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Original Paper

No Evidence of Known Types of Human Papillomavirus in Squamous Cell Cancer of the Oesophagus in a Low-Risk Area

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Controversial results regarding the presence and role of human papillomavirus in the development of oesophageal squamous cell carcinoma have been published. We used multiple broad-spectrum polymerase chain reactions to identify HPV DNA in oesophageal carcinomas from a low-incidence area. Paraffin embedded- and snap-frozen specimens from oesophageal cancer tissues of 63 patients were examined with a PCR technique with several primer pairs, capable of detecting most known HPV types. In none of the oesophagus cancer tissues could HPV DNA be detected. The role of HPV in this type of carcinoma in a low incidence area remains unclear. © 1997 Elsevier Science Ltd.

INTRODUCTION

SQUAMOUS CELL carcinoma of the oesophagus is a highly lethal disease, with striking variation in incidence in different parts of the world. From epidemiological surveys, it has been suggested that excessive alcohol intake and use of tobacco (especially in combination), and possibly certain nutritional deficiencies (vitamins A, B and C) are some of the risk factors, but these factors alone cannot explain the very high incidence in some well-defined geographical areas in North China, Iran and South Africa.

Human papillomaviruses (HPV) have been found to play a causative role in the pathogenesis of cervical dysplasia and cervical carcinomas. In 1978, a possible interaction between a bovine papillomavirus (BP4) and an environmental carcinogen (bracken fern) was regarded as an important event for the development of squamous cell carcinomas, especially of the oesophagus, in cattle grazing on the Scottish Highlands [1]. Syrjänen suggested in 1982 for the first time a possible aetiological relationship between HPV and benign proliferations of the squamous mucous membrane of the oesophagus, for instance, papillomas [2]. In the animal model of bovine papillomavirus infection, these lesions have been reported to undergo malignant transformation following exposure to carcinogens. Winkler and associates in 1985 reported the clinical, histological and morphological features of HPV infection in cases of benign oesophageal proliferations, while at the same time a role of HPV infection in carcinoma of the oesophagus in black South Africans was suggested [3,4]. In both studies, HPV antigens could be detected by means of immunoperoxidase techniques in 30% of those cases in which the histological criteria of HPV infection were met. Since then, a number of controversial studies have been published about the detection of HPV DNA in human oesophageal cancer with different techniques. Positive results were obtained mainly in high-incidence areas. In The Netherlands, squamous cell cancer of the oesophagus is a rare disease. We investigated the presence of HPV DNA in oesophageal cancer from a low-incidence area using multiple, very sensitive, broad-spectrum polymerase chain reaction (PCR) techniques.

MATERIALS AND METHODS

We investigated formalin-fixed, paraffin-embedded tumour specimens of 63 consecutive patients with operable invasive squamous or undifferentiated large cell carcinoma of the oesophagus, who participated in a phase III randomised clinical trial of surgery with ($n = 21$) or without ($n = 42$)

neoadjuvant chemotherapy. This study was carried out in the largest referral centre for oesophageal cancer patients in The Netherlands (age-adjusted death rate of oesophageal cancer: 7.9/100 000 for males and 3.2/100 000 for females). In 20 out of 42 patients treated with surgery alone, we also investigated snap-frozen specimens, collected and frozen in liquid nitrogen within 1 h after surgical removal. All available haematoxylin and eosin stained sections were reviewed, and the most representative block was selected for further studies. Patient characteristics are listed in Table 1. No patient had a history of or a presence of active papillomas of any site in the head and neck or oesophagus region.

To extract DNA from paraffin-embedded tissue, 5 μm sections were cut, taking care to prevent cross-contamination, and incubated in 300 μl 10 mM Tris-HCl (pH 8.9), 50 mM KCl, 2.5 mM MgCl$_2$ and 0.5% Tween-80 with 200 μg/ml proteinase K at 56°C for 18 h. At the end of the incubation, the aqueous phase was separated from the paraffin slurry by centrifugation, transferred to a fresh tube and the proteinase K was subsequently inactivated by boiling. Frozen tissue specimens were sliced using a cryomicrotome and two 5 μm sections were used for the preparation of DNA. To extract DNA, the slices were incubated for 4 h at 56°C with 200 μg/ml proteinase K in 200 μl 50 mM Tris-HCl (pH 8.9) and 1 mM EDTA. After completion of the digestion, the proteinase K was heat inactivated.

