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Human papillomavirus sequences are not detectable by Southern blotting or general primer-mediated polymerase chain reaction in transitional cell tumours of the bladder

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Summary. A large series of transitional cell tumours has been screened for the presence of human papillomavirus (HPV) sequences using Southern blotting and general primer-mediated polymerase chain reaction (GP-PCR). The latter technique allows the detection of a broad spectrum of both sequenced and unsequenced HPV types using two primer pairs located in the highly conserved L1 and E1 regions of the HPV genome. No evidence for HPV infection was found in 100 transitional cell tumours, 6 cases of carcinoma in situ, 2 adenocarcinomas and a squamous carcinoma of the bladder and 3 cases of cystitis. Similarly, 12 bladder tumour cell lines were HPV-negative in these assays. Cervical carcinoma cell lines containing from 1–3 to 600 copies of the HPV genome were used as positive controls and were scored positive in all assays by both Southern blotting and GP-PCR. It is concluded that despite the close proximity of the urothelium to the genital mucosa and the resemblance of some bladder tumours to known HPV-induced lesions in other tissues, HPV infection is absent or very uncommon in bladder tumours.

Key words: Human papillomavirus – Bladder tumours – General primer-mediated polymerase chain reaction

Human papilloma viruses (HPV) have been implicated as aetiological agents in human cancers of the cervix, vulva, penis and perianal region [38]. Evidence has accumulated for their causal role in the induction of premalignant anogenital lesions, and in this context, HPV DNA can be demonstrated in 80% of premalignant lesions of the vulva [16]. In addition, HPV DNA is present in more than 90% of overt cervical, vulvar and penile cancers [37]. The ubiquitous distribution of HPV in all populations studied [5, 31] and the close proximity of the bladder mucosa to the anogenital area, particularly in females, have led to the suggestion that HPV may be involved in bladder cancer [38]. At other sites, condyloma acuminatum represents a typical HPV lesion [28]. Several cases of condyloma acuminatum of the bladder have been reported [19, 21, 32, 35] and in the only case of this type analysed to date, HPV16 infection was found [18]. In this latter study, 9 other bladder tumours (1 adenocarcinoma and 8 non-invasive papillary tumours of grades 1 and 2) were studied and found to be negative for HPV types 1, 2, 6, 11, 16 and 18.

Over 60 distinct HPV types are now known, more than 20 of which are regularly detected in the human genital tract [4] and 10 of which have been associated with malignant lesions [4, 37]. In this study, 100 transitional cell tumours and 12 human bladder carcinoma cell lines have been analysed for the presence of HPV sequences using a general primer-mediated polymerase chain reaction (GP-PCR) technique which has been shown previously to permit detection of both known and unsequenced HPV types [34]. Using this sensitive technique, no evidence of HPV infection was found in this large series of tumours and it is concluded that HPV is unlikely to play a role in the development of the majority of bladder tumours.

Materials and methods

Tissue samples

Samples were collected from tumours removed at cytoscopy by diathermy or cup biopsy forceps. Tumour tissue was trimmed of debris, a sample was taken for histopathological assessment and the remainder frozen at −70°C. Tumour grade and stage were assessed according to the TNM system [33]. Peripheral blood leucocytes from patients with bladder tumours and healthy volunteers were obtained from 10 ml venous blood collected in EDTA tubes and frozen at −70°C.

Cell lines

Twelve human bladder carcinoma cell lines were used: 5637 (G. Gannon, unpublished); J.O.'N. (Human Tumor Cell Laboratory, Sloan-Kettering Cancer Center, personal communication); SCaBER [24]; VM-CUB-2 [36]; EJ [9]; RT112 (O'Toole, unpublished); SW1710 [20]; HT1376 and HT1197 [29]; SD [27]; 253J [8]; and RT4

