

# Human papillomavirus in oesophageal squamous cell carcinoma

S L Loke, L Ma, M Wong, G Srivastava, I Lo, C C Bird

## Abstract

**Thirty seven cases of oesophageal squamous cell carcinoma were studied by applying DNA slot blot analysis and in situ hybridisation using type specific probes for HPV 6, 11, 16 and 18. Cases of condyloma accuminata, cervical carcinoma, and laryngeal papilloma were used as controls. Blocks including areas of invasive carcinoma, intraepithelial neoplasia, and normal epithelium were studied in each case. No HPV genome was detectable in any of the oesophageal cases. It is concluded that these types of HPV do not have an association with oesophageal squamous cell carcinoma.**

Human papillomavirus (HPV) infection is associated with the development of papillomas or condylomas in various parts of the body,<sup>1-4</sup> and HPV types 6 and 11 are most frequently found in such benign lesions.<sup>5</sup> HPV infection has also been confirmed in oesophageal papillomas by the presence of koilocytic features,<sup>6</sup> viral antigen,<sup>7</sup> or viral genome.<sup>8</sup> There is also a suggestion that HPV infection of the oesophagus is on the increase.<sup>9</sup>

The recent detection of the HPV genome in cervical squamous cell carcinoma has prompted the search for virus in squamous cell carcinomas (SCC) in other sites of the body. HPV types 16 and 18 are most frequently detected in these malignant lesions and their association with malignancy seems to be relatively independent of site.<sup>3,4,10</sup> While there are a few reports suggesting that HPV is detectable in oesophageal SCC,<sup>11-14</sup> this has not been confirmed in other studies.<sup>4</sup>

To investigate this possibility further we applied in situ hybridisation and DNA slot-blot analysis to a series of oesophageal SCC occurring in Hong Kong. The former technique proved sufficiently sensitive to detect HPV in cervical SCC<sup>15</sup> where tumour cells are likely to contain many copies of viral genome. DNA slot-blot analysis, however, can detect virus where the genomic copies are low.

## Methods

Thirty seven cases of oesophageal SCC where total oesophagectomy had been performed were selected for study. These included a selection of 14 cases with multiple separate primary tumours in the oesophagus. The whole specimen in each case had been totally embedded for histological examination to define the extent of invasive tumour and

intraepithelial neoplasm. DNA was extracted from the tumour in 21 of these cases before wax embedding. For each case, blocks representative of invasive tumour, intraepithelial tumour, and normal adjacent epithelium were selected for analysis by in situ hybridisation.

Cases of condyloma accuminata and laryngeal papillomata containing HPV 6 or 11 and cervical SCC containing HPV 16 or 18 were chosen as positive control tissues. The human cell lines CaSki and SiHa, both containing HPV 16 genome, and HeLa, containing HPV 18, were also used as positive controls for DNA slot-blot analysis. All three cell lines were obtained from American Tissue Type Culture Collection, USA.

Plasmids containing HPV types 6, 11, 16 and 18 DNA were kindly provided by Professor zur Hausen and Dr Gissmann (German Cancer Research Centre, Heidelberg, Federal Republic of Germany).<sup>16-19</sup> HPV probes were obtained by digesting the plasmid DNA with *BamH I* for HPV 6, 11, 16, and *EcoR I* for HPV 18. The viral DNAs were labelled with digoxigenin for in situ hybridisation using a detection kit (Boehringer, Mannheim GmbH, Ponsberg, West Germany), or oligo-labelled for slot blot hybridisation according to the supplier's instructions (Bresatec, Australia).

## IN SITU HYBRIDISATION

The method used was a modification of the one previously described.<sup>20</sup> Hybridisation was performed with digoxigenin-labelled probes at a final concentration of 200 ng/ml in 50% (v/v) deionised formamide, 10% (w/v) dextran sulphate, 2 x SSC (30 mM sodium chloride, 3 mM sodium citrate) and 0.2% dried milk powder at 42°C overnight.

Post-hybridisation wash included 2 x SSC at 60°C for 10 minutes, followed by 0.2 x SSC at 42°C for 10 minutes with stirring. For sections to be washed in low stringency, the initial temperature for washing was reduced to 45°C.

Signal detection was performed using poly sheep anti-digoxigenin-Fab fragments conjugated to alkaline phosphatase (Boehringer, Mannheim, Biochema, Penzeberg, West Germany) according to the supplier's recommendations.

## SLOT-BLOT ANALYSIS<sup>21,22</sup>

DNA (5 µg) extracted from cryosections using proteinase K was blotted on to a nylon membrane (Genescreen Plus, Dupont, NEN Products, Boston, Massachusetts) according to the supplier's instructions.

