Low Detection Rate of HPV in Oral and Laryngeal Carcinomas

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The presence of human papillomaviruses (HPVs) in 38 oral and 16 laryngeal lesions (verrucous hyperplasia, carcinoma in situ and carcinomas) was investigated using the polymerase chain reaction (PCR) technique. All biopsies were fresh frozen and a set of consensus and type-specific primers was used for PCR detection and HPV typing. In oral biopsies a low proportion of HPV-positive cases was found, despite the sensitive techniques. Only one case out of 38, a carcinoma in situ was positive (2.6%). It is thought that this finding reflects a minimal presence of HPV in the oral lesions, but a transient role of virus in the induction of carcinomas cannot be ruled out. Differences in relation to other studies may be geographical and/or methodological. In laryngeal carcinomas (and dysplasias), 3 out of 16 cases were HPV positive. This frequency (19%) concurs with most other studies.

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Human papillomaviruses (HPVs) are a family of icosahedral, non-enveloped viruses with a circular, double-stranded DNA genome of 7 500–8 000 base pairs (bp), and with a special affinity for epithelial cells. So far, more than 70 different HPV types have been defined by DNA sequence analysis. Many of these are found in skin cells, where they can cause warts. HPVs are also the cause of genital warts, and certain types, especially HPV 16 and HPV 18, are involved in the development of most cervical carcinomas (1). This finding has raised the question of whether HPV infection might also be associated with carcinomas in other locations, not least in the head and neck region. Carcinomas in these sites are often papillary, and resemble virus-induced lesions.

Several studies have been carried out to screen for HPV in oral carcinomas. Studies relying on techniques such as in situ hybridization, dot blot or Southern blot, usually show an occurrence of HPV, ranging from 0 to 20% (2–5). More recently, the more sensitive polymerase chain reaction (PCR) has become the primary screening method for HPV. Several sets of consensus primers detecting a wide range of HPV types have been developed, as well as type-specific primers. In studies using PCR to screen oral carcinomas, the occurrence of HPV has generally increased markedly compared with other methods, and most studies record rates ranging from 30 to 70% (6–9). However, detection rates of 0% and 100% have been reported (10–12).

Laryngeal carcinomas have also been screened for HPV occurrence using PCR. In most cases, HPVs have been detected in 10 to 20% of squamous cell carcinomas (SCC) and in 85 to 100% of verrucous carcinomas (10, 12–15).

The aim of the present study was to use PCR to screen a relatively large number of fresh, frozen specimens from oral lesions, most of them carcinomas, for the presence of HPV. We hope thereby to obtain a more reliable evaluation of HPV prevalence. A number of laryngeal lesions were also included in the study, for comparison.

MATERIAL AND METHODS

Patient material

The material included 33 oral squamous cell carcinomas from 23 men and 10 women, oral leukoplakias from 3 men and 2 women, as well as 16 laryngeal carcinomas or dysplasias from 12 men and 4 women (see Table 1). The mean age of the patients was 64.7 years (range 35–84); for oral patients 65.9 years and for laryngeal patients 62.1 years. The distribution of oral carcinomas was: tongue 11, floor of mouth 7 , gingiva 10, bucca 2, lymph node metastasis 3. The laryngeal carcinomas were all glottic. For the distribution and classification of material, see Table 1. The material was unselected; most carcinomas

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Table 1

HPV positivity by PCR in oral and laryngeal lesions

		HLA[a]	HPV positives			HPV type					
Lesion type	n	pos	Total	Cp	Gp+	6	16	18	31	33	Other
Oral primary SCC[b]	30	30	0	0	0	0	0	0	0	0	0
Lymph node metast.	3	3	0	0	0	0	0	0	0	0	0
Oral CIS[c]	2	2	1	1	1	0	0	0	0	0	1
Oral verr. hyp.	1	1	0	0	0	0	0	0	0	0	0
Other oral	2	2	0	0	0	0	0	0	0	0	0
Total, oral	38	38	1	1	1	0	0	0	0	0	1
Laryngeal prim. ca.	9	9	2	2	1	0	0	0	–	0	2
Laryngeal CIS[c]	4	4	1	1	1	0	1	0	–	0	0
Laryngeal verr. hyp.	1	1	0	0	0	0	0	0	–	0	0
Other laryngeal	2	2	0	0	0	0	0	0	–	0	0
Total, laryngeal	16	16	3	3	2	0	1	0	–	0	2

[a] Human leukocytic antigen primers.
[b] Squamous cell carcinoma.
[c] Carcinoma in situ.

were T1 or T2. No detailed smoking- or alcohol anamnesis was available. All samples were collected at the Department of Otolaryngology, National University Hospital, Oslo, Norway.

