Human papillomavirus DNA in aerodigestive squamous carcinomas demonstrated by means of *in situ* hybridisation

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Abstract **A series of 10 oesophageal and 10 laryngeal squamous carcinomas was examined by means of immunocytochemistry and *in situ* DNA hybridisation to demonstrate human papillomavirus (HPV) infection. Changes in the epithelium adjacent to the carcinoma were found in 5 of 10 oesophageal and 7 of 10 laryngeal carcinomas. Viral antigens could not be detected with immunocytochemistry in any of the specimens. HPV 6, 11 and 16 were detected in 3 oesophageal specimens. In one of these, HPV 16 was detected in normal as well as malignant cells. HPV 7 was detected for the first time in a laryngeal carcinoma. Our results confirm previous reports of possible HPV involvement in the pathogenesis of aerodigestive carcinomas.**

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Papillomaviruses are a heterogeneous group of DNA viruses which predominantly cause benign proliferative epithelial lesions. Over the past few years, there has been good evidence to suggest an aetiological role for human papillomaviruses (HPVs) in squamous cell carcinogenesis, particularly in the lower genital tract, the larynx and in patients with epidermodysplasia verruciformis.[1,2] HPV DNA has been detected by means of DNA hybridisation in laryngeal tumours[3-5] and cell lines derived from squamous carcinomas of the head and neck.[6]

Oesophageal carcinoma is one of the aerodigestive tract carcinomas with a well-defined geographical and ethnic incidence. There is a remarkably high incidence of this carcinoma in China, Iran and South Africa.[7-9] There has been a dramatic increase in oesophageal cancer in Soweto over the last 20 years; it is now the leading cause of cancer-related deaths among black men.[9]

Previous South African studies that used epithelial morphology,[10] immunocytochemistry[11] and the polymerase chain reaction (PCR)[12] to determine the presence of HPV in oesophageal carcinomas have produced variable results. These results cannot be directly compared, however, because they originated from different geographical areas and the techniques differ vastly in sensitivity. A DNA hybridisation study has not yet been reported.

We investigated aerodigestive squamous carcinomas from the Pretoria region using the *in situ* hybridisation (ISH) technique. This sensitive technique has the advantage of localising viral DNA in tissue sections to an extent where detection of viral DNA in single cells is possible.

Material and methods

Biopsies

Biopsies were taken from the tumour and adjacent macroscopically normal tissue in 10 patients with carcinoma of the oesophagus. In the laryngeal group there were 9 laryngectomy specimens and 1 biopsy specimen taken for confirmation of clinical laryngeal carcinoma. Resected tumours were examined macroscopically and blocks were selected from both the tumour and normal mucosa.

All biopsies were fixed in 10% neutral buffered formalin and embedded in paraffin.

Light microscopy

Sections of tumour and the adjacent normal tissue were evaluated with special emphasis on epithelial changes consistent with HPV lesions.[13]

Immunocytochemistry

HPV structural proteins (group-specific papillomavirus capsid antigens) were demonstrated by means of a peroxidase-antiperoxidase (PAP) technique. All sections were stained with a polyclonal rabbit antibody to HPV (B580, Dakopatts, Denmark).

Probes

For HPV typing, the specific DNA probes of HPV 6, 7, 11, 16, 18 and 30 cloned in either pBR322 or pUC19 were used — these were kindly provided by Dr E.-M. de Villiers, Human Papillomavirus Reference Center, DKFZ, Heidelberg, Germany — and labelled with ^{32}P-dCTP (multiprime DNA labelling system, Amersham, UK). The specific activity of the probe was 2 - 5 × 10^8 counts/min/µg DNA and 5 - 10 ng of each probe were used on individual sections. Negative controls included plasmid vectors pBR322 and pUC19 hybridised on all sections investigated as well as a normal oesophageal biopsy hybridised with all HPV probes. Two paraffin tissue sections (one of cervical intraepithelial neoplasia and a carcinoma of the vulva positive for HPV 6 and 16 respectively) were used as positive control slides.

Tissue preparation

Five-micrometre thick sections were cut from paraffin wax-embedded tissues, mounted on 3-aminopropyltrieth oxysilane-coated slides[14] and baked at 60°C overnight.

In situ hybridisation

Slides were deparaffinised according to standard procedures.[14] The tissue sections were digested with a Proteinase K-containing solution (Boehringer, Mannheim, Germany), post-fixed and acetylated. The slides were prehybridised for 30 minutes at 52°C before the application of the probe solution. Heat-denatured probe solution was added to each section and the slides were incubated for 16 hours at 52°C in a humidified chamber.

After hybridisation, the slides were washed and dehydrated through graded ethanols.

Detection of hybridised probes

Slides were dipped in LM-1 emulsion (Amersham, UK). After a 4-day exposure time at 4°C, slides were developed (Ilford, Ciba Geigy), rinsed briefly in water and fixed for 5 minutes in Hypam fixative (Ilford). Sections were counterstained with haematoxylin and eosin, and mounted.

Results

HPV-suggestive changes in the epithelium adjacent to the carcinoma were found in 5 of 10 oesophageal and 7 of 10 laryngeal specimens. HPV-antigen expression could not be demonstrated with the PAP technique in any of the tumour sections or biopsies of normal epithelium.