The membranes were hybridised to the

Department of Pathology, University of Hong Kong, Queen Mary Hospital, Hong Kong

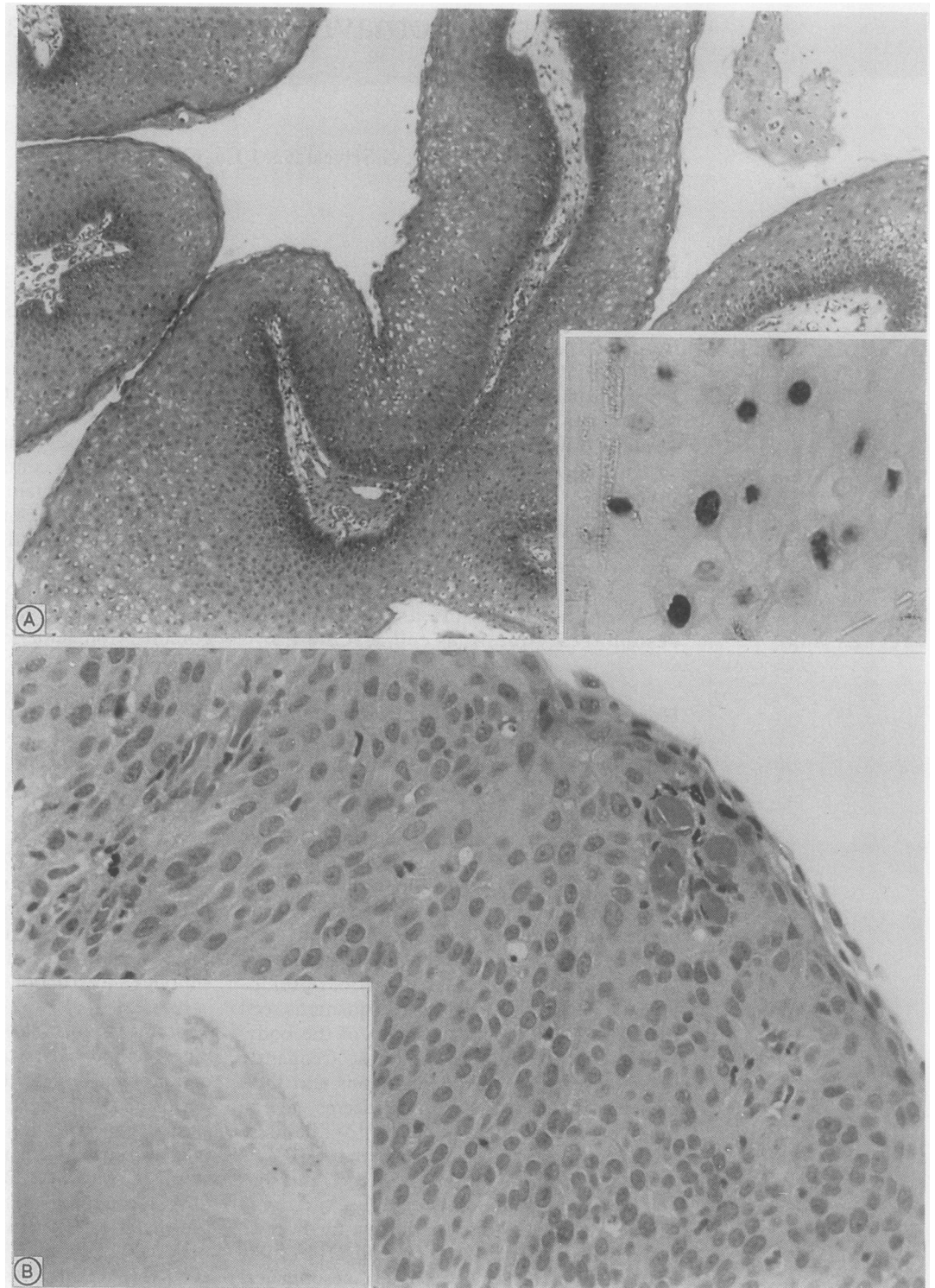
L Ma  
M Wong  
G Srivastava  
I Lo

Department of Pathology, University of Edinburgh, Scotland  
C C Bird

Correspondence to:  
Dr S L Loke

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**Figure 1** (A) Papilloma of larynx with strong signal in *in situ* hybridisation (see insert) for HPV 11 (haematoxylin and eosin). (B) Intraepithelial neoplasia of the oesophagus with no detectable signal in *in situ* hybridisation (see insert) for HPV 11 (haematoxylin and eosin).



denatured  $^{32}$ p-oligo-labelled HPV DNA probe (0.5–2 ng/ml) in 50% formamide, 1 M NaCl, 10% dextran sulphate, 1% sodium dodecyl sulphate, 0.2% polyvinyl-pyrrolidone, 0.2% Ficoll, 0.2% bovine serum albumin, 50 mM TRIS-HCl (pH 7.5), 0.1% sodium pyrophosphate (NaPPi) and denatured salmon sperm DNA at 42°C for 40 hours. The final wash of the membranes was in solution containing  $0.2 \times$  SSC, 0.1% NaPPi, 0.1 mM EDTA and 1% sodium dodecyl sulphate at 65°C for 40 minutes.