Extraction of DNA

All specimens were snap frozen immediately upon removal and stored in liquid nitrogen until examined in this study. A small piece of each tissue specimen was excised and minced, using a sterile scalpel, and transferred to a sterile microfuge tube. Each tissue specimen was suspended in 450 μl TNE buffer (50 mM Tris–Cl (pH 9), 150 mM NaCl and 5 mM EDTA), 50 μl 10% SDS and 12.5 μl proteinase K (10 mg/ml) was added, and this was incubated at 58°C overnight; 500 μl of 70% phenol/chloroform/water solution (Applied Biosystems) was added to each tube and mixed gently in a rotamixer for at least 1 h. The phases were separated by spinning at top speed in a microcentrifuge for 5 min, and the aqueous (top) phase transferred to another set of sterile microfuge tubes. This was followed by the addition of 500 μl chloroform to each tube, and the mixing, spinning and transferring steps were repeated. To each aqueous solution 1 ml absolute ethanol was added, and the tubes were placed at -20°C for at least 30 min. The precipitated DNA was pelleted by spinning at 14 000 rpm for 20 min. The pellets were washed once in 70% ethanol, placed at -20°C for 30 min, spun at 14 000 rpm for 20 min, dried at room temperature overnight and resuspended in sterile ddH_2O to a DNA concentration of about 0.2 $\mu g/\mu l$.

Detection of HPV DNA

PCR was performed with two sets of consensus primers, the E1-specific CPI/CPIIG (16) and the L1-specific Gp5 $\pm$ Gp6 + (17), as well as with type-specific primers for HPV 6, HPV 16, HPV 18, HPV 31 and HPV 33. A PCR master mix was made up of (per PCR reaction tube) 5 μl 10X PCR Buffer II (Perkin-Elmer), 8 μl dNTP mix (1.25 mM of each nucleotide), 7.2 or 4.0 μl (for the CP primers or the other primer pairs respectively) 25 mM $MgCl_2$ (Perkin-Elmer), 2.2 or 2.0 μl respectively, of each primer solution (10 pg/μl), 0.1 or 0.2 μl, respectively, of AmpliTaq DNA polymerase (Perkin-Elmer), and sterile ddH_2O to a total volume of 45 μl per tube. The master mix was, when possible, made up in a separate, DNA-free room, and always in a specially designated area, using only DNA sterile pipettes, to minimize the probability of contamination with HPV-positive DNA.

In another area, 5 μl DNA test solution was added to the PCR tubes, and the PCR was performed in a Perkin-Elmer GeneAmp PCR system 9600 or a Perkin-Elmer DNA Thermal Cycler 480, with 40 cycles as follows:

- CPI/CPIIG: 95°C (1 min), 55°C (1 min), 72°C (2 min)
- Gp5 + /Gp6 + : 95°C (30 s), 45°C (30 s), 72°C (1 min)
- Type-specific: 95°C (30 s), 55°C (30 s), 72°C (1 min)

In addition, the temperature was set to 95°C for 5 min before the first cycle, and to 72°C for 5 min after the last cycle. When a Perkin-Elmer DNA Thermal Cycler 480 was used, the reaction mixture was overlayed with a few drops of mineral oil, to prevent vaporization.

Three types of negative controls were included: to approximately every tenth tube of reaction mixture, only sterile ddH_2O was added, to detect any contamination arising in the process. One tube of reaction mixture was used as reagent control, to which nothing was added, and DNA from HPV-free tissue was added to one tube to monitor primer specificity towards HPV DNA. Solutions of the appropriate cloned HPV genomes were used as positive controls. An additional PCR using the human

leukocytic antigen (HLA)-directed primer pair Gh-26/Gh-27 was performed, in order to eliminate any non-amplifiable samples.

After the PCR, the results were monitored by performing vertical polyacrylamide gel electrophoresis (PAGE). The gels (7.5% acrylamide/bis [29:1], 1X TBE [5X TBE: 44.5 mM Tris base, 44.5 mM Boric acid, 2% (v/v) 0.5 M EDTA]) were cast according to Sambrook et al. (18) and run at 110 V for 70 min in 1X TBE. They were then stained for 30 min in SYBRgreen I gel stain (FMC Bio-Products), and any bands were visualized under UV light (300 nm).