Table 1. HPV L1 and E1-region-specific general primers

GP E1	GP1	5'TGGTACAATGGGCATATGAT3'	sense
	GP2	5'AATGGCTTTTGGAATTTACA3'	antisense
GP L1	GP5	5'TTTGTTACTGTGGTAGATAC3'	sense
	GP6	5'GAAAAATAAACTGTAAATCA3'	antisense

HPV type	Total mismatches	
	L1,2	L5,6
5	5	14
8	2	13
11	6	0
16	3	2
18	5	1
33	4	2

[30]. A breast tumour cell line SKBR-3 (Trempe and Old, 1970, unpublished) and a vulvar carcinoma cell line, A431 [14] were also used. Three human cervical carcinoma cell lines were used as controls: HeLa [13], which contains approximately 50 copies of HPV18; SiHa [11], which contains 1–3 copies of HPV16; CaSki [26], which contains 600 copies of HPV16 DNA, and C33A [1], which is HPV-negative.

DNA extraction

Tumour DNA was extracted using two methods. In the first, tissue was dissolved in guanidine isothiocyanate and centrifuged through caesium chloride [3]. Following removal of the guanidine phase, DNA was dialysed against 1×TE (10 mM TRIS-HCl, 1 mM EDTA pH 8.0), extracted with phenol, phenol:chloroform and chloroform and ethanol precipitated. In the second method, tissue was lysed in 0.75 M NaCl, 0.02 M EDTA (pH 8.0), containing 1% Sodium dodecyl sulphate (SDS) and 170 µg/ml Proteinase K (Boehringer Mannheim, Lewes, UK), followed by extraction with phenol, phenol:chloroform and chloroform and ethanol precipitation. No difference in the quality of DNA prepared by these two methods has been noted. DNA was extracted from cell lines by lysis in 1×TSE (10 mM TRIS-HCl, 0.1 M NaCl, 1 mM EDTA, pH 8.0) containing 170 µg/ml proteinase K and 1% SDS, followed by phenol:chloroform extractions and precipitation as above.

Polymerase chain reaction

Two general primer sets, GP1,2 and GP5,6 [34] were used (Table 1). These are homologous to the E1 and L1 regions of HPV respectively. Primers were made on a DNA synthesizer (Applied Biosystems 381A). PCR reactions (100 µl) contained 500–1000 ng template DNA, 50 mM KCl, 10 mM TRIS-HCl (pH 8.3), 2 mM $MgCl_2$, 0.001% gelatin, 200 µM of each deoxynucleotide triphosphate (dNTP), 0.5 µM of each primer and 1 unit of *Thermus aquaticus* (Taq) DNA polymerase (Amplitaq, Perkin Elmer/Cetus, Berkeley, Calif.). To ensure reproducibility, all components apart from DNA and enzyme were mixed and divided into aliquots. Template DNA was then added, and the reaction mixes were overlaid with one drop of light mineral oil (Sigma) and heated to 94°C for 10 min. Enzyme was then added to each tube and 40 cycles of amplification carried out using a Techne Programmable Dri-Block PHC-1. Cycling conditions were: 1 min denaturation at 94°C; 2 min annealing at 40° C; and 2 min polymerisation at 72°C. Following the last cycle, all tubes were incubated for a further 10 min at 72°C to ensure complete elongation of all amplified sequences. From each sample, 10 µl was analysed by electrophoresis in 3% Nusieve : 1% SeaKem gels (FMC Bioproducts, Risingevej, Denmark) or by polyacrylamide gel electrophoresis (PAGE) in 12% non-denaturing gels.

Southern blot analysis of tumour and cell line DNA and PCR products

Tumour and cell line DNAs were digested with *Bam*HI or *Sst*I (Gibco BRL, Paisley, UK) according to the manufacturer's instructions, separated in 0.8% agarose gels and transferred to Hybond-N membranes (Amersham, Aylesbury, UK). PCR products were transferred directly from 3% Nusieve : 1% SeaKem gels to Hybond-N. Following ultraviolet cross-linking, blots were pre-hybridised and hybridised according to the manufacturer's instructions. Probes were made by random priming [10] of plasmids containing cloned HPV sequences to a specific activity of approximately 1×10^9 dpm μg^{-1} and used at $1–2\times10^6$ dpm ml^{-1} hybridisation fluid. Following hybridisation for 18 h at 65°C, blots were washed to 2×SSC (0.3 M NaCl; 0.03 M Na citrate) 0.1% SDS and exposed to Hyperfilm-MP (Amersham) or Fuji RX film (Fuji Photofilm, London, UK).

