

ORIGINAL ARTICLE

Does human papillomavirus play a role in the development of bladder transitional cell carcinoma? A comparison of PCR and immunohistochemical analysis

S Youshya, K Purdie, J Breuer, C Proby, M T Sheaf, R T D Oliver, S Baithun

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Aim: To investigate the role of human papillomavirus (HPV) in the development of bladder transitional cell carcinoma (TCC).

Methods: Seventy eight paraffin wax embedded TCC samples were tested for the presence of HPV by two methods. First, immunohistochemistry was carried out using a polyclonal antibody capable of detecting the capsid protein of all known papillomaviruses. The second method was a consensus GP5+/6+ primer mediated polymerase chain reaction (PCR) technique, with the products analysed by both agarose gel electrophoresis and an enzyme immunoassay using type specific oligonucleotide probes for 10 different mucosal genotypes. To exclude false negative results because of the poor quality of DNA extracted from paraffin wax embedded samples, the series was extended to include 20 further blocks for which the corresponding snap frozen unfixed tissue was available.

Results: The two methods produced contrasting results, with 47 of the 78 samples positive for HPV antigen and none positive for HPV DNA. HPV DNA was not detected in the 20 additional paraffin wax embedded TCCs or in the 20 paired unfixed samples. In contrast, HPV DNA was amplified by PCR from all six of the paraffin wax embedded cervical carcinoma and anogenital wart control samples.

Conclusion: The disparity between the two sets of results is probably caused by false positives resulting from the non-specificity of the polyclonal antibody used for immunohistochemistry. These results suggest that HPV is unlikely to play an aetiological role in the development of bladder TCC.

See end of article for authors' affiliations

Correspondence to:
Dr S Baithun, Department
of Medical Oncology,
Royal London Hospital,
Whitechapel, London E1
1BB, UK; s.i.baithun@
qmul.ac.uk

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Human papillomaviruses (HPVs) are small circular DNA viruses that infect stratified squamous epithelia, namely the epidermis and mucosal epithelium. To date, more than 100 complete HPV genomes have been characterised. Although many HPV types are associated with benign lesions, certain types—such as HPV-16 and HPV-18—have an established aetiological role in tumours of the urogenital tract and anal region. HPV infection is most clearly associated with cancer of the cervix, with HPV DNA detected in over 95% of these tumours.¹ HPV has also been postulated as a carcinogen in a range of other epithelial malignancies including cancers of the skin,^{2,3} oral cavity,^{4,5} oesophagus,⁶ and conjunctiva.⁷ Indeed, it has been estimated that almost 10% of the worldwide cancer burden is linked to HPV infection.⁸

In Western countries, more than 80% of bladder cancers are transitional cell carcinomas (TCCs), with squamous cell carcinomas the second most common morphological type identified.⁹ Several risk factors have been identified for urinary bladder carcinoma including environmental carcinogens, cigarette smoking, and chronic parasitic infection.¹⁰

“It has been estimated that almost 10% of the worldwide cancer burden is linked to human papillomavirus infection”

In cattle, bovine papillomavirus has been implicated in transitional cell carcinoma formation.¹¹ Interestingly, it is the metaplastic squamous cells in these tumours that appear to contain detectable bovine papillomavirus. Previously, we have shown that squamous metaplasia is a common feature of human bladder TCC, and that its presence in muscle invasive tumours predicts a poor response to radiotherapy.^{11a}

Several previous studies have looked for an association between HPV infection and bladder TCC development. However, the wide range of frequency detected, varying between 0% and 81%,¹² has meant that the role of HPV in bladder carcinogenesis remains controversial. In our current study, we set out to examine bladder TCCs for the presence of HPV by two methods: (1) immunohistochemistry with an antibody against HPV capsid protein and (2) a polymerase chain reaction (PCR) enzyme immunoassay (EIA)¹³ using GP5+/6+ consensus primers, which are capable of detecting low copy number DNA from a wide spectrum of HPVs associated with mucosal epithelia.¹⁴