Autoradiography was carried out using an X-OMAT AR X-ray film (Kodak).

## Results

### IN SITU HYBRIDISATION

With post-hybridisation washing in high stringency, strong signal was detectable in the control tissues for each HPV type. Similar washing conditions gave no detectable signal in the cases tested. A representative pair of control condyloma and oesophageal SCC hybridised with HPV 11 probe is shown in fig 1. When washing was performed in low stringency, there was still no signal in all of the cases tested and the background was the same between the sections hybridised to the HPV probes and the plasmid without the viral

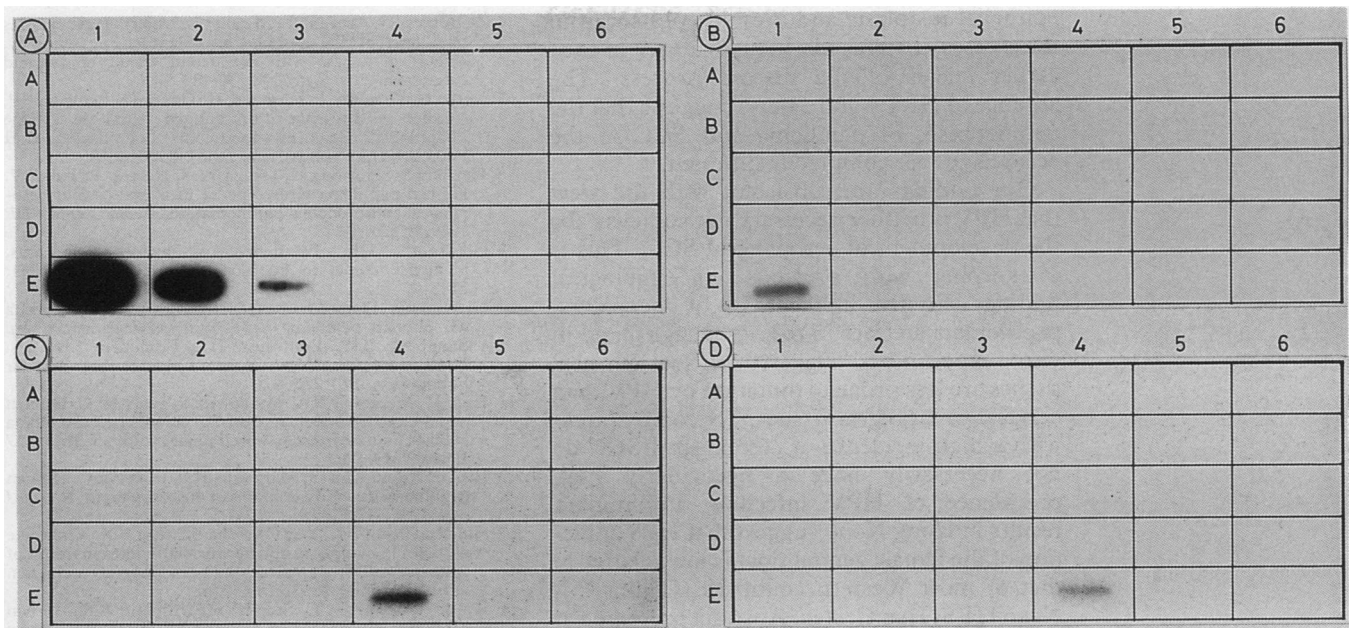


Figure 2 No signal was detected in DNA slot-blot using 5  $\mu$ g total DNA from each of the 21 cases of invasive carcinoma (A, 1–6; B, 1–6; C, 1–6 and D, 1–3):

- (A) Hybridisation with HPV16, with 1  $\mu$ g, 100 ng, 10 ng, 1 ng, 100 pg and 10 pg DNA from CaSki cell line (E, 1–6) as control.  
 (B) Hybridisation with HPV18, with 1  $\mu$ g, 100 ng, 10 ng, 1 ng, 100 pg and 10 pg of DNA from HeLa cell line (E, 1–6) as control.  
 (C) Hybridisation with HPV6, with 10  $\mu$ g DNA from CaSki (E, 1), SiHa (E, 2), HeLa (E, 3) and laryngeal papilloma (E, 4) as controls.  
 (D) Hybridisation with HPV11, with 10  $\mu$ g DNA from CaSki (E, 1), SiHa (E, 2), HeLa (E, 3) and laryngeal papilloma (E, 4) as controls.

inserts. Similar negativity was noted for the invasive carcinomas, intraepithelial neoplasia, and normal mucosa. This indicated that there were no focal collections of cells containing abundant virions of these HPV types.

