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Detection of Human Papillomavirus DNA in Cancer of the Urinary Bladder by *in situ* Hybridisation

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Summary—The association of the human papillomavirus (HPV) with cancer of the urinary bladder was assessed by *in situ* hybridisation using probes selective for HPV types 6/11 and 16/18 DNA. No hybridisation signal was detected with the type 6/11 probe on 100 formalin-fixed, paraffin-embedded bladder tumours sampled. However, when the same samples were hybridised with the HPV type 16/18 DNA probe, 11 of 66 (16.6%) papillary and 1 of 10 (10%) solid transitional cell carcinomas gave positive signals. These results suggest the involvement of HPV in cancer of the bladder, although the frequency of multiple HPV types in these tumours is uncertain.

While viral studies on mammalian urothelium have shown an association between the bovine papillomavirus (BPV) and cancer of the bladder in cattle (Olson *et al.*, 1965; Jarrett, 1987), the evidence for HPV involvement in urinary bladder cancer in man is less clear. Sixty types of HPV are known to infect man and although most of these proliferations are benign, some may convert to malignancies (Syrjanen, 1990). This conversion is related to HPV type: for example, in the genitourinary tract, HPV types 6 and 11 are most commonly associated with genital warts (condylomata acuminata), whereas types 16 and 18 are associated with more severe dysplasias and carcinomas (zur Hausen, 1987). Genital condylomata can extend to the urethra and urinary bladder and also remain in close association with cancer of the bladder (Murphy *et al.*, 1983; Walther *et al.*, 1986; Shirai *et al.*, 1988). The presence of HPV antigen in cancer of the bladder has previously been established (Bryant *et al.*, 1987). However, although HPV DNA types 6 and 11 have been identified in condylomata and cancers of the bladder and urethra (Del Mistro *et al.*, 1988; Querci Della Rovere *et al.*, 1988; Mevorach *et al.*, 1990; Wilson *et al.*, 1990), the presence of HPV types 16 and 18 in cancers of the urinary bladder has yet to be substantiated.

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In order to investigate further the role of HPV in these tumours, 100 paraffin sections of urinary bladder tumours were analysed for the presence of HPV types 6/11 and 16/18 DNA using a non-isotopic *in situ* hybridisation technique. The findings showed the presence of HPV type 16/18 DNA in transitional cell carcinomas of the bladder, and the significance of these results is discussed.

Materials and Methods

A total of 100 transurethrally resected urinary bladder samples were fixed in 10% formalin and embedded in paraffin wax. Sections (3 μm) were stained with haematoxylin and eosin and examined microscopically (Table). The samples were then examined for HPV DNA by *in situ* hybridisation. Formalin-fixed and paraffin-embedded genital condylomata and positive HPV 16 control slides (Bio-Nuclear Services, Reading) were used as controls.

Probes

The DNA probes studied were plasmid pUC19 (Gibco Ltd, Paisley) for use as a negative control probe, and HPV DNA types 6b, 11, 16 and 18, kindly donated by Professor H. zur Hausen and Dr E. M. de Villiers, DKFZ, Heidelberg, Germany. The probes were biotinylated with biotin 11-dUTP (Gibco Ltd) using a nick translation kit (Gibco Ltd)

Table *In situ* Hybridisation of Urinary Bladder Samples using HPV DNA Probes

Histopathology	*No. of patients*	*HPV 6/11*	*HPV 16/18*
Papillary TCC	66	—	11
Solid TCC	10	—	1
Mixed papillary/solid TCC	2	—	—
Squamous cell carcinoma	3	—	—
Adenocarcinoma	4	—	—
Undifferentiated carcinoma	7	—	—
Dysplasia	3	—	—
Benign	5	—	—

TCC—transitional cell carcinoma.

and following the recommended protocol. Unincorporated dNTPs were separated from the biotinylated DNA by the spun column technique (Maniatis *et al.*, 1982).

The concentration of each of the stored biotinylated probes was 10 ng/μl. For use, the HPV type 6/11 DNA probe cocktail was prepared by adding 40 μl each of HPV types 6 and 11 biotinylated probes to 2 ml of hybridisation buffer [2 × SSC (1 × SSC is 150 mM sodium chloride, 15 mM tri-sodium citrate), 5% (w/v) dextran sulphate, 0.2% (w/v) milk powder, 50% (v/v) formamide]. This was sufficient to treat 25 paraffin sections. The HPV type 16/18 probe was prepared similarly.

