas of squamous differentiation with at least some areas of classic urothelial carcinoma present. All the cases were reviewed and the diagnoses confirmed. None of our cases in this study were known or suspected of having a history of infection with *S. haematobium.* One case of 'urinary bladder squamous cell carcinoma' from a 57-year-old woman was positive for HPV. However, after reviewing the clinical chart, the patient had a history of uterine cervical squamous cell carcinoma. Therefore, the correct diagnosis for this case was uterine cervical squamous cell carcinoma involving the urinary bladder, and the case was excluded from the study.

Immunohistochemical studies were conducted on 5-μm formalin-fixed, paraffin-embedded tissue sections using an anti-HPV mouse monoclonal antibody (Clone K1H8, 1:50; Dako, Carpinteria, CA, USA) and an anti-p16 mouse monoclonal antibody using the CINtec Histology kit (CINtec Histology, Westborough, MA, USA). The HPV antibody can detect HPV subtypes 6, 11, 16, 18, 31, 33, 42, 51, 52, 56 and 58. Nuclear staining was considered positive for HPV. HPV *in situ* hybridization was performed using Inform HPV II Family 6 Probe (detecting HPV genotypes 6 and 11) and HPV III Family 16 Probe (detecting HPV genotypes 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, and 66) (Ventana Medical Systems, Tucson, AZ, USA).

The CINtec Histology kit involves a two-step antibody method for the detection of the human p16 antibody. The first antibody is a monoclonal mouse antibody (E6H4) directed towards the human p16 protein. A secondary antibody, for visualization purposes, involves a polyclonal goat anti-mouse antibody conjugated with horseradish peroxidase. These steps were performed and have only been validated on formalin-fixed, paraffin-embedded tissue after heat-induced epitope retrieval at 95–99°C in the included epitope retrieval solution. For p16 immunohistochemistry, any nuclear immunoreactivity of p16 was deemed positive. Only nuclear staining was considered as true immunoreactivity, but the presence of cytoplasmic staining was recorded. Appropriate negative and positive controls were used.

Results

The clinicopathological information is summarized in Table 1. Most cystectomy cases were found to be of high stage (pT3–pT4) for both squamous cell carcinoma (23 of 31, 74%) and urothelial carcinoma with squamous differentiation (15 of 21, 71%). Of the 17 noncystectomy specimens, 14 were pT2.

Table 1 Clinicopathological information for urinary bladder squamous cell carcinoma and urothelial carcinoma with squamous cell differentiation

	Urinary bladder squamous cell carcinoma n = *42*	*Urothelial carcinoma with squamous differentiation* n = *27*
Age (year)	37–96 (mean, 64)	49–101 (mean, 75)
Male/female (ratio)	15/27 (1.8 F:M)	21/6 (3.5 M:F)
Tumor size[a] (cm)	1.6–14 (mean, 6)	0.3–8.5 (4.4)
Tumor stage		
Cystectomy specimens (n = *52*)		
pTa	0	2
pT1	1	1
pT2	7	3
pT3	14	10
pT4	9	5
TUR and biopsy specimens[b] (n = *17*)		
Ta	0	0
T1	3	0
T2	8	6

[a]Tumor size statistics only include cases from resection specimens.
[b]Transurethral resections (TUR) and biopsy specimens can only be pathologically staged as high as pT2.

There was a 1:1 male-to-female ratio in these patients and they had a mean age of 63 years (range of 37–84 years). Staging characteristics were similar to that seen in the overall study population.

The results of immunohistochemical staining for HPV and p16 and *in situ* hybridization for HPV are summarized in Table 2. HPV DNA and protein were not detected in any of the 42 cases of squamous cell carcinoma of the urinary bladder (0%, 0/42) or in any of the 27 cases of urothelial carcinoma with squamous differentiation of the urinary bladder (0%, 0/27). p16 expression was detected in 13 cases (31%, 13/42) of squamous cell carcinoma and 9 cases (33%, 9/27) of urothelial carcinoma with squamous differentiation (Figures 1–6 and Table 2). The staining extent is summarized in Table 3. While nuclear staining was the only parameter used in scoring, only two cases demonstrated pure nuclear staining. The majority showed focal to diffuse cytoplasmic staining coupled with nuclear staining. Pure cytoplasmic staining was not identified in any examined case. Concordant staining between the urothelial and squamous components of the urothelial carcinoma with squamous differ-

Table 2 Results of HPV *in situ* hybridization (ISH) and immunohistochemical (IHC) staining and p16 IHC staining in urinary bladder squamous cell carcinoma and urothelial carcinoma with squamous differentiation