For analysis of the presence of HPV DNA, 5 μl of each sample was subjected to 40 cycles of PCR amplification in 50 μl reactions using each of the following primer sets: (1) MY09 (5'CGT CC(A/C) A(A/G)A GGG A(A/T)A CTG ATC) and MY11 (5'GC(A/C) CAG GG(A/T) CAT AA(C/T) AAT GG) [5]; (2) GP5 (5'TTT GTT ACT GTG CTA GAT AC) and GP6 (5'TGA TTT ACA GTT TAT TTT TC) [6]; (3) CPI (5'TTA TCA (T/A)AT GCC CA(T/C) TGT ACC AT) and CPIIG (5'ATG TTA AT(A/T) (G/C)AG CC(A/T) CCA AAA TT) [7,8]; (4) CPI and CPIIS (5'ATA TTG TCT GAG CCT CC(A/T) AA(A/G) TT) [9] and (5) Pulm (5'TGT CAA AAA CCG TTG TGT CC) and Pu2r (5'GAG CTG TCG GCT TAA TTG CTC) [10]. Amplification was also done using a nested PCR approach using the MY09/11 primer set in the first and the GP5/6 primer set in the second PCR. Each of these primer pairs was designed for the amplification of spectra of genital HPV types

Table 1. Patient and tumour characteristics (n = 63)

Median age	63.5 years (35–77 years)
Gender	
male	48
female	15
Race	
Caucasian	60
Black	2
Chinese	1
Grade of differentiation	
well	7
moderately	38
poor	17
undifferentiated	1
Stage (UICC)	
I	6 (10%)
II	26 (41%)
III	10 (16%)
IV	21 (33%)

with the exception of the CPI/IIS primers set, which was developed for the detection of HPV types present in the skin. The MY09/11, GP5/6, Pulm/2r and CPI/IIG PCRs were performed under the conditions described in the original publications including the appropriate positive (SiHa DNA) and negative controls [5–7,9,10]. In addition, we used a nested PCR method directed by two novel primer pairs specifically designed for the sensitive amplification of HPV types present in the skin lesions of epidermodysplasia veruciformis patients [11]. The nucleotide sequence of the two primers used in the first PCR are 5'CA(A/G) GGT CA(C/T) AA(C/T) AAT GG(C/T) AT (CP65) and 5'AA(C/T) TTT CGT CC(C/T) A(A/G)A G(A/G)A (A/T)AT (CP70), and those of the two primers used in the nested PCR are 5'AAT CA(A/G) (A/C)TG TTT (A/G)TT AC(A/T) GT (CP66) and 5'G(A/T)T AGA TC(A/T) ACA T(C/T)C CA(A/G) AA (CP69).

In a separate reaction, a β-globin PCR was carried out using two pairs of primers; either PC03 (glo-1) and RS42 amplifying a 441 bp fragment or glo-1 (5'ACACAACTGT-GTTCACTACC) and glo-3 (5'TCTATTGGTCTCC-TTAAACC) amplifying a 172 bp fragment. Successful amplification of the β-globin fragment, visualised on an ethidium-stained agarose gel, indicated that the sample was adequate for PCR analysis.

RESULTS

None of the different PCR reactions resulted in the detection of HPV DNA in the oesophageal carcinoma specimens when the PCR products were analysed by ethidium bromide stained agarose gels (Figure 1). Also no specific signals were detected after blotting and hybridisation of the PCR products of the MY09/11 PCR, the GP5/6 PCR and the CPI/IIG PCR with mixtures of PCR labelled probes. These primer pairs combined are capable of detecting most known HPV types. 78 and 79 of a total of 83 samples were positive when analysed by PCR with the two β-globin primer pairs producing a 441 and 171 bp fragment, respectively (Figure 1(d)). This indicates that almost all DNA preparations were adequate for PCR analysis.

