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## ORIGINAL ARTICLE

# HPV and other risk factors of oral cavity/oropharyngeal cancer in the Czech Republic

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**OBJECTIVE: An association between high-risk human papillomavirus (HR HPV) infection and a risk of development of a subgroup of head and neck cancers has been proposed recently. The main risk factors of oral and oropharyngal cancer observed in our population are smoking and alcohol consumption. The incidence of oral/oropharyngeal tumours in the Czech Republic is relatively high and there are no data available about the prevalence of HPV DNA presence in these tumours.**

**MATERIALS AND METHODS: Eighty patients with a primary oropharyngeal cancer were enrolled. The presence of HPV DNA has been evaluated by polymerase chain reaction in 68 cases from which the tumour tissue and demographical and clinical data were available. The typing of HPV was performed by nucleotide DNA sequencing.**

**RESULTS: The HPV DNA was detected in 51.5% of samples tested. Among the HPV DNA positive tumours, 80% contained HPV16. In the analysed group there were 54 men and 14 women. The prevalence of HPV DNA was lower in oral (25%) than in oropharyngeal (57%) tumours, and higher in never smokers (100%) and never drinkers (68.8%). HPV DNA presence was not related to gender, age, number of lifetime sexual partners or practice of oral-genital sex, size of tumour or presence of regional metastases.**

**CONCLUSIONS: The difference in the prevalence of HPV DNA positive tumours between cases of oral cavity and oropharyngeal carcinoma exposed and not exposed to tobacco or alcohol support the theory that HPV DNA positive tumours form an aetiologically distinct subgroup of head and neck tumours.**

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## Introduction

The aetiology of head and neck squamous cell cancer is multifactorial with alcohol and tobacco consumption considered to be the main risk factors (Franceschi *et al*, 1990; Castellsague *et al*, 2004). The association between high-risk human papillomavirus (HR HPV) infection and the risk of head and neck cancer development dates to 1985, when HPV16 was detected in oral squamous cell tumours (Loning *et al*, 1985). Since then, HPV DNA has repeatedly been found in a variable proportion of head and neck cancers (Gillison *et al*, 2000; Gillison and Shah, 2001; Mork *et al*, 2001; Ritchie *et al*, 2003).

It has been proposed that HPV DNA positive tumours form an aetiologically distinct group of oral cavity/oropharyngeal tumours. In this group of tumours, distinct clinical and epidemiological characteristics can be found (Smith *et al*, 2004a).

Similar to other Central European countries, incidence rates of cancers of the oropharynx and oral cavity (ICD-10: C01–C06, C9–10) in the Czech Republic are relatively high: $11.2/10^5$ for males and $2.4/10^5$ for females. Unlike the incidence of laryngeal cancer, the incidence of oral cavity/oropharyngeal cancer has increased steadily in recent years. In 1999 mortality figures were $6.4/10^5$ for men and $1.3/10^5$ for women (Cancer Incidence, 2000, 2003). Five-year survival in patients with oropharyngeal and oral cancer (all stages combined) is only about 50%.

Because there are no data available on the prevalence of HPV DNA in the head and neck cancers in the Czech Republic, the aim of the present study was to determine the prevalence and types of HPV DNA in

histology specimens of oropharyngeal and oral cavity cancer cases and to determine, whether HPV DNA positive and negative cases differ in epidemiological and clinical features such as gender, age, tobacco and alcohol, sexual behaviour, location and extent of tumour.

## Materials and methods

*Population studied*

Subjects enrolled in our study were selected from patients treated in the Department of Otolaryngology Head and Neck Surgery, 1st Medical Faculty Charles University, Prague during 2000–2003. All patients with a primary cancer of the oral cavity or oropharynx who agreed to sign the information consent form were enrolled. Data regarding demographics, risk factors for oral cavity and oropharyngeal cancer, and risks related to HPV exposure were accessed by a patient questionnaire. A computerized medical report was completed for each patient.

Eighty patients were enrolled and 11 cases were excluded: two patients died before treatment, in two cases the location of the tumour was revaluated, in one case the histological analysis did not confirm the presence of the tumour, and in six patients there was not enough material for the analyses. Additionally one sample did not amplify with beta-globin primers and was excluded from the final analyses. Altogether HPV-DNA presence was evaluated in tumours of 68 patients.

*Tumour specimens*

Most patients were surgically treated and cancer tissue taken during surgery was sent on dry ice to the surgical pathology laboratory. For a few cases not treated by surgery, biopsy specimens were available for evaluation of HPV.