All hybridisations with pBR322 and pUC19 were negative. Three of 10 oesophageal carcinomas and only 1 laryngeal carcinoma contained HPV DNA. HPV 6, 11 and 16 were detected in the oesophageal carcinoma group; 1 patient showed a double infection (HPV 11 and 16); HPV 16 was detected in the tumour as well as the adjacent normal epithelium (Fig. 1). HPV 7 was detected in another patient with laryngeal carcinoma in a biopsy taken from macroscopically normal epithelium adjacent to the tumour; this was later classified as intraepithelial neoplasia.

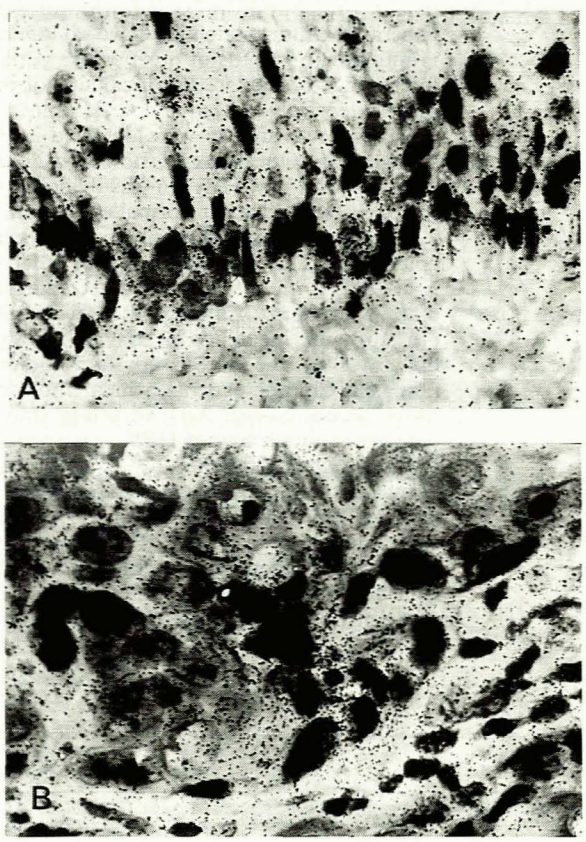

FIG. 1.
***In situ* hybridisation of radiolabelled HPV 16 DNA to adjacent normal epithelium** (A) **as well as in tumour cells** (B) **of an oesophageal carcinoma. Accumulation of developed grains on the nuclei of cells indicates the presence of viral DNA.**

Discussion

Our results confirm the presence of HPV DNA in oesophageal and laryngeal carcinomas. Viral antigens were not demonstrated in any of our cases where immunocytochemistry was used. Similar findings were reported in a previous study, which evaluated 20 oesophageal specimens.[11] The low or negative detection rates may be due to the fact that the technique only identifies a productive viral infection, while the degree of neoplasia is inversely related to viral expression. Furthermore, as the nature of the test depends on an antigen-antibody interaction, target antigenic determinants may be distorted by fixation in formalin, digestion by trypsin or heating in paraffin.[15] The temperature of the paraffin wax ovens, though, was carefully controlled and never exceeded 60°C.

The epithelium adjacent to the tumours showed HPV-suggestive changes in 5 oesophageal and 7 laryngeal carcinomas. Viral DNA could only be detected in 1 of these oesophageal tumours. The low detection rate of HPV in epithelial lesions thought to be specifically induced by the virus may be due to a low copy number of viruses present, undetectable by the ISH technique, or other HPV types with low sequence homology to the types used in this study.

Viral DNA was only detected in 4 patients. We failed to detect HPV 30 in any of the laryngeal carcinomas, but it is noteworthy that this is the first detection, to our knowledge, of HPV 7 in a laryngeal carcinoma. This virus is usually found in warts in the hands of butchers and meat handlers, from which it was originally isolated.[16,17] It was also detected in oral lesions of 7 HIV-infected patients, but so far has not been demonstrated in any oral papillomas of immunologically normal patients.[18] Unfortunately we have no information on the immunological status of our positive patient. It may be interesting to evaluate the incidence of this HPV type more extensively in head and neck tumours.

HPV 6, 11 and 16 were detected in 3 oesophageal carcinomas. One patient showed a double infection (HPV 11 and 16). HPV 16 was demonstrated in malignant cells and adjacent normal epithelium. In the other 2 patients viral DNA was only found in adjacent normal epithelium. This is in keeping with other studies where viral DNA is more frequently detected in adjacent tissue than in tumours.[19] Although there were only 10 cases in our study the incidence of HPV DNA was only a little higher (43%) in a larger study of 51 oesophageal carcinomas in Chinese patients, which employed ISH.[19] The more recent South African study that used PCR[12] detected a much higher HPV incidence (71%); this is similar to the finding of a PCR study on laryngeal carcinomas with a 75% detection rate.[20] Both these studies involved only 14 and 16 specimens respectively. Being such a sensitive technique, PCR can potentially detect single genes; cross-referencing with Southern blot or ISH and other control techniques is therefore essential to avoid misleading results. Larger studies, of both oesophageal and laryngeal carcinomas, that employ different techniques in tandem are needed for optimal evaluation.

In addition to physical, chemical and nutritional factors previously related to the development of oesophageal and laryngeal carcinomas, this study supports the theory that HPV infection is important in this multistep pathogenesis.

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