Human papillomavirus and schistosomiasis associated bladder cancer

K Cooper, Z Haffajee, L Taylor

Abstract
Aims—To determine the human papillomavirus DNA status of schistosomal associated squamous cell carcinoma of the urinary bladder in South Africa.

Methods—Twenty five archival samples of bladder squamous cell carcinoma associated with Schistosoma haematobium were subjected to non-isotopic in situ hybridisation and the polymerase chain reaction for the detection of human papillomavirus 6, 11, 16, 18, 31, and 33 genotypes.

Results—Using these two techniques, none of the 25 cases was shown to harbour human papillomavirus DNA.

Conclusions—This study abrogates the role of human papillomavirus in schistosomal associated bladder carcinoma in South Africa. It is suggested that other factors including nitrosamine exposure, p53 mutation, and additional unknown chromosomal events play a major role in the development of this parasite associated neoplasm.

Keywords: Schistosoma haematobium; urinary bladder; squamous cell carcinoma; human papillomavirus

In Africa, schistosomiasis associated bladder cancer in Africa tends to present as squamous cell carcinomas in relatively young individuals. This association is clearly defined with Schistosoma haematobium, the causative agent of urinary or bladder schistosomiasis. In a previous study in South Africa, p53 mutations were recorded in 57% of schistosomal associated squamous cell carcinomas of the urinary bladder. The majority was detected in exon 8 with multiple mutations, a fact that is suggestive of the involvement of a carcinogenic agent.

The association of human papillomavirus (HPV) and non-schistosomal bladder squamous cell cancers has been reported in isolated cases in the USA. The first case occurred in a 61 year old female who was immunocompetent and had previous evidence of papillomavirus associated disease. The second was a renal transplant recipient on chronic immunosuppression. A recent Egyptian study has also demonstrated HPV DNA in six of 16 (38%) bladder squamous cell cancers. In addition, the majority of the HPV positive cases in this study harboured schistosomiasis.

The present study was undertaken to investigate a possible association between HPV and schistosomiasis in squamous cell cancers of the urinary bladder in South Africa and to correlate this with the previous demonstration of p53 mutational analysis. In addition, a multistep carcinogenic pathway for schistosomal associated bladder squamous cell carcinoma is proposed.

Methods and materials
Tissue samples
Twenty five paraffin wax embedded tissue samples of bladder carcinomas from individuals infected with S haematobium were obtained from the departmental archives. All tumours were reviewed and classified as invasive squamous cell carcinomas, closely associated with ova of S haematobium.

Non-isotopic in situ hybridisation
Non-isotopic in situ hybridisation (NISH) was performed using a technique described previously. Sections (4 μm) from the paraffin wax embedded tissue samples were cut on to slides pretreated with 3% aminopropyltriethoxysilane (Sigma, St Louis, Missouri, USA). The sections were allowed to dry overnight at 42°C and then dewaxed and rehydrated according to standard protocols. The slides were treated with 3% hydrogen peroxide in methanol to reduce residual non-specific peroxidase activity. Unmasking of nucleic acids was achieved by a limited proteolysis in proteinase K (500 μg/ml) at 37°C and the reaction stopped in distilled water after 15 minutes. The slides were air dried prior to the addition of aliquots of hybridisation mix (6 μl) containing 2 ng/μl of digoxigenin labelled HPV 6, 11, 16, 18, 31, or 33 (a gift from Professor J0’D McGee, Oxford, UK). Each section was covered with a coverslip to prevent drying. The slides were then placed in a moist Petri dish. Target DNA and probe were denatured in a hot air oven at 95°C for 15 minutes and then allowed to hybridise at 42°C for two hours. The sections were then subjected to two post-hybridisation washes of five minutes each in 4x standard saline citrate buffer and incubated in Tris buffered saline containing 5% (wt/vol) bovine serum albumin and 5% (vol/vol) Triton X-100 (TBT) for 10 minutes.

Detection of hybridised probe followed conventional immunohistochemical techniques. Sections were incubated for 30 minutes with monoclonal anti-digoxigenin (1/10 000; Sigma) followed by biotinylated rabbit antimouse F(ab’)2 fragment (1/200; Dako, Glostrup, Denmark) for 30 minutes. The final incubation was in avidin-peroxidase conjugate (1/75; Dako) containing powdered non-fat milk (0.05 mg/μl) as a further means of...
Table 1  PCR primer sequences for the HPV E6 gene and β globin gene*  

<table>
<thead>
<tr>
<th>Name</th>
<th>5’→3’ nucleotide sequence†</th>
<th>Target</th>
<th>Strand</th>
</tr>
</thead>
<tbody>
<tr>
<td>WD72</td>
<td>CCGTCCGGACGCGAAACGG</td>
<td>HPV E6</td>
<td>+</td>
</tr>
<tr>
<td>WD76</td>
<td>CCGTTTAAACCGAAMCGG</td>
<td>HPV E6</td>
<td>+</td>
</tr>
<tr>
<td>WD66</td>
<td>AGCATCGGGTATACTGCTC</td>
<td>HPV E6</td>
<td>-</td>
</tr>
<tr>
<td>WD67</td>
<td>WGCAWATGAMWGCGTCTC</td>
<td>HPV E6</td>
<td>-</td>
</tr>
<tr>
<td>WD154</td>
<td>TCCGTTGTTGTGGTCGTC</td>
<td>HPV E6</td>
<td>-</td>
</tr>
<tr>
<td>GH204</td>
<td>GAAGAGCAAGGACGGTAC</td>
<td>β globin</td>
<td>+</td>
</tr>
<tr>
<td>PC04</td>
<td>CACCTTCACTACGTTCCC</td>
<td>β globin</td>
<td>-</td>
</tr>
</tbody>
</table>