#### DNA SLOT-BLOT ANALYSIS

By titrating the DNA extracted from cell lines, we determined our sensitivity in HPV detection. HPV 16 genome was detectable in 10 ng of total DNA from CaSki cell lines but not in 5  $\mu$ g of total DNA from each of the cases when hybridised in the same conditions (fig 2A). As there was an average of 400–500 copies of HPV 16 per cell in CaSki cell lines, our DNA slot-blot should be able to detect HPV 16 genome with a sensitivity of up to an average of one copy per cell. This was further supported by our ability to detect the genome in 2  $\mu$ g of total DNA from the SiHa cell line which contained one to two copies per cell. Similarly, HPV 18 genome was detectable in 1  $\mu$ g of total DNA from HeLa cell lines but not in 5  $\mu$ g total DNA from SCC (fig 2B). As HeLa cell lines contained four to 50 copies of viral genome per cell, we should have been able to detect HPV 18 genome with a sensitivity of up to 0.8–10 copies per cell in SCC.

HPV 6 or 11 genome was readily detectable in 5  $\mu$ g of total DNA from the cervical condylomata and laryngeal papillomata, but not in a similar amount of total DNA from the oesophageal SCC. As the copy numbers of viral genome in the condylomata and papillomata were unknown, the sensitivity for detection of HPV 6 and 11 was not determined (fig 2C, D). Our inability to detect any HPV genome of any type in the 21 cases studied indicated that either these HPV types were not present in the

invasive tumours, or that, there were too few copies of the viral genome.

#### Discussion

SCC, the most common malignancy of the oesophagus,<sup>23</sup> is common among Southern Chinese, and ranks fifth in the league table of deaths from cancer in Hong Kong.<sup>24</sup> The recent interest in HPV and its association with cervical SCC has led to the investigation of its possible aetiological role at other sites of the body. As in cervical SCC, detection of viral genome has been reported in several other mucosal sites.<sup>1–4</sup>

Hille *et al* reported detection of HPV antigen by immunostaining oesophageal SCC in South African patients in about a third of the cases.<sup>12</sup> HPV genome has been detected in three cases of oesophageal SCC in Australian patients by Kulski *et al* using filter in situ hybridisation with a probe mixture containing HPV types 11, 13, 16 and 18.<sup>14</sup> These workers found no detectable viral antigen in these cases, suggesting that detection of the genome is more sensitive than immunostaining.

By contrast, Kiyabu *et al* were unable to detect HPV 16 or 18 in invasive SCC of the oesophagus in North American patients using the polymerase chain reaction.<sup>4</sup> The possible role of other types of the virus was not investigated, and no mention was made of intraepithelial neoplasia or areas of adjacent normal epithelium.

Our inability to detect the presence of HPV in cases of SCC of the oesophagus is in keeping with the findings of Kiyabu *et al*.<sup>4</sup> Moreover, by performing in situ hybridisation, we can correlate histology with viral genome detection. As no HPV was detectable in intra-

epithelial neoplasia and normal epithelium in these cases, it seems that virus is absent even at earlier stages of the disease process. The absence of HPV 6 and 11 also suggests that the pathogenesis of papilloma and SCC of the oesophagus are completely different.

Our findings are consistent with the view that HPV is neither necessary nor sufficient for the development of oesophageal SCC. This is in keeping with previous epidemiological findings. Firstly, unlike SCC of the cervix, papillomata are rare in the oesophagus, and in vitro experiments suggest that oesophageal tissues are less prone to mutation by HPV than cervical or laryngeal tissues.<sup>25</sup> Secondly, areas with a high incidence of oesophageal SCC do not necessarily have a particularly high prevalence of HPV infection. Preliminary results in Hong Kong suggest that HPV infection of the female genital tract is low relative to that of most Western countries (Collins RJ, Ngan HYS, Hsu C, *et al.* Abstract presented at the thirty fourth annual scientific meeting of the Royal College of Pathologists of Australia, 1989), and yet we have a high incidence of cervical SCC. Thirdly, other environmental factors such as diet or exposure to chemical carcinogens are epidemiologically related to SCC of the oesophagus. It is, however, still possible for HPV to act as an additional promoter after tumour induction by these carcinogens.<sup>26 27</sup>

In conclusion, we were unable to detect HPV genome in oesophageal SCC in Hong Kong using techniques that could detect small copy number of virions in tumour cells. This strongly suggests that HPV does not have a similar role in SCC of the oesophagus as is the case in comparable malignancies of other mucosal sites.

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