Human papillomavirus associated with oesophageal cancer

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HPV was implicated for the first time in the pathogenesis of oesophageal cancer in a study reported in 1982. These organisms are small non-enveloped DNA viruses classified as belonging to the Papovaviridae family, and more than 70 papillomavirus types have been identified on the basis of sequence divergence. The association of certain types of HPV primarily with normal tissue or benign lesions, as opposed to the cancer associated types, has led to the concept of low and high oncogenic risk HPVs.

Studies on the presence of HPV in oesophageal cancer have generated conflicting results, with the prevalence rates ranging from 0% to 71%. These differences probably result from variations in the specificity and sensitivity of the analytical techniques used. Compared with other techniques, the polymerase chain reaction (PCR) is a simple, rapid, and sensitive method for the detection of HPV DNA in tissue samples. Furthermore, the use of consensus primers is an advantage in PCR based studies because these primers can detect a wide spectrum of HPV types.

In our study, we have determined the incidence and type of HPV in tumours from patients with oesophageal cancer in the Transkei, a high risk area for this cancer, using nested PCR with degenerate HPV L1 consensus primers that can detect a wide variety of genotypes. The major HPV detected in these patients was HPV-11.

MATERIALS AND METHODS

Tissue collection

The Umtata General Hospital is a 1000 bed facility and the major tertiary care centre for the Transkei, to which all cases of oesophageal cancer are referred. Fifty paraffin wax embedded blocks of histologically confirmed squamous cell carcinoma of the oesophageal region, upper respiratory tract, and digestive tract.
was prepared for PCR essentially as described previously. DNA was isolated from formalin fixed, paraffin wax embedded tissue sections of the DNA was checked by agarose gel electrophoresis. DNA degradation during all steps of the procedure. Care was taken to avoid contamination procedures. Pellets were placed into 1.5 ml microcentrifuge tubes and dewaxed with sequential washes of xylene and 95% ethanol. The oesophagae were randomly chosen from archival material in the department of anatomical pathology of the Umtata General Hospital during the period 1995 to 1998. The demographic data of each of the patients were retrieved from their respective records. The biopsies were taken from black patients presenting with dysphagia and histopathological examination of samples during all steps of the procedure. Care was taken to avoid contamination. The quality of DNA extraction and PCR carry over were rigorously observed. A new microtome blade was used for each biopsy specimen, with DNA extraction and PCR analysis being carried out in small batches.

PCR amplification
The degenerate MY09/MY11 primer set was synthesised on a Beckman Oligo 1000 DNA synthesiser (Beckman, California, USA). The primer set (table 1) was capable of amplifying a wide range of HPV types to produce a PCR product of 450 bp. The amplification mixture consisted of 1x PCR buffer, (10mM Tris/HCl, pH 8.3, 50mM KCl, and 1.5mM MgCl₂), 200µM of each dNTP, 100 pmol of each primer, 2.5 units of Taq DNA polymerase (Roche Biochemicals) and 500 ng of DNA in a final volume of 50 µl. Forty amplification cycles were completed in an Eppendorf Mastercycler as follows: one minute at 94°C, one minute at 55°C, and 1.5 minutes at 72°C. The initial denaturation step was for three minutes at 94°C and the final extension step was prolonged to five minutes at 72°C. Each batch of samples included negative controls containing water and positive control DNA from an HPV positive cervical carcinoma. PCR products were analysed on a 2% agarose gel and visualised by ethidium bromide staining.

The GP5+/GP6+ primer set is a non-degenerate primer set (table 1) that detects a wide range of HPV types using a lower annealing temperature during PCR and produces a PCR product of approximately 150 bp. The amplification mixture consisted of 1x PCR buffer, 200µM of each dNTP 50 pmol of each primer, 2.5 units of Taq polymerase (Roche, Mannheim, Germany), and 500 ng of DNA in a final volume of 50 µl. Forty amplification cycles were completed in an Eppendorf Mastercycler according to the following protocol: initial denaturation was for five minutes at 94°C, followed by denaturation for one minute at 94°C, annealing for two minutes at 40°C, extension for 1.5 minutes at 72°C, and a final extension step of five minutes at 72°C. The WD66, WD67, WD72, WD76, and WD154 primers are consensus primers that target the E6 and E7 transforming genes.

Sequencing of PCR products
To identify the HPV types, all the positive PCR products were subjected to direct DNA sequence analysis using the T7 Sequenase version 2.0 DNA PCR product sequencing kit (Amersham, Little Chalfont, Buckinghamshire, UK). The nucleotide sequences were subsequently subjected to Basic Local Alignment Search (BLAST), which is a set of similarity search programmes designed to explore all of the available sequence databases (ncbi.nlm.nih.gov).