Probes

Plasmids containing cloned HPV 5, 8, 11, 16, 18, and 33 were kindly provided by Dr. K. Vousden. In each case, the entire plasmid was labelled by random priming for use as a probe for Southern analysis.

Results

Southern analysis of bladder tumours and cell lines

Aliquots of DNA (10 µg) extracted from 44 bladder tumour specimens (20 grade 1, 17 grade 2 and 5 grade 3 transitional cell tumours, 1 adenocarcinoma and 1 case of carcinoma in situ) were digested with *Bam*HI or with *Sst*I. *Bam*HI cleaves HPV16 DNA once and *Sst*I does not cut within the HPV16 genome. In benign cervical lesions, HPV DNA is commonly present in episomal form, whereas in many cervical carcinomas integrated HPV sequences can be detected [7]. Analysis of DNA cut with these two enzymes therefore allows the physical state of the HPV16 genome to be assessed.

Southern blots of tumour DNA specimens were hybridised to probes for HPV 5, 8, 11, 16, 18 and 33, used either singly or in combinations of up to three probes simultaneously. HeLa DNA was included as a positive control on all blots and showed hybridisation to the HPV18 probe which could be detected following overnight exposure of the autoradiograph in each case. No hybridisation of bladder tumour DNAs to any of these probes was detected after exposure times up to 28 days. Similarly, DNA from the 12 bladder tumour cell lines, SKBR-3 and A431 showed no hybridisation to these HPV probes.

General primer-mediated PCR

The PCR primers GP5,6 and GP1,2 have been used previously to amplify sequences from both known and unsequenced HPV types present in cervical scrapes and

Table 2. Tissues analysed

Tumour/tissue type	Number of specimens
Transitional cell carcinoma	
Grade 1	38
Grade 2	42
Grade 3	20
Total	100
Carcinoma in situ	6
Adenocarcinoma	2
Squamous carcinoma	1
Bladder tumours unclassified[a]	11
Cystitis	3

[a]Tumour specimens for which no histopathology report was available

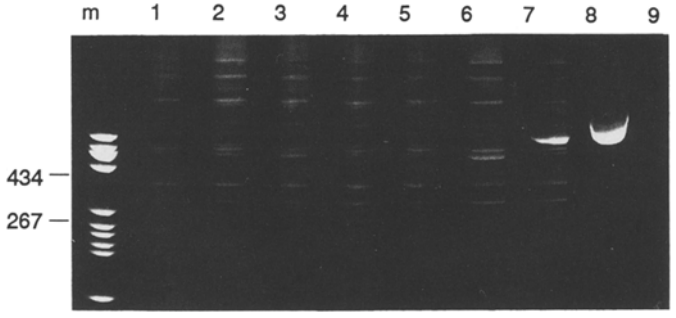

Fig. 1. PCR products obtained after amplification with GP1,2. From each reaction 10 µl was electrophoresed on a 12% non-denaturing polyacrylamide gel and stained with ethidium bromide. m, pBR322 cut with *Hae*III; template DNA: lanes 1–6, bladder tumour DNAs; lane 7, SiHa; lane 8, CaSki; lane 9, control (no template DNA)