MATERIALS AND METHODS

Tissue samples and immunohistochemistry

In total, 98 cases of TCC were retrieved from the hospital files as paraffin wax embedded biopsies. The mean age of the patients was 73 years, with a range of 21–95. For 20 of these samples, non-fixed TCC tissue that had been snap frozen in liquid nitrogen was also available. Four paraffin wax embedded cervical carcinoma biopsies from patients with a mean age of 42.75 years, together with two paraffin wax embedded anogenital wart samples were used as positive controls for the PCR detection technique.

HPV antigen detection was performed on 5 µm thick sections of paraffin wax embedded tissue using routine immunological procedures. A polyclonal rabbit antibody against HPV capsid protein (Biogenex, San Ramon, California, USA) was used as the primary antibody at a dilution of 1/3000, with horseradish peroxidase conjugated

Abbreviations: EIA, enzyme immunoassay; DIG, digoxigenin; HPV, human papillomavirus; PCR, polymerase chain reaction; TCC, transitional cell carcinoma

Table 1 Summary of HPV detection results

Sample	Immunohistochemistry	PCR
Fixed TCC		
SM	35/45 (78%)	0/98 (0)
NSM	12/33 (36%)	
Total	47/78 (60%)	
Non-fixed TCC	ND	0/20 (0)
Fixed cervical carcinoma	ND	4/4 (100%)
Fixed anogenital wart	ND	2/2 (100%)

ND, not done; NSM, non-squamous metaplasia samples; PCR, polymerase chain reaction; SM, squamous metaplasia samples; TCC, transitional cell carcinoma.

antirabbit secondary antibody and diaminobenzidine chromogen used for visualisation of the results. HPV positive cervical carcinoma sections were used as positive controls.

DNA preparation, PCR, and sequencing

Genomic DNA was extracted from snap frozen biopsies and 5–10 µm paraffin wax embedded tissue sections using the QIAamp DNA mini kit (Qiagen, Hilden, Germany), according to the manufacturer's protocol, with the modification that paraffin wax embedded tissue was digested with proteinase K for five days. The quality of the isolated DNA was tested by PCR amplification of the β globin gene using the primers B1 and B19.¹⁵

Mucosal HPV plasmid clones were used as positive controls. HPV types 6, 11, 16, 18, and 40 were kindly provided by E-M de Villiers (Heidelberg, Germany), HPV types 31 and 35 by A Lorincz (Gaithersburg, Maryland, USA), and HPV types 33, 34, and 39 by G Orth (Paris, France). A series of 10 fold dilutions ranging from 20 to 2×10^{-13} ng/µl was made for each plasmid DNA.

PCR amplification using GP5+/6+ biotinylated primers¹⁴ was carried out on plasmid dilutions and β globin positive clinical samples as follows. Briefly, PCR was performed in a final reaction volume of 50 µl containing either 10 µl of diluted plasmid DNA with 200 ng human placental DNA or 200 ng genomic DNA, 5 µl buffer II 10× (supplied with AmpliTaq Gold; Applied Biosystems, Foster City, California, USA), 3.5mM MgCl₂, 50 µM of each deoxynucleoside triphosphate, 50 pmole of each forward and reverse primer, and 1 U of AmpliTaq Gold. Thermal cycler conditions were as follows: initial denaturation for four minutes at 94°C, followed by 40 cycles of one minute at 94°C, two minutes at 48°C, and 1.5 minutes at 72°C, with a final extension for four minutes at 72°C. Two negative controls—200 ng placental DNA and water—were included for every three genomic DNA samples. Aliquots (5 µl) from each PCR reaction were run on a 2% agarose gel, stained with 0.3 µg/ml ethidium bromide, and visualised on an ultraviolet imager.