		HPV-IHC		*HPV-ISH*		*p16*	
	n	*+ (%)*	*– (%)*	*+ (%)*	*– (%)*	*+ (%)*	*– (%)*
Urinary bladder squamous cell carcinoma	42	0 (0%)	42 (100%)	0 (0%)	42 (100%)	13 (31%)	29 (69%)
Urothelial carcinoma with squamous differentiation	27	0 (0%)	27 (100%)	0 (0%)	27 (100%)	9 (33%)	18 (67%)

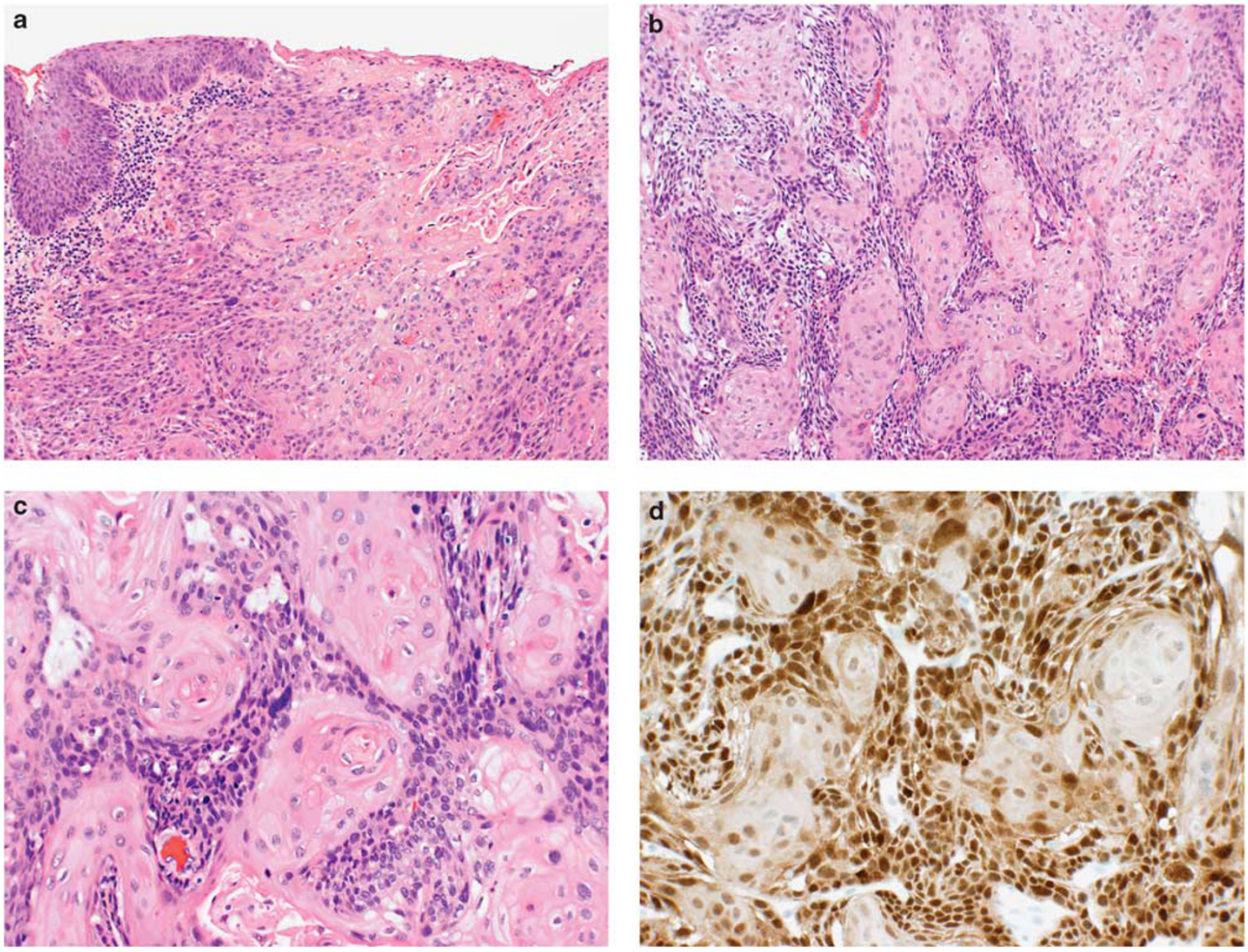

Figure 1 Squamous cell carcinoma of the urinary bladder (**a–c**). p16 immunostaining of the same case demonstrating strong nuclear staining and less intense cytoplasmic staining (**d**).

entiation was seen. The p16 expression in all cases occurred in the absence of demonstrable HPV DNA or HPV protein expression. p16 immunoreactivity did not correlate with any clinicopathological characteristics.