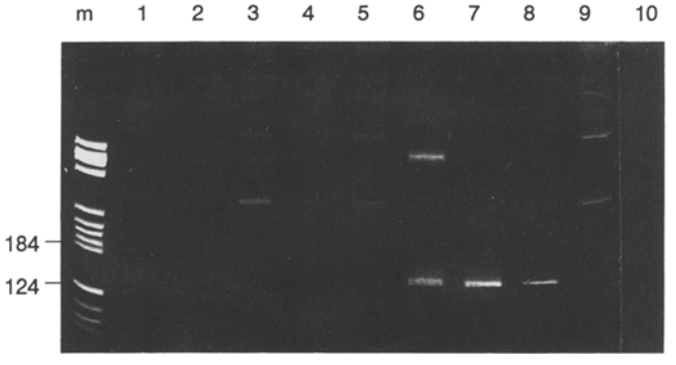

Fig. 2. PCR products obtained after amplification with GP5,6. Gel as in Fig. 1. m, pBR322 cut with *Hae*III; template DNA: lanes 1–5, bladder tumour DNAs; lane 6, HeLa; lane 7, CaSki; lane 8, SiHa; lane 9, C33a; lane 10, control (no template DNA)

carcinomas [34]. Primers GP5,6 are L1-region-specific and allow amplification of a DNA fragment of approximately 140 bp from HPV 1, 6, 8, 11, 13, 16, 18, 30, 31, 32, 33, 45 and 51. These primers have no more than two mismatches with sequenced HPV DNAs specific for genital mucosa but show more divergence from the L1

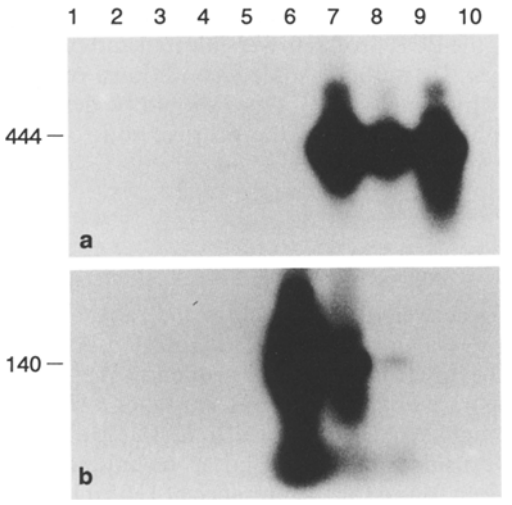

Fig. 3. Southern blots of PCR products hybridised to HPV16 and HPV18 probes simultaneously. Aliquots (10 µl) of the same PCR reactions shown in Figs. 1 and 2 were electrophoresed in 1.5% agarose gels and blotted. After hybridisation, blots were washed to 2× standard saline citrate, 0.1% SDS. (a) Products generated with GP1,2; lanes 1–6, bladder tumour DNAs; lane 7, HeLa; lane 8, SiHa; lane 9, CaSki; lane 10, control (no template DNA). (b) Products generated with GP5,6; lanes 1–5, bladder tumour DNAs; lane 6, HeLa; lane 7, CaSki; lane 8, SiHa; lane 9, C33a; lane 10, control (no template DNA)

region of cutaneous HPV types 1, 5 and 8. The E1-region-specific primers GP1,2 show fewer mismatches with HPV types 5 and 8 and retain adequate homology for amplification of a 444 bp fragment from HPV types 6, 11, 13, 16, 18, 30–33, 45 and 51 (Table 1) [34].

DNA from bladder tumours was subjected to 40 cycles of amplification using each set of primer pairs under non-stringent conditions with annealing at 40°C to allow amplification from mismatched primers. Details of the tissues analysed are shown in Table 2. These included 100 transitional cell carcinomas and 6 cases of carcinoma in situ. The expected PCR product of approximately 400–500 bp was observed following amplification of SiHa and CaSki cell line DNAs with GP1,2 primers (Fig. 1, lanes 7 and 8). No product was obtained from C33a, which contains no HPV sequences (not shown), or from any of the bladder tumours (Fig. 1, lanes 1–6). A product of 140–150 bp was amplified using GP5,6 from HeLa, CaSki and SiHa (Fig. 2, lanes 6–8) but not from C33a (Fig. 2, lane 9) or any of the bladder tumours tested (Fig. 2, lanes 1–5). In all cases where no specific product was obtained, many minor product bands, believed to represent the result of non-specific priming events, were detected. In positive control samples these bands were present in inverse proportion to the amount of specific product, so that it appears that in the presence of a specific template, primers were unavailable for non-specific priming. These appearances are similar to those described by van den Brule et al. [34] for cervical scrapes and carcinomas.