The pathologist obtained two adjacent samples of tumour from the primary site. One of the paired sections from each anatomical localization was then labelled, snap frozen in liquid nitrogen and stored for eventual future analysis. Each remaining paired section from each anatomic site was fixed in 10% neutral formalin and paraffin embedded. From each paraffin block, the first section was histologically analysed, two subsequent sections were assigned for HPV DNA detection by polymerase chain reaction (PCR), and the next section was again histologically analysed. Histological analysis confirmed that the material for HPV DNA detection contained enough tumour cells (at least 10% of the volume of the sample). Only samples which met this criterion were included in the study. Two 20-$\mu$m sections for PCR detection of HPV DNA were put in a sterile microtube. Control paraffin blocks (blocks without tissue or with HPV-free tissue) were inserted after every fifth block with biopsies to monitor for possible carry-over contamination. The knife of the microtome, all tools and the surrounding area were cleaned with ethanol after processing each sample.

*DNA extraction*

Paraffin was removed with xylene, and DNA was extracted by incubation with proteinase K (Sigma, St Louis, MO, USA) at final concentration 200 $\mu$g ml$^{-1}$ in lysis buffer (50 mM Tris-HCl, pH 8; 5 mM EDTA, pH 8; 1% Tween 20) for 2 h at 55°C. Proteinase K was inactivated at 95°C for 10 min and samples were stored at −20°C. For every nine samples one negative control was included in the process of DNA preparation (lysis buffer with proteinase K and no paraffin).

*PCR*

Ten microlitres of the crude lysate of each sample was amplified in a thermocycler (PTC 100; MJ Research, Inc., Waltham, MA, USA). After amplification, 10 $\mu$l of PCR product was analysed on a 3% horizontal agarose gel (NuSieve 3:1, FMC BioProduct).

*PCR of the control gene*

A fragment of the human beta-globin gene was amplified with primers PC 03 (5′ACACAACTGTGTTCAC TAGC 3′) and PC 04 (5′CAACTTCATCCACGTT CACC 3′) (Saiki *et al*, 1985). A positive beta-globin amplification proved that the sample contains enough DNA and that no PCR inhibitors are present. If the result of the control gene amplification was negative, the sample was phenol/chloroform-extracted and the amplification was repeated. Fifty microlitres of the reaction mixture contained 1x concentrated reaction buffer (Fermentas, Viznius, Lithuania), 4.0 mmol l$^{-1}$ $MgCl_2$, 0.2 mmol dNTPs, 0.05 pmol of each primer (PC 03 and PC 04), and 2.5 U Taq-polymerase (Gibco). After an initial denaturation for 5 min at 95°C, each of the 40 cycles consisted of a 1 min of denaturation at 95°C, primer annealing for 2 min at 55°C, and chain elongation for 2 min at 72°C. At the end, an extra incubation for 3 min at 72°C was carried out.

*HPV detection by PCR*

Samples with positive amplification of the control gene were subjected to HPV DNA detection with general GP5+ (5′ TTTGTTACTGTGGTAGATACTAC 3′) and GP6+ (5′ GAAAAATAAACTGTAAATCATA TTC 3′) primers, which generate a 150-bp long fragment of the L1 gene (de Roda Husman *et al*, 1995). Hundred microlitres of the reaction mixture contained 1x concentrated reaction buffer (Gibco), 3.5 mmol l$^{-1}$ $MgCl_2$, 0.2 mmol dNTPs, 50 pmol of each primer (GP5+ and GP6+) and 2 U Taq-polymerase (Gibco). After an initial denaturation for 5 min at 95°C, each of the 40 cycles consisted of denaturation for 1 min at 94°C, primer annealing for 2 min at 40°C, and chain elongation for 2 min at 72°C. The last cycle was followed by the incubation for 3 min at 72°C.

Sterile water and HPV-negative DNA of LEP cells, a human lung embryonic fibroblast cell line, were used as negative controls. DNA from SiHa, a human cervical cancer cell line containing one or two copies of the HPV 16 genome per cell, was included in every run as a positive control.

*HPV typing by a nucleotide DNA sequencing*

Samples positive by PCR which reveal a clear band on the agarose gel were subjected to a DNA nucleotide sequencing in order to determine the exact HPV genotype. Fifty microlitres of the PCR product was extracted from the 2% agarose gel (NuSieve GTG agarose; FMC BioProducts, Philadelphia, PA, USA) by the phenol/chloroform-method, and sequenced with the 'BigDye Terminator Primer Cycle Sequencing kit' (Applied Biosystems, Foster City, CA, USA). The analysis was done on the automatic ABI PRISM 310 sequencing system (Applied Biosystems).