DISCUSSION

We investigated a possible role of HPV in the pathogenesis of squamous cell carcinoma of the oesophagus in patients from a low-incidence area. In this material, of which 95% of the specimens were proven to be suitable for PCR analysis, we could not detect any HPV DNA. These results strongly suggest that HPV DNA of the known HPV types is not present in at least the majority of the oesophageal squamous cell carcinomas in The Netherlands. We cannot exclude the possibility that novel HPV types, which do not match the PCR primers used, are present. Recently, we succeeded in detecting novel HPV types in skin lesions of immunocompromised patients by PCR amplification, employing a nested PCR approach using the primer sets CP65/66 and CP69/70 [11].

Data from the literature on HPV, relating to benign and malignant oesophageal lesions, are conflicting [12]. In a study from Australia, with DNA hybridisation techniques, using a mixed probe of HPV types 11, 13, 16 and 18, an overt positive reaction in 2, and a weak positive reaction in 3 out of 10 cases of oesophageal squamous cell carcinomas could be demonstrated, whereas no detectable viral antigen in these cases was found, suggesting that detection of the genome is more sensitive than immunostaining [13]. In 1990 the same
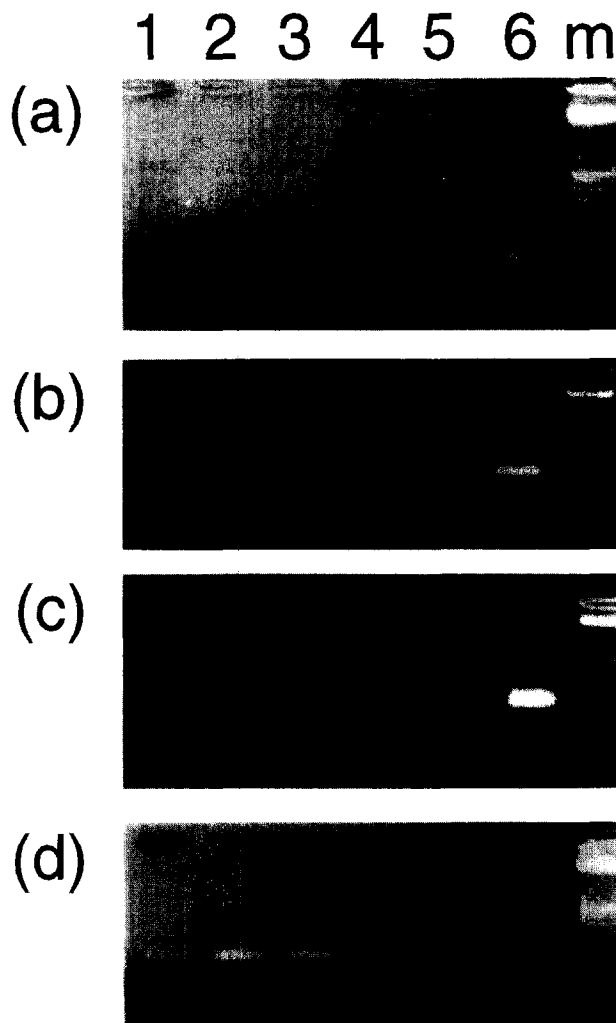
Figure 1. Absence of HPV DNA from oesophageal carcinomas. Ten µl of the PCR products of 5 tumours (lanes 1–5) and SiHa cells containing 1–2 copies of HPV-16 DNA per cell (lane 6) were run on a ethidium bromide stained agarose gel. Lane m: 100 bp ladder marker. The band of the positive control (lane 6) indicates the position of expected HPV amplimers. Amplimers of the PCR directed by (a) GP5/6 primers, (b) CPI/IIG primers, (c) Pulm/2r primers, (d) Glo-1/Glo-3 primers.