Discussion

Information regarding an association between HPV infection and urinary bladder squamous cell carcinoma is limited. In this study, we were unable to detect either HPV DNA by *in situ* hybridization or HPV protein expression by immunohistochemical staining in 42 cases of primary urinary bladder squamous cell carcinoma or in 27 cases of urothelial carcinoma with squamous differentiation. These findings indicate that HPV does not play a demonstrable role in the development of urinary bladder squamous cell carcinoma or urothelial carcinoma with squamous differentiation.

The role of HPV infection in the genesis of urothelial neoplasms has been a subject of controversy. Many investigators have found little or no correlation between HPV infection and urothelial carcinoma and have concluded that the virus does

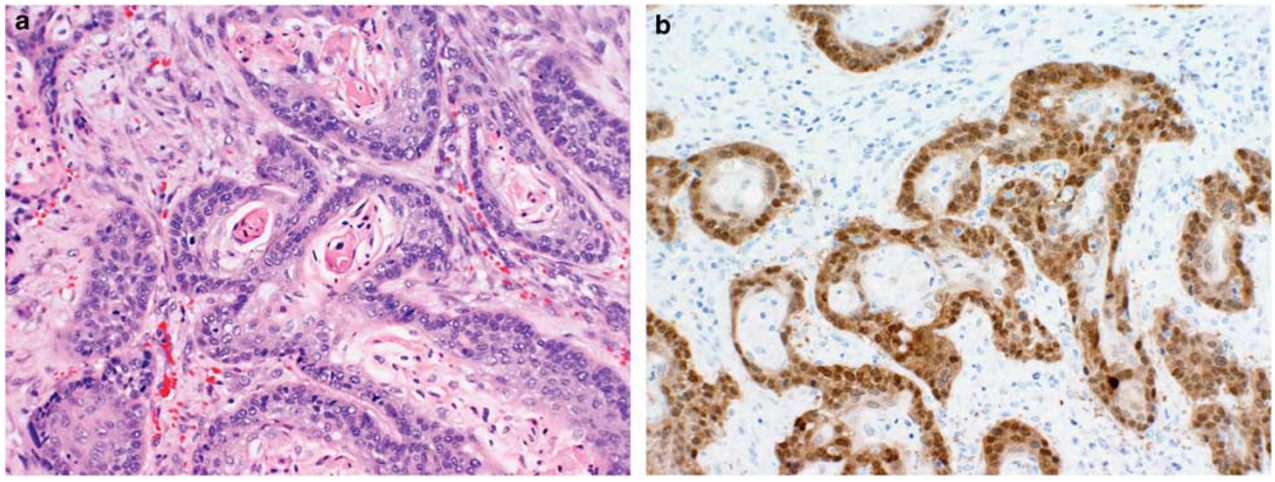

Figure 2 Squamous cell carcinoma of the urinary bladder showing well-formed keratin pearls (**a**) and corresponding immunoreactivity with p16 (**b**).