* Reference 8; † Degenerate code, W = A + T; Y = C + T; S = G + C; ‖ Product size 268 base pairs.

reducing non-specific nuclear staining. All dilutions of antisera were made up in TBT. Sections were incubated in diaminobenzidine (DAB) as chromogen substrate and counterstained lightly in Mayer’s haematoxylin. Positive control sections obtained from paraffin embedded penile squamous cell carcinoma tissue blocks known to contain HPV 16 (by both NISH and polymerase chain reaction (PCR)) were included in each experiment. Negative controls comprised similar tissue sections and steps with the omission of HPV DNA probes.

POLYMERASE CHAIN REACTION

Two 10 μm sections of tissue were cut from each specimen and placed on a clean glass slide. Proper PCR protocol was observed at all times to prevent contamination and cross contamination of samples. The sections were dewaxed and rehydrated as per standard protocol, rinsed in sterile distilled water, and allowed to air dry. DNA was extracted from the samples using the QUAGEN QIAamp tissue kit (QUAGEN Ltd, Dorking, UK). The samples were suspended in a lysis buffer containing proteinase K and allowed to incubate overnight at 55°C. DNA was precipitated with ethanol in a spin column and finally eluted in water. A volume of 10 μl was amplified for each DNA sample using a mixture of positive and negative strand primers to the E6 region. Each 50 μl reaction contained 10 μl TrisHCl, 50 mM KCl, 4 mM MgCl₂, 200 μM dNTP; 2.5 U Taq polymerase (Boehringer Mannheim, Mannheim). Extracted DNA from paraffin sections known to contain HPV DNA was used as a positive control. Two negative control tubes were set up using the same PCR method but sample DNA was omitted and extracted DNA from tissue without HPV was used. Gels were viewed with an ultraviolet transilluminator and photographed on Polaroid film.

Results

CLINICAL DETAILS

Haematoxylin and eosin sections of all 25 tumours were reviewed and classified as squamous cell carcinomas. Invasive islands of tumour tissue were closely associated with schistosomal ova within the stroma in all cases. The age of the patients varied from 29–72 years (18 males and seven females), the median age being 47 years. This is similar to that reported in the literature, with the majority of patients with schistosomal associated bladder cancer being younger than 50.

NON-ISOTOPIC IN SITU HYBRIDISATION

None of the 25 cases harboured HPV DNA within nuclei of squamous cell carcinomas. Positive and negative control sections on tissue with and without HPV DNA demonstrated positive and negative NISH signals, respectively.

POLYMERASE CHAIN REACTION

Amplifiers of the HPV E6 gene (240 base pairs) were not present in any of the 25 tumours, even though intact DNA was demonstrated in all cases with the amplification of the β globin gene (268 base pairs). Both positive and negative controls produced expected results with respect to HPV DNA and β globin gene amplification (that is, positive and negative, respectively).

Discussion

The association between urinary schistosomiasis and squamous cell carcinoma of the bladder in Africa is well established. Epidemiological studies have demonstrated clearly that there is a high incidence of bladder cancer in endemic areas of urinary schistosomiasis. The majority of these cancers tend to be squamous cell carcinoma in young individuals and are associated with a heavy schistosomal egg load.

A persistent secondary bacterial infection in schistosomal bladders has also been demonstrated. Organisms, such as Escherichia coli and Proteus spp, capable of reducing nitrites to nitrous forms with formation of N-nitrosoamines have been the focus of attention. However, bacterial nitrate reductase is not the only enzyme implicated in schistosomal associated bladder carcinogenesis. Production of carcinogenic metabolites from tryptophan and elevated levels of the deconjugating enzyme glucuronidase has also been cited as cofactors. An important animal study has shown that baboons infected with S haematobium and then fed with the N-nitroso compound, BBN, developed bladder tumours. However, uninfected control baboons fed on BBN did not develop tumours more than two
infections involving oncogenic HPV types were present, especially in the Japanese series. A recent study from Egypt demonstrated HPV DNA in six of 16 (38%) bladder squamous cell cancers, the majority of which was associated with schistosomiasis. HPV types 16 or 18 were present in the majority of these tumours. However, the present study failed to demonstrate HPV in schistosomal associated squamous cell cancers of the bladder; this discrepancy may be related to geographical differences, as has been described for bladder transitional cell carcinoma. Although HPV has been shown to be associated with uterine cervical and oesophageal cancer in South Africa, the present study did not demonstrate the virus in schistosomal associated bladder cancer in this country. Hence, an alternative pathway for schistosomal associated bladder squamous cell carcinoma is proposed. Further molecular investigation is necessary to elucidate the later carcinogenetic events of this unique association.
24 Knowles MA. Human papillomavirus sequences are not detectable by Southern blotting or general primer-mediated polymerase chain reaction in transitional cell tumours of the bladder. Urol Res 1992;20:297-301.