The cervical cell lines used as positive controls all contain multiple copies of HPV. In order to exclude the possibility that small amounts of PCR product were

produced by HPV present in bladder tumours at one copy or less per cell, the PCR products were blotted and probed for HPV types 16 and 18 under conditions of low stringency in which many HPV types should be detected with these probes. Apart from the positive controls, no signal was detected (Fig. 3).

Discussion

The association between HPV infection and cancer of the cervix, vulva, vagina and penis is well established. At these tissue sites, the presence of HPV sequences has been readily demonstrated by a variety of techniques including Southern blotting and PCR methods [6, 12, 22, 25]. In the present study, using both these latter techniques, no evidence has been found for the presence of HPV DNA in a large series of bladder tumours.

One case has been reported previously in which HPV16 was detected in bladder carcinoma in situ in a patient with mild immunodeficiency [18]. In this case, the bladder lesions showed a marked similarity to condyloma accuminata and the patient had concurrent Bowen's disease of the vulva and severe dysplasia of the vaginal wall. HPV16 DNA was demonstrated in the bladder tumour and in vulval and vaginal lesions of this patient by hybridisation of Southern blots to mixed probes for HPV types 1, 2, 6, 11, 16 and 18. Tumours from 9 other patients examined showed no evidence of infection with these viruses. Two other studies [15, 17] examined small numbers of bladder tumours for the presence of HPV types 30 and types 1 and 2 respectively, and found none. Since these latter studies concerned HPV types not commonly associated with human cancer, and the study of Kitamura et al. [18] included only 9 tumours from immunocompetent patients, the present study aimed to survey a large series of bladder tumours for as large a number of HPV types as possible.

The physical proximity of the bladder to the genital area, the presence of multifocal disease in some patients and the existence of carcinoma in situ, which may resemble some HPV-induced genital lesions, has previously led to the suggestion that HPV may play a role in the development of at least some bladder tumours. In addition a number of studies have reported an increased risk of bladder cancer following cancer of the cervix [2, 23]. It was considered that the most likely HPV types to be present were the cutaneous HPV types 5 and 8 and the genital types 6, 11, 16, 18, and 33. Southern analysis failed to reveal the presence of any of these types. However, the possibility existed that other known or unknown HPV types might be present or that low copy number HPV infection might not be detected by this technique. The development of a sensitive PCR method to detect a broad spectrum of HPV genotypes using only two sets of primers [34] provides a useful method to screen large numbers of tissue specimens for a wide range of HPV types. Such a PCR-based technique has the additional advantage that only a very small DNA sample is required. This has allowed several cases of carcinoma in situ, which yielded only small tissue samples, to be included in this study. Positive results were obtained consistently from HPV-positive cervical carcinoma cell lines using both primer pairs, confirming that HPV-specific amplification occurred under the reaction conditions used. No amplification products of the expected sizes were obtained from any of the bladder tumour DNA samples. Additional confirmation of these negative results was obtained by Southern blotting of the PCR products and hybridisation to mixed HPV probes under non-stringent conditions, which sould allow detection of not only the HPV types for which specific probes were included, but other related types as well [34].

Short urethral length and the prevalence of HPV in the female genital tract might suggest a more likely role for HPV in bladder tumours in females than in males. Certainly, based on physical grounds alone it is likely that these viruses reach the bladder more frequently in women than in men. This series included 32 samples from females and in none was HPV DNA found. Similarly, the absence of HPV sequences in 6 cases of carcinoma in situ suggests that HPV is not present in the majority of cases, despite some histopathological similarities to known HPV-induced lesions elsewhere. The previous finding of HPV16 in one case of carcinoma in situ has demonstrated that the virus can gain access to the bladder and infect urothelial cells. However, it is likely that the immunodeficiency of the patient concerned represented a permissive factor in this case. Such cases are likely to represent only a very small subset in whom HPV may be present.