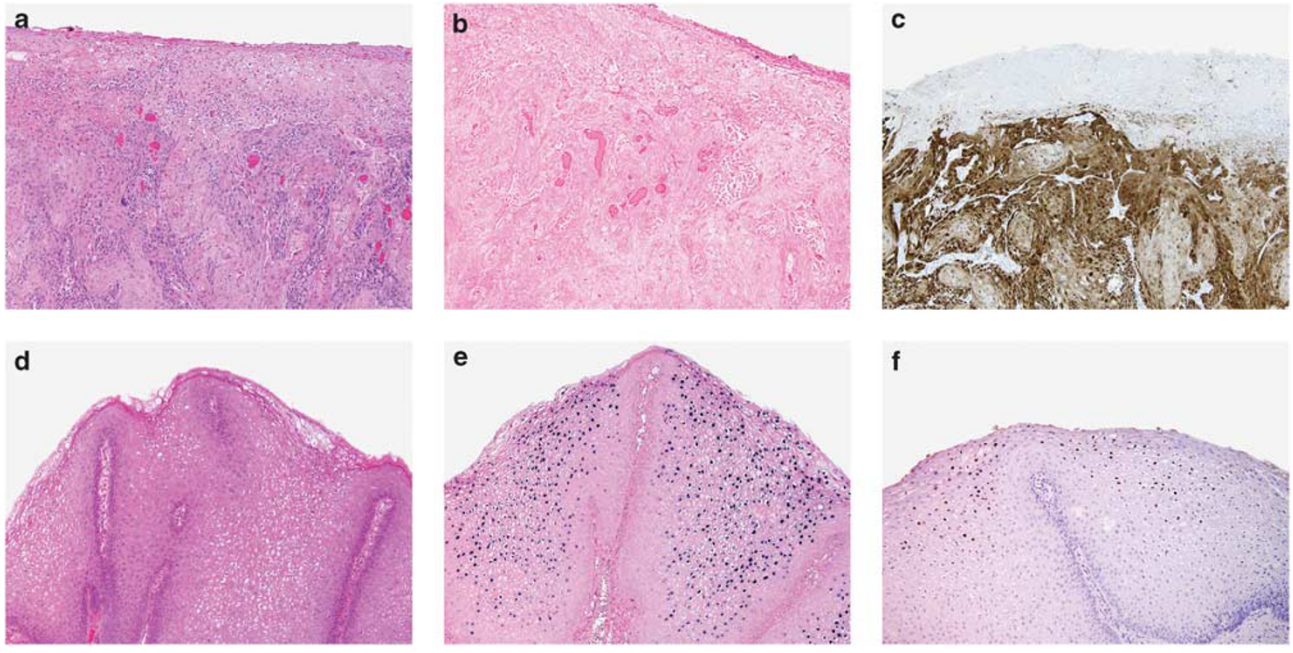

Figure 3 Squamous cell carcinoma of the urinary bladder (**a**) with negative human papillomavirus (HPV) *in situ* hybridization (ISH) (**b**) and positive p16 staining (**c**). A case of confirmed HPV-infected uterine cervical squamous cell dysplasia was used as positive control (**d**) with HPV-ISH (**e**) and HPV immunostaining (**f**), both displaying strong nuclear positivity.

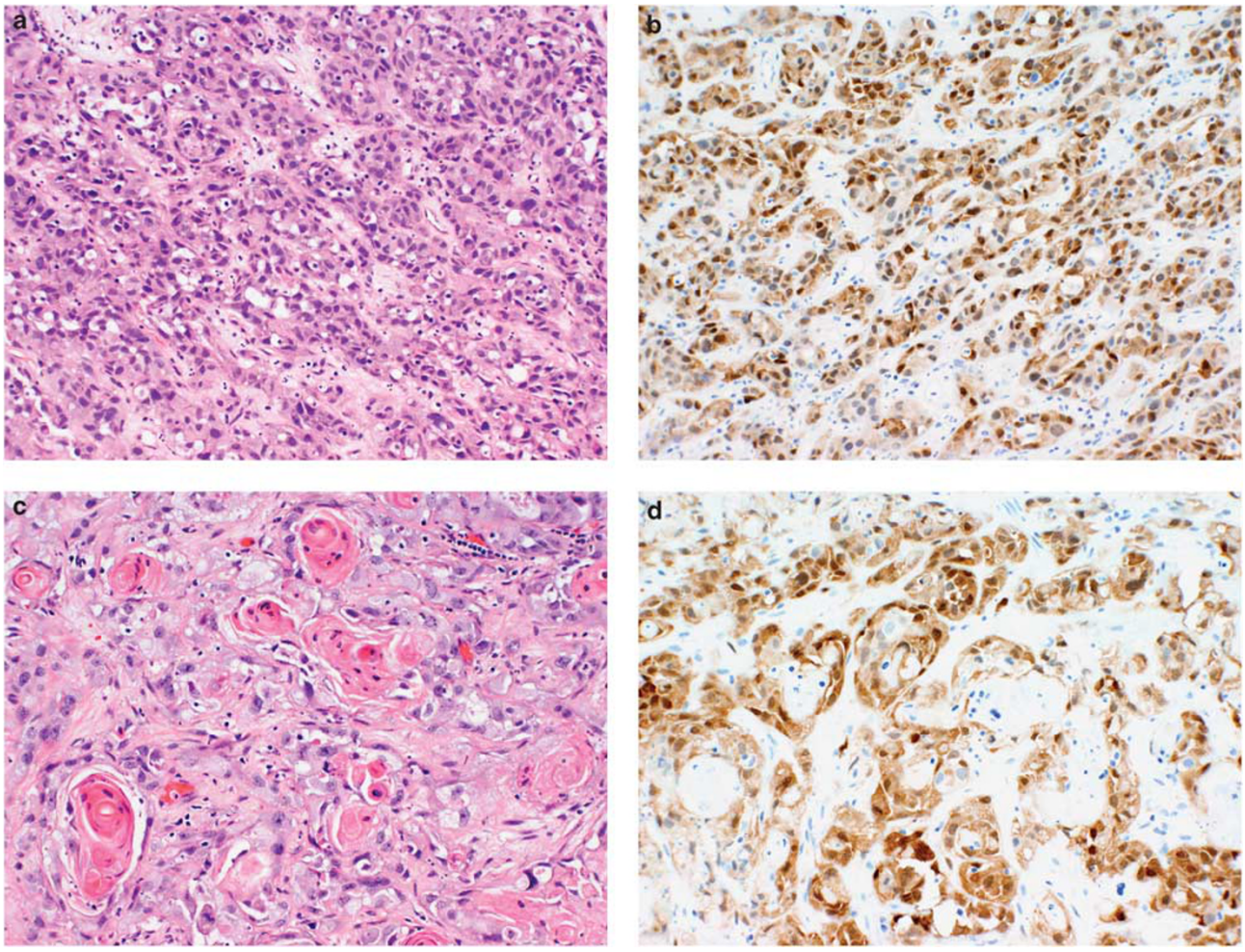

Figure 4 Urothelial carcinoma with squamous differentiation. The classic urothelial component is demonstrated in part (**a**) with its corresponding p16 immunostaining in (**b**). Well-defined squamous differentiation from the same case is seen in part (**c**) with its corresponding p16 immunostaining in part (**d**).