PCR products of positive samples were gel purified using a QIAquick gel extraction kit (Qiagen) and sequenced using an ABI Prism dRhodamine terminator cycle sequencing kit (Applied Biosystems), according to the manufacturer's protocol. Products were analysed on a Perkin Elmer 377 ABI Prism automated sequencer and BioEdit Sequence Alignment software¹⁶ was used to align the forward and reverse complement sequences. The resulting consensus sequence was compared with those of known HPV types in the GenBank database using the Blast programme on the website of the National Centre for Biotechnology Information (National Institutes of Health, Bethesda, Maryland, USA).

Enzyme immunoassay

HPV type specific oligonucleotide probes¹⁷ corresponding to the above plasmids were 3' end labelled with digoxigenin labelled dideoxyuridine triphosphate (DIG-ddUTP) using a

DIG oligonucleotide 3' end labelling kit (Roche Applied Sciences, Mannheim, Germany), according to the manufacturer's protocol. DNA was precipitated with 0.1 volumes LiCl and six volumes of absolute ethanol to remove unincorporated DIG-ddUTP. Labelled DNA probes were resuspended in 20 µl distilled H₂O and the labelling efficiency tested using a DIG nucleic acid detection kit (Roche), according to the manufacturer's instructions.

Before their use on PCR products from clinical samples, HPV probes were first tested by EIA against GP5+/bioGP6+ PCR products from the dilution series of the relevant HPV plasmid DNA to verify sensitivity. Low risk (HPV types 6, 11, 34, and 40) and high risk (HPV types 16, 18, 31, 33, 35, and 39) HPV probes were then pooled and tested on the PCR products of LR and HR HPV plasmids to ensure the probe cocktails were group specific.

EIA was performed on GP5+/bioGP6+ PCR products from clinical samples and PCR negative controls (placental DNA and water), as described previously.¹⁸ In brief, PCR products were immobilised in a streptavidin coated microtitre plate and hybridised with probe cocktails containing 10 pmol/ml of each labelled oligonucleotide probe. Hybrids were detected by anti-DIG antibodies conjugated with alkaline phosphatase and *p*-nitrophenyl phosphate was used as the colorimetric substrate. Optical density was measured at 405 nm against 620 nm and a cutoff value of three times the mean optical density of the PCR negative controls was used.

RESULTS

Immunohistochemical detection of HPV capsid antigen

Immunohistochemistry was carried out on 78 paraffin wax embedded bladder TCCs. Forty seven of these samples were positive using a polyclonal antibody against HPV capsid protein (table 1; fig 1).

Detection of HPV DNA by PCR

Consensus GP5+/6+ primer mediated PCR followed by agarose gel electrophoresis was used to detect HPV DNA in 98 paraffin wax embedded bladder TCCs, 20 paired snap frozen TCC biopsies, and six positive control samples, comprising four paraffin wax embedded cervical carcinoma biopsies and two paraffin wax embedded anogenital warts. The ability of the technique to amplify HPV DNA from paraffin wax embedded tissue was demonstrated by the finding that all cervical carcinoma and anogenital wart samples were positive (table 1). The sequencing of PCR products later revealed that three of the cervical carcinoma samples were positive for HPV-16 and one was positive for HPV-18 DNA, whereas the two wart samples both contained HPV-6 DNA. In contrast, none of the paraffin wax embedded or snap frozen bladder TCCs was positive. There was no discrepancy between the results obtained using fixed and fresh frozen tissue, suggesting that the failure to detect HPV DNA in these samples was not the result of the technical difficulty of extracting good quality genomic DNA from paraffin wax embedded samples.