author published his results on the positive detection of HPV type 6, 11, 16 and 18 DNA by filter *in situ* hybridisation (FISH) techniques in 9 out of 39 oesophageal cancers from a low-incidence area [14]. Chang and associates reported in 1990 positive results with the same method in oesophageal cytologic specimens from a high-risk area, ranging from atypia to invasive squamous cell carcinoma, including lesions adjacent to the tumour area [15]. In 75% of positive cases, HPV type 16 and/or 18 DNA sequences were found. These findings were confirmed in several other reports, especially from high-risk areas, such as South Africa, Japan, China and France [16–18]. The largest series to date was published in 1993 by Chang and associates [19]. In this study, biopsy specimens of tumour, adjacent epithelia, regional lymph nodes and surgically resected margins were collected from 363 patients surgically treated with oesophagectomy for invasive squamous cell carcinoma in the high-risk area of Linxian, China. In 20% of cases, positive signals were found

in the nuclei of cancer cells by *in situ* hybridisation, in 4% only in the adjacent epithelium with hyperplastic or dysplastic changes, and in only 1 case in the resected margin. HPV type 16 was the most common finding in the 85 positive cases [20]. The presence of positive hybridisation signals in regional lymph node metastases (12.3%), exclusively confined to the nuclei of metastatic cancer cells, suggests a causal association of HPV and oesophageal carcinoma in these patients, more so because the same viral type was invariably detected in both the primary tumour and the metastatic lesions. Cooper and associates demonstrated the presence of HPV DNA in 25 of 48 oesophageal cancers from a high-risk area (South Africa), utilising non-isotopic *in situ* hybridisation with HPV DNA probes to HPV 6, 11, 16 18, 31 and 33 [21]. Very recently, Dillner and associates found an association between seropositivity to HPV type 16 and the risk of oesophageal cancer in Finland [22].

In contrast to these data, negative results have also been reported, especially in low-risk areas, but also in high-risk areas [23–26]. Loke and associates applied both *in situ* hybridisation and DNA slot blot analysis to a series of 37 cases where total oesophagectomy was performed for squamous cell carcinoma in the high-incidence area of Hong Kong. With both techniques no HPV was detectable in cancer cells nor in intra-epithelial neoplasia nor in normal oesophageal mucosa. Kiyabu and associates used *in vitro* gene amplification by the polymerase chain reaction to look for HPV type 16 and 18 DNA in invasive squamous cell cancers of various types [23]. While 70% of the ano-genital carcinomas, and 36% of the oropharyngeal carcinomas contained HPV DNA sequences, none of 13 oesophageal carcinomas were found to be positive. Our own results from a low-incidence area are consistent with these studies.

There are several complicating issues when comparing studies about HPV detection in benign and malignant tissues. In high-risk areas, a possible influence of screening methods regarding the stage of the tumour at the time of diagnosis could exist. It is important to realise that several authors found HPV DNA in the epithelium, adjacent to the carcinoma, more than in the cancer itself [16]. This phenomenon is consistent with the earlier findings that bovine papillomavirus type 4 DNA in high copy number could be readily identified in bovine papillomas, but no viral DNA nor viral antigens could be detected in malignant lesions, indicating that viral genomes are not necessary to maintain a malignant state.

A second issue is the methodology. Using amplification by the polymerase chain reaction, it is possible to detect HPV genomes with high sensitivity, for instance up to less than 10^{-1} copies of viral genome per cell, which is superior compared to the older hybridisation techniques and immunostaining techniques [14, 15]. We used in our study several pairs of general primer sequences, which are conserved among a broad spectrum of HPV types, and which permit the detection of more HPV types in a single sample. Also potentially new HPV types can be identified by these methods.

In conclusion, in our study with oesophageal squamous cell cancer specimens from a low-incidence area, we could not confirm the results of some other investigators, identifying HPV DNA in oesophageal carcinomas, mainly type 16 and 18. In this respect, our results are identical to those described by Loke, Kiyabu, Sugimachi and Akutsu [22, 24–26].

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