*Statistical analysis*

The standard chi-square test and in some instances the Fisher exact test were used. Odds ratios with 95% confidence intervals and two-tailed *P*-values were calculated in $2 \times 2$ tables using the EPI INFO statistical package (CDC, Atlanta, GA, USA) and GraphPad InStat (version 3.00) (GraphPad Software, San Diego, CA, USA). All tests were two sided and the significance level was $P = 0.05$.

In the risk factor analyses the patients were divided into never smoker, former smokers (smoked regularly at least half a pack of cigarettes a week for a year or longer, but were not currently smokers at the time of diagnosis), and current smokers. Similar definitions were applied for alcohol consumption; abstainers (never drank), former drinkers (drank on average of one drink or more a week for a year or longer, but were no longer drinking at the time of diagnosis), and current alcohol users.

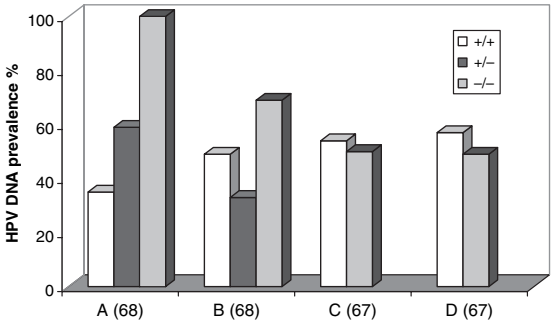
## Results

In the analysed group there were 54 men (mean age 58.6 years; age range 40–77) and 14 women (mean age 55.3 years; age range 44–71), 50% of patients were current smokers (median number of years of smoking 37), 40% formers smokers (median number of years of smoking 31) and 10% never smokers, and 63% were current alcohol users (median number of years of drinking 38), 13% were former drinkers (median number of years of drinking 26) and 24% never drank alcohol.

Fifty-six of the tumours were located in the oropharynx (82%), while only 12 (18%) were located in the oral cavity. Tumour sites included tongue ($n = 5$), base of tongue ($n = 8$), floor of mouth ($n = 6$), other unspecified parts of mouth ($n = 1$), tonsils ($n = 28$), other unspecified parts of oropharynx ($n = 19$) and pharynx ($n = 1$).

The HPV DNA presence was detected in 51.5% of tumours from 68 patients. Eighty per cent of HPV DNA positive tumours contained HPV16. HPV33 was detected in three samples and three samples contained uncharacterized HPV type.

The HPV DNA positive tumours were found in 42.3% of subjects younger than 56 years and in 57.1% of subjects older than 56 years ($P = 0.3$). The prevalence of HPV DNA was 64.3% in women and 48.1% in men ($P = 0.4$). We found no differences between HPV DNA presence by tumour size or frequency of regional metastases. None of these patient or tumour factors were significant.




| | A | | | B | | | C | | D | |
|---|---|---|---|---|---|---|---|---|---|---|
| +/– | +/+ | +/– | –/– | +/+ | +/– | –/– | + | – | ≤6 | >6 |
| No | 34 | 27 | 7 | 43 | 9 | 16 | 43 | 24 | 30 | 37 |
| HPV DNA+ | 12 | 16 | 7 | 21 | 3 | 11 | 23 | 12 | 17 | 18 |
| % + | 35.3 | 59.3 | 100 | 48.8 | 33.3 | 68.8 | 53.5 | 50.0 | 56.7 | 48.6 |

**Figure 1** Human papillomavirus DNA prevalence in tumours and relationship to other risk factors. A, smoking (+/+ current smoker, +/– former smoker, –/– never smoker); B, alcohol (+/+ current alcohol user, +/– former alcohol user, –/– never alcohol user); C, practice orogenital sex; D, lifetime number of sexual partners

The relation between HPV prevalence and other risk factors is shown in Figure 1. Tumours of never smokers and never drinkers were more often HPV DNA positive than were tumours of cases with tobacco (*P* for trend = 0.001) and alcohol (*P* for trend = 0.3) history. The prevalence of HPV DNA in tumours was similar in the group of patients with <6 and 6 or more lifetime sexual partners (56.7% *vs* 48.6%; $P = 0.6$) as well as in the group of patients who practice oral sex or who did not (53.5% *vs* 50%; $P = 0.8$). The most significant association was found between HPV DNA positivity and anatomical localization of the tumour. Tumours of oropharynx were more often HPV DNA positive (57%) than tumours of the oral cavity (25%) ($P = 0.06$).