not play an etiological role.[10–12] However, in a study involving Iranian patients, HPV DNA was detected in slightly over 35% of urothelial neoplasia cases, and 81% of the viral strains identified were HPV type 18, which is considered a high-risk strain for the development of uterine cervical neoplasia.[34] More recently, Li *et al*[7] have also shown the presence of HPV infection in bladder cancer. They implicated HPV type 16, which is also considered a high-risk strain for the development of uterine cervical neoplasia. Shigehara *et al*[9] have also shown HPV infection in bladder cancer. Their study showed a predilection for the virus in low-grade tumors in young patients (<60 years of age). Interestingly, they also reported p16 expression in 94% of cases with HPV infection.[9]

The signal-transduction pathways in HPV-related squamous cell carcinoma have been well studied in the uterine cervix.[28] The HPV E7 protein inactivates the retinoblastoma tumor suppressor gene, which normally inhibits p16. Cervical cancer associated with HPV, therefore, has increased p16 expression[29,30] and its expression is used by some as a surrogate marker for HPV infection in cervical lesions.[13–31] It has been hypothesized that HPV infection might similarly play a role in the development of urinary bladder squamous cell carcinoma. In contrast to our findings and the previously mentioned reports, others have shown a positive correlation between high-risk HPV infection and high-grade urothelial cancers of the bladder.[7–9,35,36] Cai *et al*[37] have proposed that HPV may have a causative role in the genesis of urothelial neoplasms, particularly in nonmuscle-invasive bladder cancer. In our study, we detected p16 expression in 31% (13/42) of cases of urinary bladder squamous cell carcinoma and in 33% (9/27) of cases of urinary bladder urothelial carcinoma with squamous differentiation. However, despite the presence of p16 expression, none of these tumors had evidence of HPV infection at either the DNA level or the protein level, as measured by *in situ* hybridization or immunohistochemical staining, respectively. In separate studies using *in situ* hybridization, Guo