Pre-treatment

Paraffin sections were cut on to single-well slides (C. A. Hendley, Loughton) coated with 3-aminopropyltriethoxysilane (Sigma Chemical Company, Poole) as previously described (Burns *et al.*, 1987), and incubated for several days at 56°C to ensure maximum adhesion. The sections were dewaxed in xylene for 2 × 5 min and washed in absolute alcohol for 2 × 5 min. The sections were then hydrated through to distilled water and digested in 1% (w/v) diastase (Sigma) for 30 min at 37°C. Following a rinse in distilled water, each slide was incubated in 100 μl of proteinase K (Sigma) at a concentration of 100 μg/ml in 20 mM calcium chloride, 20 mM Tris hydrochloride, pH 7.4, for 1 h at 37°C, rinsed in distilled water, dehydrated in alcohol and air dried for 1 h.

Hybridisation

The slides were placed on a tray, 75 μl of each probe mixture were applied to duplicate sections, and covered with Gelbond (Miles Laboratories, Slough) with the hydrophobic side down. The slides were incubated on a metal tray at 90°C for 6 min and then transferred to a humidified tray overnight at 42°C. Following incubation, the Gelbond was removed and the slides rinsed in 2 × SSC for 2 × 20 min at room temperature and then in 0.1 × SSC containing 0.1% (v/v) Triton X100, 0.04% (w/v) magnesium chloride, at 42°C for 30 min.

Detection

To each slide was added an aliquot of 0.01% (v/v) streptavidin (Dako Limited, High Wycombe) in TBS buffer, pH 7.6 [50 mM Tris, 150 mM sodium chloride, 2 mM magnesium chloride, 0.1% (w/v) bovine serum albumin] and the slides incubated at room temperature for 30 min. Following a rinse in TBS buffer for 5 min, the slides were incubated in an aliquot of 0.1% (v/v) biotinylated alkaline phosphatase (Dako) in TBS buffer for 30 min at room temperature. The slides were rinsed in TBS buffer, washed for 5 min in veronal acetate buffer, pH 9.2 (30 mM sodium acetate, 30 mM sodium barbitone, 100 mM sodium chloride, 50 mM magnesium chloride) and developed for 60 min in a filtered solution of 0.05% (w/v) fast red TR, 0.05% (w/v) naphthol AS BI phosphate (pre-diluted in 100 μl dimethylformamide) and 0.024% (w/v) levamisole in veronal acetate buffer. The slides were rinsed in water, counterstained in Mayer's haemalum, washed in water for 5 min and mounted in Apathy's medium.

Results

Of the 100 urinary bladder samples analysed by *in situ* hybridisation, no signal was detected using the HPV type 6/11 DNA probe. However, positive hybridisation signals were detected in 12 samples when the sections were hybridised with the HPV type 16/18 DNA probe. Of these, 11 of 66 (16.6%) were papillary and 1 of 10 (10%) was a solid transitional cell carcinoma. Hybridisation using the pUC19 probe and all other samples were negative (Table). The positive hybridisation signals were seen as red, water-insoluble precipitates confined to isolated nuclei in well differentiated areas of the tumours. In the papillary tumours, the signals were primarily found in the clear, koilocyte-like or adjacent cells (Fig. 1). However, the positive signal in the solid carcinoma was found in isolated tumour cells and not in the koilocyte-type cells found in adjacent urothelium (Fig. 2). Background staining due to the affinity of avidin for glycogen (Grody *et al.*, 1987) was eliminated by pre-treatment of the sections with diastase. Over-development of the colour reaction can produce a high level of

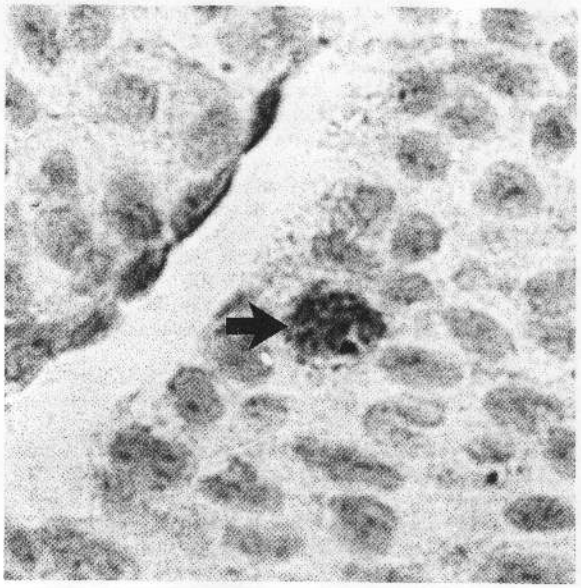

Fig. 1 *In situ* hybridisation of a low grade papillary transitional cell carcinoma showing an isolated nucleus (arrowed) positive with the HPV 16/18 biotinylated DNA probe. (Fast red-naphthol capture method ×920).