It is concluded that HPV infection is very uncommon in neoplastic lesions of the bladder. In areas of the world where transitional tumours (representing 100 of 103 overt tumours in this study) are the major histopathological tumour type, it appears that HPV does not contribute to the neoplastic process.

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References

1. Auersperg N (1964) Long-term cultivation of hypodiploid human-tumor cells. J Natl Cancer Inst 32:135
2. Bailar JC (1962) The incidence of independent tumors among uterine cancer patients. Cancer 16:842
3. Chirgwin JM, Przybyla AE, MacDonald RJ, Rutter WJ (1979) Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. Biochemistry 18:5294
4. De Villiers EM (1989) Heterogeneity of the human papillomavirus group. J Virol 63:4898
5. De Villiers EM, Wagner D, Schneider A, Wesch H, Miklaw H, Wahrenderf J, Papendick U, zur Hausen H (1987) Human papillomavirus infections in women with and without abnormal cervical cytology. Lancet II:703
6. Durst M, Gissman L, Ikenberg H, zur Hausen HA (1983) A papillomavirus DNA from a cervical carcinoma and its prevalence in cancer biopsy samples from different geographic regions. Proc Natl Acad Sci USA 80:3812
7. Durst M, Kleinheinz A, Hotz M, Gissman L (1985) The physical state of human papillomavirus type 16 DNA in benign and malignant genital tumors. J Gen Virol 66:1515