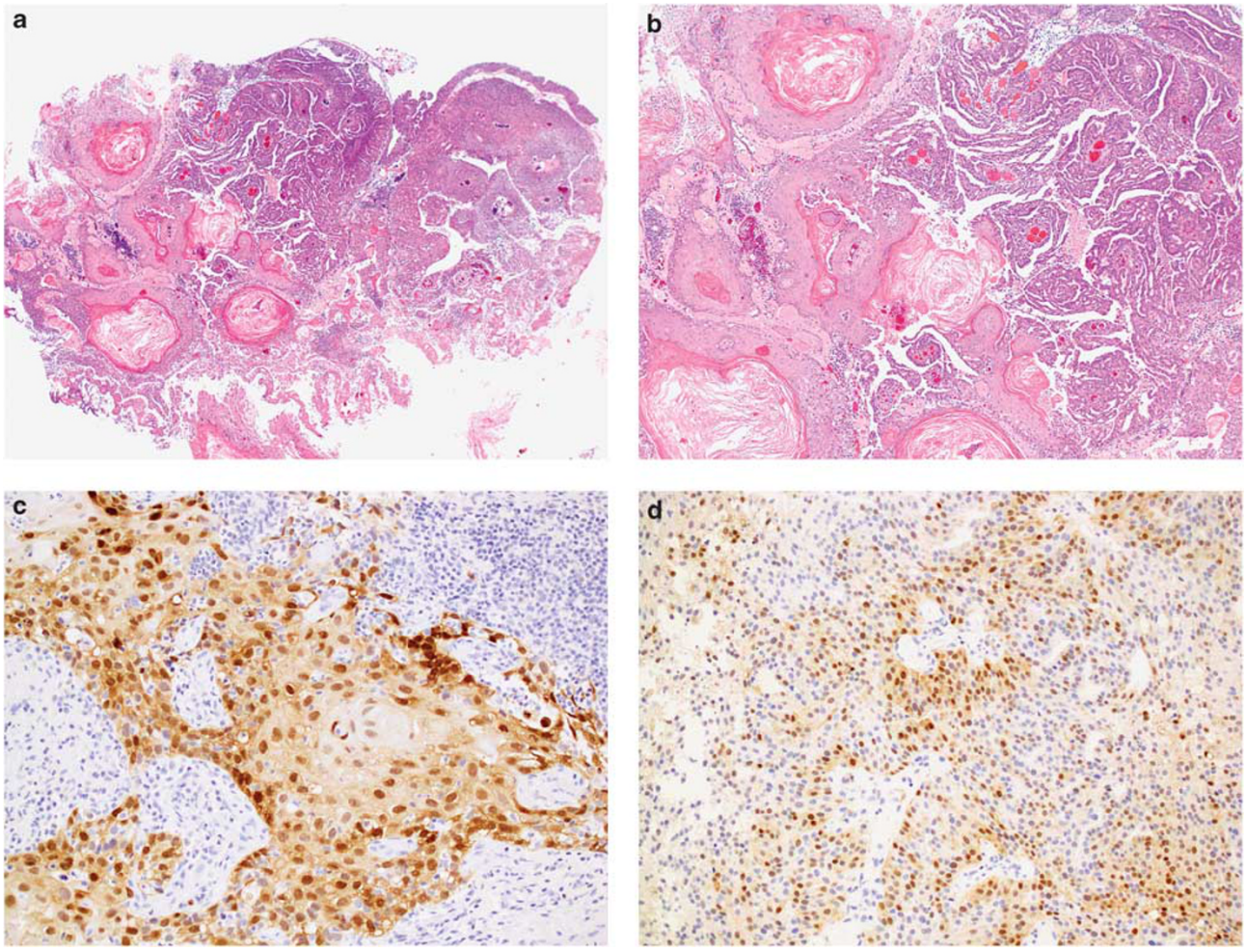

Figure 5 The mixed morphology comprising the classic urothelial and squamous differentiation components of urothelial carcinoma with squamous differentiation are demonstrated clearly in this case (**a** and **b**). p16 immunostaining is shown in both the squamous (**c**) and the urothelial (**d**) components from this case.

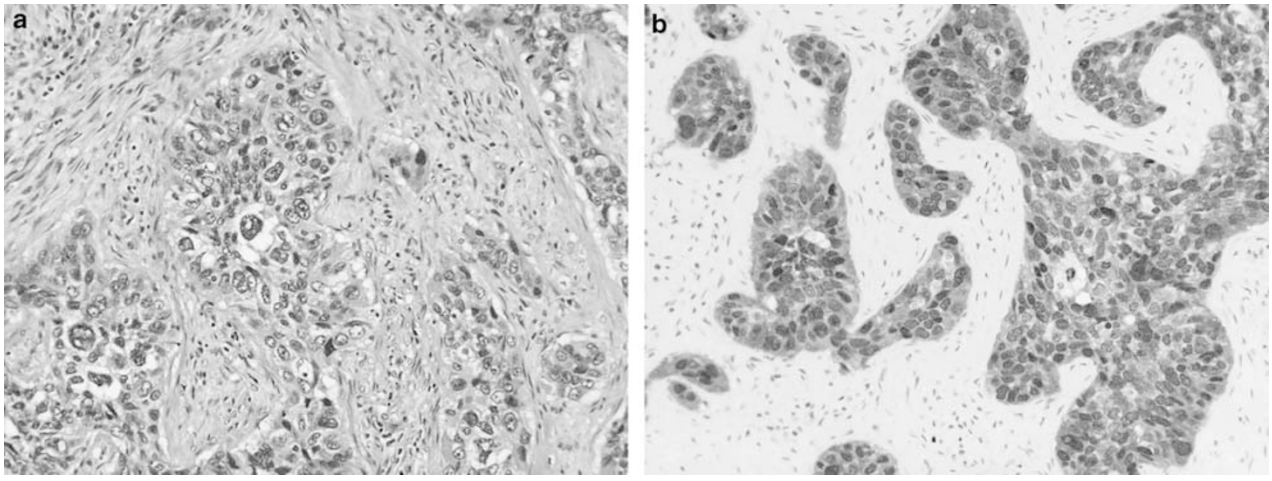

Figure 6 High-grade invasive urothelial carcinoma with strong p16 immunostaining (**a** and **b**).

et al[38] and Westenend *et al*[39] found no HPV DNA in any of the 32 cases of urinary bladder squamous cell carcinoma that they evaluated. More recently, using three different polymerase chain reaction techniques, Ben Selma *et al*[40] did not find HPV DNA in 125 cases of urinary bladder carcinoma, including five cases of squamous cell carcinoma. These findings, in concordance with our findings, further

Table 3 Extent of p16 immunostaining in urinary bladder squamous cell carcinoma and urothelial carcinoma with squamous differentiation

		Percentage of p16 staining			
	n	*0%*	*1–10%*	*10.1–74.9%*	*75–100%*
Urinary bladder squamous cell carcinoma	42	29	3	2	8
Urothelial carcinoma with squamous differentiation	27	18	1	1	7

emphasize the notion that HPV infection does not play a role in the pathogenesis of urinary bladder squamous cell carcinoma.