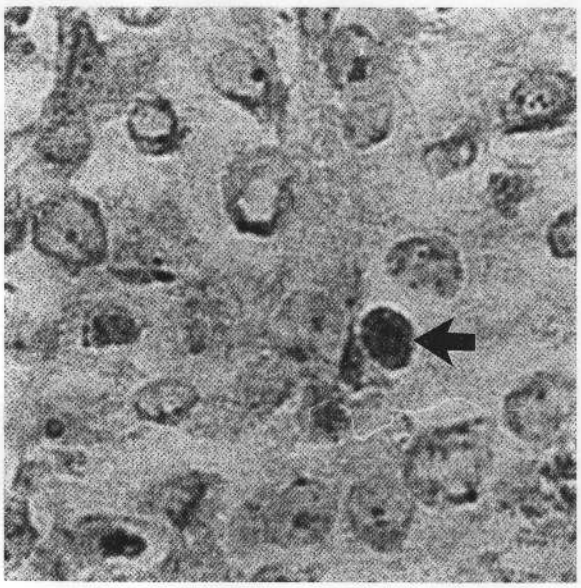

Fig. 2 High grade solid transitional cell carcinoma showing nuclear positivity (arrowed) for HPV 16/18 DNA using *in situ* hybridisation and a biotinylated probe. (Fast red-naphthol capture method ×920).

background staining, but this was controlled by monitoring the reaction after the first 30 min.

Incidentally, using the immunocytochemical method previously described (Bryant *et al.*, 1987), 6 of the 12 tumours (50%) showing positive hybridisation signals with the HPV 16/18 DNA probe were also positive for PV structural antigen.

Discussion

Cancer of the urinary bladder is one of the most common malignancies in males in the occidental population and several factors have been implicated in the development of these tumours (Morrison, 1984; Hartge *et al.*, 1985). The association of HPV with pre-malignant and malignant lesions of epithelium has long been recognised and such lesions are found throughout the genitourinary tract (Syrjanen, 1990).

The presence of condylomata in the bladder is well documented (Walther *et al.*, 1986; Shirai *et al.*, 1988). Because these lesions are typical of HPV infection (Krzyzek *et al.*, 1980), their presence suggests that infection by HPV may lead to carcinoma of the bladder. However, while there was no evidence to suggest the presence of condylomata in the patients in this study, the question arises as to whether HPV is regularly associated with other tumours of the urinary bladder. This study has shown that all of the samples analysed for HPV type 6/11 DNA were negative. This supports the concept that HPV types 6 and 11 are closely associated with benign lesions. In contrast, HPV types 16/18, which are more commonly associated with severe dysplasias and carcinomas (zur Hausen, 1987), were identified in 16.6% of papillary and 10% of solid transitional cell carcinomas. Previous studies have shown that while cancer of the bladder has been associated with HPV types 11 (Querci Della Rovere *et al.*, 1988) and 16 (Kitamura *et al.*, 1988; D. J. McCance, unpublished observations), several studies have failed to demonstrate other HPV types in them (Green *et al.*, 1981; Kahn *et al.*, 1986; T. Kitamura, personal communication). Furthermore, HPV type 6 has been identified in a papillary transitional cell carcinoma of the urethra but not in a histologically similar papillary cancer of the bladder in the same patient (Mevorach *et al.*, 1990). It has also been suggested that since HPV-associated lesions have been identified in the male urethra (Melchers *et al.*, 1989), bladder tumours obtained by transurethral resection could include HPV-infected urethral cells. In fact, bladder cancer patients have been found to have a higher incidence of HPV infection than the general population (Hartveit and Maehle, 1989). However, although this study has shown the presence of HPV in transitional cell carcinomas of the bladder, further studies will be necessary in

order to assess more fully the role that these and other HPV types may have in the genesis of these tumours.

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