## Discussion

Our study examined the prevalence and types of HPV infection in oral cavity and oropharyngeal tumours and the association between HR HPV tumours and other risk factors of head and neck cancer. Many studies of HPV infection of head and neck carcinogenesis report conflicting results because of the differences in the detection methods and epidemiological characteristics of the patient group. Most consistent are the data regarding the HPV DNA prevalence at different anatomical locations. The results of our study are in agreement with numerous others, which have reported higher prevalence of HPV DNA in the oropharyngeal than in oral cancers (Gillison *et al*, 2000; Miller and Johnstone, 2001). We used PCR HPV DNA detection technique based on general primers comparable with other recently published large-scale studies (Herrero *et al*., 2003; Smith *et al*, 2004b). For tumours of the oral cavity, HPV DNA prevalence ranges between 0 and 21% (Mineta *et al*,

1998; Klussmann *et al*, 2001; El Mofty and Lu, 2003; Herrero *et al*, 2003), and for oropharyngeal tumours between 18 and 45% (Klussmann *et al*, 2001; Herrero *et al*, 2003; Ritchie *et al*, 2003). In our study HPV DNA was detected in 25% of oral cavity and in 57% of oropharyngeal tumours. The overall higher prevalence of HPV DNA in our patients (51.5%) might be explained by the high proportion of tumours located in the oropharynx (82%), particularly in the tonsils (50% of all tumours located in the oropharynx) and other parts of the oropharynx (34%) which are known to be the site with the highest prevalence of HPV markers (Herrero *et al*, 2003).

Like Lindel *et al* (2001) we found a higher rate of HPV infection in female patients, but the number of females in our cohort was small and the difference was not statistically significant. Badaracco *et al* (2000) found that HPV infection was not related to gender whereas Ritchie *et al* (2003) reported a higher prevalence of HPV DNA in males.

A higher percentage of HPV positive tumours has been reported both in younger (Strome *et al*, 2002; El Mofty and Lu, 2003) and older (Lindel *et al*, 2001) patients. Other studies, including ours, did not find any age-related difference in the distribution pattern of HPV DNA (Sisk *et al*, 2000).

Smoking and alcohol consumption are the most important risk factors of oral cavity and oropharyngeal cancer in the Central European population. Consequently, the lower proportion of smokers in patients with HPV DNA positive tumours suggests that these tumours might form a distinct epidemiological entity. Oral cancers in young people with no smoking histories may also be HPV induced (Llewellyn *et al*, 2001). In agreement with other studies (Gillison *et al*, 2000; Lindel *et al*, 2001; Herrero *et al*, 2003; Ritchie *et al*, 2003), we detected HPV DNA less frequently among both current and former tobacco and alcohol users. The tumours of former smokers were more often HR HPV positive than tumours of current smokers and less likely to be HPV positive than in tumours of never smokers and the difference was statistically significant.

In contrast to other studies (Herrero *et al*, 2003; Ritchie *et al*, 2003), where HPV DNA presence was positively related to the number of sexual partners or practice of oral sex, we found no difference in our study. Another study has supported the theory of sexual transmission reporting an excess of tonsillar cancer among husbands of women with HPV-associated neoplastic lesions of the cervix (Hemminki *et al*, 2000).

We found no relation between tumour size and presence of regional metastases in HPV HR positive *vs* negative tumours. These results are in contrast to the study of Mellin *et al* (2000), who reported a strong association between T classification and HPV DNA positivity.

In numerous studies better prognosis of HPV DNA positive tumours has been reported (Gillison *et al*, 2000; Mellin *et al*, 2000; Lindel *et al*, 2001; Li *et al*, 2003; Ritchie *et al*, 2003). The more favourable outcome could be explained by a better response of HPV DNA positive tumours to radiotherapy (Lindel *et al*, 2001), although this survival benefit was not observed by another (Friesland *et al*, 2001). Even though the follow-up period of the patients in our study was very short (on average 1 year), there were fewer recurrences in patients with HPV DNA positive tumours (data not shown). The association between HPV DNA status and disease prognosis may be answered after completion of the ongoing longitudinal study.

## Conclusions

Even though the difference in the HPV DNA prevalence in tumours related to age, gender and sexual behaviour, tumour size and regional lymph nodes status was not statistically significant, most probably due to a small number of cases, the differences in the prevalence of HPV DNA positive tumours between subjects exposed and not exposed to tobacco and alcohol supports the theory that HPV DNA positive tumours form an aetiologically distinct subgroup of head and neck tumours.

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