RESULTS
PCR using the MY09/MY11 primer pair resulted in very weak amplification of the appropriately sized DNA fragment, whereas subsequent PCR with the internal primer pair GP5+/GP6+ yielded intense bands of the correct size (150 bp). Of the 50 specimens tested, 23 were positive for HPV, as seen by

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Primers used in PCR amplification of human papillomavirus DNA showing the corresponding annealing temperatures and PCR product sizes</th>
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<tbody>
<tr>
<td>Primers</td>
<td>Annealing temperature</td>
</tr>
<tr>
<td>GP6+: 3′-CTTACACTGTGATACATGTC-5′</td>
<td>40°C</td>
</tr>
<tr>
<td>GP5+: 3′-CTTACACTGTGATACATGTC-5′</td>
<td>40°C</td>
</tr>
<tr>
<td>MY09: 5′-CGGCGGAGTGAGATTGAT-3′</td>
<td>55°C</td>
</tr>
<tr>
<td>MY11: 5′-GTGCTGTGATGATGATGAT-3′</td>
<td>55°C</td>
</tr>
</tbody>
</table>

Figure 1: Nested PCR amplification. DNA was isolated from biopsies obtained from patients with oesophageal cancer and subjected to nested PCR using the conditions shown in table 1 and in the materials and methods section. Lanes 1 and 2 are the negative controls and lane 3 is a positive control derived from a patient with cervical carcinoma known to be human papillomavirus (HPV) positive. The samples in lanes 4, 6, 7, and 10 were positive for HPV, whereas the samples in lanes 5, 8, 9, 11, and 12 were negative for HPV.
the presence of the 150 bp PCR fragment (fig 1). All samples that were positive with the L1 primers also yielded a band of the correct size with the E6 and E7 primers (data not shown).

The PCR products were subjected to DNA sequence analysis and aligned with the known HPV sequences in the DNA database using BLAST (National Institutes of Health). The biopsies were shown to contain DNA to HPV types 11, 16, 39, and 52. The low risk HPV-11 was the most common subtype (48%) whereas the intermediate and high risk HPV-52 and HPV-16 occurred at a much lower frequency (13%) (table 2). The correlations between the histological data and the HPV subtype are shown in table 3.

**DISCUSSION**

Several studies during the past two decades have shown the presence of HPV in DNA isolated from patients with oesophageal cancer. 3, 10-14 Studies have generated conflicting and often contradictory data, which may be attributed to the geographical location with respect to either low or high incidence areas. 15 In addition, variations in the prevalence rates of HPV from the same geographical areas have also been reported. For example, the presence of HPV DNA has been confirmed in 23.4% of patients with oesophageal cancer in China, 16 whereas another study carried out in the same area 17 found no evidence of HPV DNA. Similarly, prevalence rates of 30% and 23% have been reported in patients with oesophageal cancer from Australia; in both instances the same technique was used. 18, 19

In South Africa, squamous cell carcinoma of the oesophagus is the most common cancer among black men. In 1989, Sagar reported the incidence of oesophageal cancer to be nearly 400/100 000 in the age group 35–65 years. 20 Various studies have reported the existence of high and low incidence areas of oesophageal cancer in Transkei. Rose, 21 using the Bantu Cancer Registry, reported that the Lusikisiki and Bizana districts in northeastern Transkei had lower incidence rates (8/100 000 and 3/100 000, respectively) than the southwestern districts, of Butterworth and Cenatane, which had incidences of 103/100 000 and 54/100 000, respectively. 22

The earlier studies that associated HPV with 30% of cases of oesophageal cancer in South Africa used immuno-histochemistry. 23 Subsequent studies have reported prevalence rates ranging between 17% and 71%, 24-27 supporting the notion that HPV infection may be an integral part of a multistep process leading to oesophageal cancer in high risk areas. However, none of these studies targeted the Transkei region of South Africa, a region with a very high incidence of oesophageal cancer. In our current study, the prevalence of HPV infection was found to be very high in that 46% of patients were HPV positive, supporting the earlier findings that HPV may play an important role in the development of oesophageal cancer in South Africa. 28

Several PCR based studies have reported on the presence of HPV in oesophageal cancer, although the detection rates differ between these studies. 29-33 A variety of factors, mainly technical, may account for the observed differences in these studies.