Screening of samples by PCR-EIA

All PCR products from clinical samples were also subjected to EIA to investigate the efficacy of PCR-EIA as a screening technique. All six of the cervical carcinoma and anogenital wart control samples in which HPV DNA was detected by PCR followed by agarose gel electrophoresis were also positive by PCR-EIA. The PCR products from the carcinoma samples were positive with the high risk but not the low risk probe cocktail, whereas the reverse was true for the wart samples, demonstrating the accuracy of EIA in distinguishing between high and low risk HPV DNA. All of the TCC samples

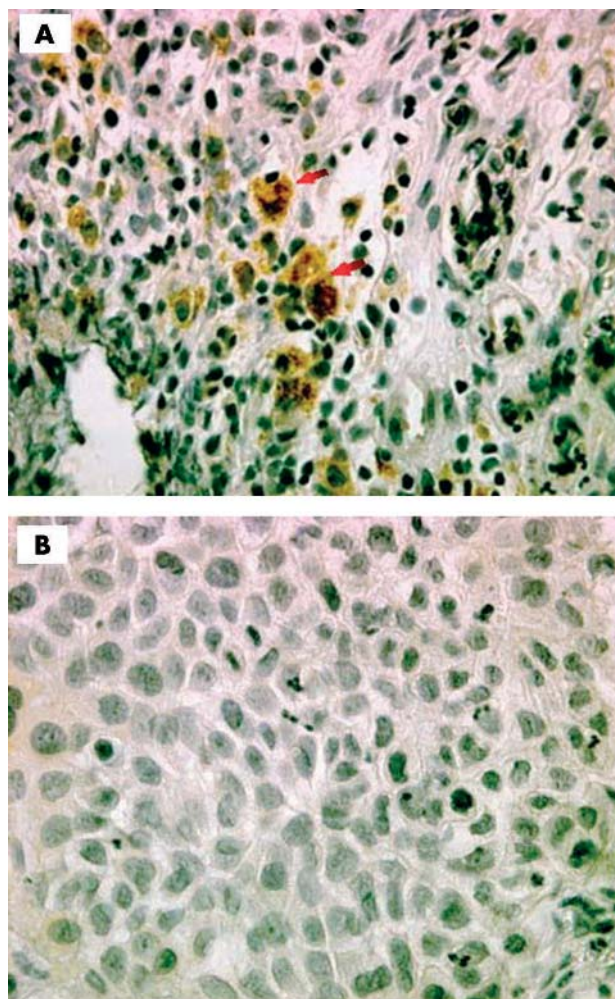


Figure 1 Immunohistochemical staining for human papillomavirus (HPV) capsid protein in bladder transitional cell carcinoma. Original magnification, $\times 40$. (A) HPV positive section indicated by nuclear and cytoplasmic staining of the tumour tissue (arrowheads). (B) HPV negative section.

were negative by PCR-EIA, confirming the results obtained by agarose gel electrophoresis of PCR products.

DISCUSSION

HPV has been shown to play a role in the development of anogenital cancers, particularly cervical cancer.¹ In our study, we examined the possible aetiological role of HPV in bladder carcinogenesis using both immunohistochemical and PCR based detection methods. Forty seven of the 78 TCC samples screened were positive by immunohistochemistry with a polyclonal antibody against HPV L1 capsid protein. In contrast, all samples were negative for HPV DNA by PCR amplification using the consensus GP5+/6+ primer set, followed by agarose gel electrophoresis and EIA with type specific probes. The ability of the GP5+/6+ primers to amplify low copy number HPV DNA has been clearly demonstrated by de Roda Husman *et al*,¹⁴ who reported a detection threshold of below 100 fg HPV-16 DNA in 100 ng of human placental DNA, equivalent to less than one copy for each cellular genome. In addition, we have shown that this technique reproducibly amplified HPV DNA from all six paraffin wax embedded cervical cancer and anogenital wart control samples. Nevertheless, to exclude the possibility of false negative results as a result of the poor quality of DNA

extracted from paraffin wax embedded samples, another 20 paraffin wax embedded TCCs were selected for which snap frozen tissue was also available. Negative results were also obtained after PCR amplification of DNA extracted from the fixed and frozen samples, confirming that no HPV DNA was present.