8. Elliot AY, Cleveland P, Cervenka J, Castro AE, Stein N, Hakala TR, Fraley EE (1974) Characterization of a cell line from human transitional cell cancer of the urinary tract. J Natl Cancer Inst 53:1341
9. Evans DR, Irwin RJ, Havre PA, Bouchard JG, Kato T, Prout GR (1977) The activity of the pyrimidine biosynthetic pathway in MGH-U1 transitional carcinoma cells grown in tissue culture. J Urol 117:712
10. Feinberg AP, Vogelstein B (1983) A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. Anal Biochem 132:6
11. Friedl F, Kimura I, Osako T, Ito Y (1970) Studies on a new human cell line (SiHa) derived from carcinoma of the uterus. 1. Its establishment and morphology. Proc Soc Exp Biol Med 135:543
12. Fukushima M, Okagaki T, Twiggs LB, Clark BA, Zachow KR, Ostrow RS, Faras AJ (1985) Histological types of carcinoma of the cervix and detectability of human papillomavirus DNA. Cancer Res 45:3252
13. Gey GO, Coffman WO, Kubicek MT (1952) Tissue culture studies of the proliferative capacity of cervical carcinoma and normal epithelium. Cancer Res 12:264
14. Giard DJ, Aaronson SA, Todaro GJ, Arnstein P, Kersey JH, Dosik H, Parks WP (1973) In vitro cultivation of human tumors: establishment of cell lines derived from a series of solid tumors. J Natl Cancer Inst 51:1417
15. Green M, Orth G, Wold WSM, Sanders PR, Mackey JK, Favre M, Croissant O (1981) Analysis of human cancers, normal tissues, and verrucae plantares for DNA sequences of human papillomaviruses types 1 and 2. Virology 110:176
16. Ikenberg H, Gissmann L, Gross G, Grussendorf E-I, zur Hausen H (1983) Human papillomavirus type 16-related DNA in genital Bowen's disease and in Bowenoid papulosis. Int J Cancer 32:563
17. Kahn T, Schwartz E, zur Hausen H (1986) Molecular cloning and characterisation of a new human papillomavirus from a laryngeal carcinoma. Int J Cancer 37:61
18. Kitamura T, Yogo Y, Ueki T, Murakami S, Aso Y (1988) Presence of human papillomavirus type 16 genome in bladder carcinoma in situ of a patient with mild immunodeficiency. Cancer Res 48:7207
19. Kleiman H, Lancaster Y (1962) Condyloma acuminata of the bladder. J Urol 88:52
20. Kyriazis AA, Kyriazis AP, McCombs WB III, Peterson WDJ (1984) Morphological, biological and biochemical characteristics of human bladder transitional cell carcinomas grown in tissue culture and in nude mice. Cancer Res 44:3997
21. Lewis HY, Wolf PL, Pierce JM (1962) Condyloma acuminatum of the bladder. J Urol 88:248
22. Melchers W, van den Brule A, Walboomers J, de Bruin M, Burger M, Herbrink P, Meijer C, Lindeman J, Quint W (1989) Increased detection rate of human papillomavirus in cervical scrapes by the polymerase chain reaction as compared to the modified FISH and Southern-blot analysis J Med Virol 27:329
23. Newell GR, Krementz ET, Roberts JD (1975) Excess occurrence of cancer of the oral cavity, lung and bladder following cancer of the cervix. Cancer 36:2155
24. O'Toole C, Nayak S, Price Z, Gilbert WH, Waisman J (1976) A cell line (SCaBER) derived from squamous cell carcinoma of the human urinary bladder. Int J Cancer 17:707
25. Palefsky JM, Holly EA, Gonzales J, Berline J, Ahn DK, Greenspan JS (1991) Detection of human papillomavirus DNA in anal intraepithelial neoplasia and anal cancer Cancer Res 51:1014
26. Patillo RA, Hussa RO, Story MT, Ruckert ACF, Shalaby MP, Mattingly RF (1977) Tumor antigen and human chorionic gonadotropin in CaSki cells: a new epidermoid cervical cancer cell line. Science 196:1456
27. Paulie S, Hansson Y, Lundblad M-L, Perlmann P (1983) Lectins as probes for identification of tumor-associated antigens on urothelial and colonic carcinoma cell lines. Int J Cancer 31:297
28. Pfister H (1987) Human papillomaviruses and genital cancer. Adv Cancer Res 48:113
29. Rasheed S, Gardner MB, Rongey RW, Nelson-Rees WA, Arnstein P (1977) Human bladder carcinoma: characterization of two new tumor cell lines and search for tumor viruses. J Natl Cancer Inst 58:881
30. Rigby CC, Franks LM (1970) A human tissue culture cell line from a transitional cell tumour of the urinary bladder: growth, chromosome pattern and ultrastructure. Br J Cancer 24:746
31. Schneider A, Hotz M, Gissmann L (1987) Increased prevalence of human papilloma viruses in the lower genital gract of pregnant women. Int J Cancer 40:198
32. Shirai T, Yamamoto K, Adachi T, Imaida K, Masui T, Ito N (1988) Condyloma acuminatum of the bladder in two autopsy cases. Acta Pathol Jpn 38:399
33. Union Internationale Contre le Cancer (UICC) (1978) TNM classification of malignant tumours, 3rd edn. International Union against Cancer, Geneva
34. Van den Brule AJC, Snijders PJF, Gordijn RLJ, Bleker OP, Meijer CJLM, Walboomers JMM (1990) General primer-mediated polymerase chain reaction permits the detection of sequenced and still unsequenced human papillomavirus genotypes in cervical scrapes and carcinomas. Int J Cancer 45:644
35. Walther M, O'Brien DP III, Birch HW (1986) Condylomata acuminata of the bladder: case report and literature review. J Urol 135:362
36. Williams RD (1980) Human urologic cancer cell lines. Invest Urol 17:359
37. Zur Hausen H, Schneider A (1987) The role fo papillomaviruses in anogenital cancer. In: Salzman NP, Howley PM (eds) The papovaviridae 2. Plenum, New York, p 245
38. Zur Hausen H (1989) Papillomaviruses in anogenital cancer as a model to understand the role of viruses in human cancers. Cancer Res 49:4677

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