RESULTS AND DISCUSSION

The results of the screening are presented in Table 1. We did not detect HPVs in any of the 33 oral squamous cell carcinomas (SCCs) studied, but HPVs were detected in 1 out of 2 oral carcinomas in situ (CIS) (50%). In the laryngeal material, we detected HPVs in 2 out of 9 SCCs (22%) and in 1 out of 4 CIS (25%).

Earlier studies on oral SCCs have shown an HPV positivity between 0 and 100% on examination using PCR (6, 7, 12, 19). Methodological and technical differences in detection and typing may play a role in explaining these discrepant results. First of all, there are more than 70 different HPV types, and no set of primers exists that can detect them all. Most HPV consensus primers are primarily designed to detect the HPV types common in cervical samples. Other HPV types may play a role in oral carcinomas, but we used specific primers for those HPV types that have most commonly been detected in oral lesions. Inclusion of sufficient negative controls, preferably both with and without DNA, is important in order to detect any possibility of false positive results, arising from contamination or lack of primer specificity. The PCR procedure is notoriously vulnerable for contamination. Likewise, positive controls should be added to eliminate the possibility of false negatives. Finally, to eliminate any non-amplifiable samples, an additional PCR should be carried out, directed against a DNA sequence present in all cells, e.g. in the gene coding for the human leukocytic antigen (HLA).

Another uncertainty is due to possible integration of viral DNA into the cell genome. This is shown to be relatively common in malignant tumours (20, 21). When the viral genome integrates, deletions occur in the L1 ORF, to which most consensus primers are directed. If all HPV DNA is integrated, this deletion leads to false negative PCR results. We have used consensus primers from both the L1 and E1 regions (16, 17). Since a part of the E1 ORF is also commonly disrupted at integration, neither of our consensus primer pairs will detect completely any integrated cases. It is worth noting, however, that in one of the laryngeal carcinomas HPV was detected with the E1-specific primers, and not with the L1-specific primers. Integration is likely to occur in similar frequency in different series of carcinoma, however, and this is therefore not a likely explanation for discrepant results.

Formaline fixation introduces artefacts that can lead to negative PCR results (22). This is particularly true if the amplificate is large (>200 bp). The most commonly used consensus primers, MY09/MY11, give a product of 450 base pairs, whereas the Gp5+/Gp6+ product is only 150 bp, and the CPI/CPIIG product is 188 bp. In this study, only frozen tissue samples and consensus primers giving relatively short products were used, so the problems associated with formaline fixation should not be applicable. All controls described above were included in this study. It is still possible that the choice of primers and deletions may have caused false negatives, but it is unlikely that this explains the total lack of HPV positivity in oral carcinomas. Hence, the very low degree of HPV positivity detected probably reflects a realistic figure for this population. Admittedly, however, HPV may occur focally in lesions (23), and in future studies the inclusion of multiple tissue samples may be warranted.

In a recent study (24), HPV was detected in 13% of oral, and 3.6% of laryngeal carcinomas, using snap-frozen tissue and a protocol similar to the one used by us. Although a number of positive oral carcinomas were found, the positivity rate is lower than that for most other studies. This supports the notion that false positive PCR results must be excluded by carefully controlled procedures and use of adequate controls.

Geographical and ethnical differences may explain discrepant results (12). Results from Finland have, for instance, shown HPV positivity in up to 30% of oral carcinomas (4). It would indeed merit further study to establish the cause of such differences in prevalence rates in defined clinical materials with all adequate controls included.

The results regarding laryngeal carcinomas were in accordance with previous studies, where HPVs are generally detected in 4 to 20% of the cases (10, 14, 24–26). The exception is verrucous carcinomas, where HPVs in most studies are detected in 85–100% of the cases (13, 15). However, no verrucous carcinomas were included in this study. Type 16 is the most common HPV type found in laryngeal carcinoma, irrespective of histological type.

In summary, in a Norwegian material of oral and laryngeal carcinomas and dysplasias, we found a low detection rate for HPV. We think this reflects the true involvement, but would suggest that more early lesions are studied with the same methodology to determine whether HPV can act as an initiator in carcinogenesis and then disappear (hit-and-run theory, (27)). Our finding of one positive out of two oral CIS, and one positive out of four laryngeal CIS can point in this direction. This suggestion is further strengthened by the finding by others of a slightly higher

HPV positivity in CIS (30–40%) than in SCC (20–30%) (28, 29).

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