Consensus primers have an advantage in PCR studies because they can detect a wide spectrum of HPV types, including novel types. The HPV E1 and L1 genes are suitable targets for consensus primers as long as these genes are not lost or disrupted after viral integration. In contrast, the E6 and E7 genes are thought to be retained in all carcinomas but are too variable to be targeted with consensus primers. 34, 35

Studies using paraffin wax embedded tissue sections resulted in successful amplification of several genes, ruling out the possibility that tissue fixation or the age of the blocks might affect the results of the PCR. Nested PCR with the MY09/MY11 and GP5+/GP6+ primers was sufficiently sensitive for the detection of HPV in paraffin wax embedded biopsies in our study.

**Table 3** Correlation between the presence of HPV subtype and the histopathological data in SCC of the oesophagus

<table>
<thead>
<tr>
<th>No. of patients</th>
<th>HPV type</th>
<th>Histopathology</th>
</tr>
</thead>
<tbody>
<tr>
<td>8</td>
<td>11</td>
<td>SCC, keratinising type</td>
</tr>
<tr>
<td>11</td>
<td>11</td>
<td>SCC, papillary keratinising type</td>
</tr>
<tr>
<td>2</td>
<td>16</td>
<td>SCC, non-keratinising type</td>
</tr>
<tr>
<td>2</td>
<td>39</td>
<td>SCC, keratinising</td>
</tr>
<tr>
<td>1</td>
<td>39</td>
<td>SCC, keratinising</td>
</tr>
<tr>
<td>2</td>
<td>Unknown</td>
<td>SCC, keratinising</td>
</tr>
</tbody>
</table>

HPV, human papillomavirus; SCC, squamous cell carcinoma.

**Table 2** Frequency of HPV types in oesophageal cancer biopsies

<table>
<thead>
<tr>
<th>HPV type</th>
<th>No. of patients</th>
</tr>
</thead>
<tbody>
<tr>
<td>11</td>
<td>11</td>
</tr>
<tr>
<td>39</td>
<td>7</td>
</tr>
<tr>
<td>16</td>
<td>2</td>
</tr>
<tr>
<td>52</td>
<td>1</td>
</tr>
<tr>
<td>Unknown</td>
<td>2</td>
</tr>
<tr>
<td>Total</td>
<td>23</td>
</tr>
</tbody>
</table>

DNA was isolated from oesophageal cancer biopsies and subjected to PCR amplification using the primers indicated in table 1 and under the conditions described in the material and methods section.

"We also detected HPV type 39, which has never before been shown to be present in oesophageal cancer"

The earlier studies discussed above have shown that HPV types 16 and 18 were more commonly detected in oesophageal cancer. In our study, we found HPV type 16 DNA to be present in only two of the 50 samples, whereas HPV type 18 DNA was not detected. HPV type 52 DNA was present in only one sample whereas HPV type 11 was the most predominant. Chang and colleagues 23 also detected HPV-6 and HPV-11 in oesophageal cancer biopsies by in situ hybridisation using a mixed HP6/11 probe. HPV-6 and HPV-11 are generally found in benign genital condylomata. These two subtypes also account for almost all cases of juvenile onset and adult onset respiratory papillomatosis. 36, 37 Although found almost exclusively in benign lesions, these HPV subtypes have occasionally been detected in malignant lesions. One study has shown that women with a history of condylomata acuminata had a 3.8-fold increased incidence of carcinoma in situ compared with the total female population residing in the same city. 38 HPV type 11 has been implicated in the malignant progression of laryngopharyngeal lesions, 39 cancer of the anus, 40 and penile carcinoma. 41 We also detected HPV type 39, which has never been shown to be present in oesophageal cancer. According to sequence comparison data, HPV-39 most closely resembles HPV-18 and is phylogenetically classified together with HPV-45 in the mucogenital high risk group. 42, 43 These HPV types have been detected in a disproportionately high percentage of rapidly progressive invasive cervical carcinomas. HPV-39 has been detected in erythroplasia of Queyrat, 44 a carcinoma in situ that mainly occurs on the glans, the prepuce, or the urethral meatus of elderly men.

In conclusion, a potential role of HPV in the development of oesophageal cancer has emerged as a result of the HPV-like histological changes in the mucosa of patients with oesophageal cancer and the presence of HPV antigens in tumours. Although various types of HPV have been detected by PCR amplification, clear evidence for a role in oesophageal cancer is still lacking.
The high prevalence of HPV DNA in oesophageal cancer in patients from the Transkei region of South Africa provides further evidence for a role of HPV in this cancer.

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