In summary, our findings indicate that HPV infection does not play a role in the development of squamous cell carcinoma of the urinary bladder or urothelial carcinoma with squamous differentiation. These findings also emphasize that p16 expression, although demonstrable in about one-third of our cases, should not be used as a surrogate marker for evidence of HPV infection in either squamous cell carcinoma of the urinary bladder or urothelial carcinoma with squamous differentiation, as neither HPV DNA nor protein was detected in these neoplasms.

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Disclosure/conflict of interest

The authors declare no conflict of interest.

References

1 Lagwinski N, Thomas A, Stephenson AJ, *et al.* Squamous cell carcinoma of the bladder: a clinicopathologic analysis of 45 cases. Am J Surg Pathol 2007; 31:1777–1787.
2 Kantor AF, Hartge P, Hoover RN, *et al.* Epidemiological characteristics of squamous cell carcinoma and adenocarcinoma of the bladder. Cancer Res 1988;48: 3853–3855.
3 Dahm P, Gschwend JE. Malignant non-urothelial neoplasms of the urinary bladder: a review. Eur Urol 2003;44:672–681.
4 Kassouf W, Spiess PE, Siefker-Radtke A, *et al.* Outcome and patterns of recurrence of nonbilharzial pure squamous cell carcinoma of the bladder: a contemporary review of The University of Texas MD Anderson Cancer Center experience. Cancer 2007;110:764–769.
5 Mostafa MH, Sheweita SA, O'Connor PJ. Relationship between schistosomiasis and bladder cancer. Clin Microbiol Rev 1999;12:97–111.
6 Shokeir AA. Squamous cell carcinoma of the bladder: pathology, diagnosis and treatment. BJU Int 2004;93:216–220.
7 Li N, Yang L, Zhang Y, *et al.* Human papillomavirus infection and bladder cancer risk: a meta-analysis. J Infect Dis 2011;204:217–223.
8 Lopez-Beltran A, Escudero AL. Human papillomavirus and bladder cancer. Biomed Pharmacother 1997;51: 252–257.
9 Shigehara K, Sasagawa T, Kawaguchi S, *et al.* Etiologic role of human papillomavirus infection in bladder carcinoma. Cancer 2011;117:2067–2076.
10 Boucher NR, Anderson JB. Human papillomavirus and bladder cancer. Int Urogynecol J Pelvic Floor Dysfunct 1997;8:354–357.
11 Boucher NR, Scholefield JH, Anderson JB. The aetiological significance of human papillomavirus in bladder cancer. Br J Urol 1996;78:866–869.
12 Youshya S, Purdie K, Breuer J, *et al.* Does human papillomavirus play a role in the development of bladder transitional cell carcinoma? A comparison of PCR and immunohistochemical analysis. J Clin Pathol 2005;58:207–210.
13 Agoff SN, Lin P, Morihara J, *et al.* 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;16:665–673.
14 Benevolo M, Mottolese M, Marandino F, *et al.* Immunohistochemical expression of p16(INK4a) is predictive of HR-HPV infection in cervical low-grade lesions. Mod Pathol 2006;19:384–391.
15 Dehn D, Torkko KC, Shroyer KR. Human papillomavirus testing and molecular markers of cervical dysplasia and carcinoma. Cancer 2007;111:1–14.
16 Doxtader EE, Katzenstein AL. The relationship between p16 expression and high-risk human papillomavirus infection in squamous cell carcinomas from sites other than uterine cervix: a study of 137 cases. Hum Pathol 2012;43:327–332.
17 Horn LC, Lindner K, Szepankiewicz G, *et al.* P16, p14, p53, and cyclin D1 expression and HPV analysis in small cell carcinomas of the uterine cervix. Int J Gynecol Pathol 2006;25:182–186.
18 Keating JT, Cviko A, Riethdorf S, *et al.* Ki-67, cyclin E, and p16INK4 are complimentary surrogate biomarkers for human papilloma virus-related cervical neoplasia. Am J Surg Pathol 2001;25:884–891.
19 Klaes R, Benner A, Friedrich T, *et al.* P16INK4a immunohistochemistry improves interobserver agreement in the diagnosis of cervical intraepithelial neoplasia. Am J Surg Pathol 2002;26:1389–1399.
20 Klaes R, Friedrich T, Spitkovsky D, *et al.* Overexpression of p16(INK4A) as a specific marker for dysplastic and neoplastic epithelial cells of the cervix uteri. Int J Cancer 2001;92:276–284.
21 Kong CS, Balzer BL, Troxell ML, *et al.* P16INK4A immunohistochemistry is superior to HPV *in situ* hybridization for the detection of high-risk HPV in atypical squamous metaplasia. Am J Surg Pathol 2007;31:33–43.
22 Negri G, Egarter-Vigl E, Kasal A, *et al.* P16INK4a is a useful marker for the diagnosis of adenocarcinoma of the cervix uteri and its precursors: an immunohistochemical study with immunocytochemical correlations. Am J Surg Pathol 2003;27:187–193.
23 Nieh S, Chen SF, Chu TY, *et al.* Is p16(INK4A) expression more useful than human papillomavirus