“The association of human papillomavirus with bladder transitional cell carcinoma may vary with different geographical locations”

The disparity between the above results casts doubt on the accuracy of the methods used, because it appeared unlikely that samples containing HPV DNA at levels below the PCR detection threshold would express L1 capsid protein in sufficient quantities for immunohistochemical detection. Although samples containing HPV DNA may appear as PCR false negatives if integration of the HPV genome into chromosomal DNA during carcinogenesis leads to disruption of the PCR primer binding sites, this seems unlikely to have occurred here because the GP5+/6+ primers are located within the L1 open reading frame. Indeed, we are not the first to observe a lower rate of positivity for HPV DNA compared with HPV capsid antigen in bladder TCC samples. Lopez-Beltran *et al* reported that 32% of 76 bladder TCCs were positive for HPV capsid antigen using a polyclonal antibody, whereas only 9% were positive for HPV DNA by PCR.^{19, 20} These investigators suggested that the difference might have been caused by the use of a restricted range of type specific PCR primers, meaning that samples containing DNA from less common HPV types may have been overlooked. However, in view of the ability of the GP5+/6+ consensus PCR primer mediated technique to detect a range of HPV DNAs in paraffin wax embedded samples, as shown here and previously, the discrepancy between the two sets of experimental data in our present study is most probably attributable to false positives resulting from the immunohistochemical protocol, particularly because a polyclonal rather than monoclonal antibody was used.

Many earlier investigations have reported a low prevalence of HPV in TCC. For example, using a PCR methodology identical to our present study, Sur *et al* detected HPV DNA in only one of 64 paraffin wax embedded TCCs screened,¹² and Aynaud *et al* found no HPV DNA in 58 bladder TCCs examined.²¹ Chetsanga *et al* detected HPV DNA in only one of 44 TCCs using a degenerate PCR technique followed by dot blot analysis with type specific probes for six HPV types commonly detected in anogenital lesions.²² Nevertheless, a

Take home messages

- Immunohistochemistry and the polymerase chain reaction were used to investigate human papillomavirus (HPV) in transitional cell carcinoma (TCC) with very different results
- Forty seven of 78 samples were positive for HPV antigen yet none was positive for HPV DNA
- Because HPV DNA was not detected in unfixed TCC samples but was found in all six paraffin wax embedded cervical carcinoma and anogenital wart controls, the disparity is probably caused by the non-specificity of the polyclonal antibody used for immunohistochemistry
- HPV is unlikely to play an aetiological role in the development of bladder TCC

few studies have reported higher incidences of HPV positivity among bladder TCCs. De Gaetani and colleagues²³ detected HPV DNA in 39.5% of samples screened, whereas Chan and colleagues²⁴ detected HPV DNA in six of 20 TCCs by dot blot analysis using type specific PCR primers and probes. Interestingly, although HPV type 6, 11, 16, 18, 31, and 33 type specific probes were used, all positive samples were found to contain HPV-18 DNA. Elsewhere, two Japanese studies^{25–26} found incidences of 81% and 31%, respectively. The dissimilarity in HPV prevalence reported by these investigations suggests that the association of HPV with bladder TCC may vary with different geographical locations. This is supported by the fact that most studies reporting a high incidence of HPV positive samples were performed in southern Europe or Asia,²⁷ whereas most carried out in northern Europe and America reported an extremely low rate of HPV positivity.²⁸ Therefore, our finding of a lack of association between HPV and bladder TCCs is not surprising.

Authors' affiliations

S Youshya, K Purdie, J Breuer, C Proby, Centre for Cutaneous Research, 2 Newark Street, Whitechapel, London E1 2AT, UK
S Baihuth, M T Sheaf, Department of Histopathology, Royal London Hospital, Whitechapel, London E1 1BB, UK
R T D Oliver, Department of Medical Oncology, Royal London Hospital

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