test to determine the outcome of atypical squamous cells of undetermined significance-categorized Pap smear? A comparative analysis using abnormal cervical smears with follow-up biopsies. Gynecol Oncol 2005;97:35–40.
24 Pientong C, Ekalaksananan T, Kongyingyoes B, *et al.* Immunocytochemical staining of p16INK4a protein from conventional Pap test and its association with human papillomavirus infection. Diagn Cytopathol 2004;31:235–242.
25 Samarawardana P, Dehn DL, Singh M, *et al.* P16(INK4a) is superior to high-risk human papillomavirus testing in cervical cytology for the prediction of underlying high-grade dysplasia. Cancer Cytopathol 2010;118:146–156.
26 Tsoumpou I, Arbyn M, Kyrgiou M, *et al.* p16(INK4a) immunostaining in cytological and histological specimens from the uterine cervix: a systematic review and meta-analysis. Cancer Treat Rev 2009;35: 210–220.
27 Wang JL, Zheng BY, Li XD, *et al.* P16INK4A and p14ARF expression pattern by immunohistochemistry in human papillomavirus-related cervical neoplasia. Mod Pathol 2005;18:629–637.
28 Munoz N, Bosch FX, de Sanjose S, *et al.* Epidemiologic classification of human papillomavirus types associated with cervical cancer. N Engl J Med 2003; 348:518–527.
29 zur Hausen H. Molecular pathogenesis of cancer of the cervix and its causation by specific human papillomavirus types. Curr Top Microbiol Immunol 1994; 186:131–156.
30 Subhawong AP, Subhawong T, Nassar H, *et al.* Most basal-like breast carcinomas demonstrate the same Rb−/p16+ immunophenotype as the HPV-related poorly differentiated squamous cell carcinomas which they resemble morphologically. Am J Surg Pathol 2009;33:163–175.
31 Cheng L, Leibovich BC, Cheville JC, *et al.* Squamous papilloma of the urinary tract is unrelated to condyloma acuminata. Cancer 2000;88:1679–1686.
32 Chaux A, Pfannl R, Lloveras B, *et al.* Distinctive association of p16INK4a overexpression with penile intraepithelial neoplasia depicting warty and/or basaloid features: a study of 141 cases evaluating a new nomenclature. Am J Surg Pathol 2010;34:385–392.
33 Cubilla AL, Lloveras B, Alejo M, *et al.* Value of p16(INK)(a) in the pathology of invasive penile squamous cell carcinomas: a report of 202 cases. Am J Surg Pathol 2011;35:253–261.
34 Barghi MR, Hajimohammadmehdiarbab A, Moghaddam SM, *et al.* Correlation between human papillomavirus infection and bladder transitional cell carcinoma. BMC Infect Dis 2005;5:102.
35 Shigehara K, Sasagawa T, Doorbar J, *et al.* Etiological role of human papillomavirus infection for inverted papilloma of the bladder. J Med Virol 2011;83:277–285.
36 Moonen PM, Bakkers JM, Kiemeney LA, *et al.* Human papilloma virus DNA and p53 mutation analysis on bladder washes in relation to clinical outcome of bladder cancer. Eur Urol 2007;52:464–468.
37 Cai T, Mazzoli S, Meacci F, *et al.* Human papillomavirus and non-muscle invasive urothelial bladder cancer: potential relationship from a pilot study. Oncol Rep 2011;25:485–489.
38 Guo CC, Gomez E, Tamboli P, *et al.* Squamous cell carcinoma of the urinary bladder: a clinicopathologic and immunohistochemical study of 16 cases. Hum Pathol 2009;40:1448–1452.
39 Westenend PJ, Stoop JA, Hendriks JG. Human papillomaviruses 6/11, 16/18 and 31/33/51 are not associated with squamous cell carcinoma of the urinary bladder. BJU Int 2001;88:198–201.
40 Ben Selma W, Ziadi S, Ben Gacem R, *et al.* Investigation of human papillomavirus in bladder cancer in a series of Tunisian patients. Pathol Res Pract 2010;206: 740–743.