



## Smoking, alcohol consumption and human papillomavirus infection as risk factors for oral cavity and oropharyngeal tumors in Serbia – A pilot study

Pušenje, alkohol i humani papiloma virus kao faktori rizika od razvoja oralnih i orofaringealnih tumora u Srbiji – pilot studija

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### Abstract

**Background/Aim.** The oral cavity and oropharyngeal cancers are among the most common cancers worldwide with the multifactorial etiology. The aim of this study was to determine the major risk factors among patients with oral cavity and oropharyngeal tumors in Serbia. **Methods.** A total of 63 patients with biopsy proven malignant (33 patients) or benign (30 patients) oral cavity or oropharyngeal lesions were included in this study. The data about gender, age, smoking habits and alcohol consumption were obtained from the routine medical files. The detection and genotyping of human papillomavirus (HPV) was done in paraffin embedded tissue samples using *in situ* hybridization. **Results.** Malignant lesions were more frequent in men, smokers and patients who consume alcohol with a statistically significant difference compared to the patients with benign lesions. The prevalence of HPV infection was higher in patients with malignant lesions compared to patients with benign lesions, but without statistically significant difference. High risk genotypes were detected only in patients with malignant lesions of tonsils and base tongue cancer, while low risk types were demonstrated in patients with benign lesions with a highly statistically significant difference. **Conclusion.** The results point to the significant association of tobacco smoking, alcohol consumption and high risk HPV genotypes as risk factors for oral cavity and oropharyngeal carcinomas in Serbian patients.

**Key words:** alcohol drinking; carcinoma, squamous cell; human papillomavirus; mouth neoplasms; pharyngeal neoplasms; risk factors; serbia; smoking.

### Apstrakt

**Uvod/Cilj.** Karcinomi usne duplje i orofaringealne regije su među najčešćim malignitetima u svetu. Cilj ove studije bio je da utvrdi faktore rizika od pojave oralnih i orofaringealnih tumora kod bolesnika u Srbiji. **Metode.** Studijom su bila obuhvaćena 63 bolesnika sa patohistološkom potvrdom malignih lezija (33 bolesnika) i benignih lezija (30 bolesnika) u usnoj duplji/orofaringealnoj regiji. Uvidom u medicinsku dokumentaciju dobijeni su podaci o demografskim karakteristikama (pol i starost) i navikama bolesnika (pušenje i konzumacija alkohola). Tehnikom *in situ* hibridizacije identifikovan je humani papilomavirus (HPV) u tkivima tumora oralne i orofaringealne regije, fiksiranim u formalinu i ukalupljenim u parafin. **Rezultati.** Maligne lezije su bile statistički značajno češće kod muškaraca koji su konzumirali alkohol i duvan. Infekcija HPV bila je češća kod bolesnika sa malignim lezijama u odnosu na bolesnike sa benignim lezijama, ali bez statistički značajne razlike. Visokoonkogeni tipovi 16/18 otkriveni su samo kod bolesnika sa malignim lezijama i to tonzila i baze jezika, dok su niskoonkogeni tipovi 6/11 identifikovani kod bolesnika sa benignim lezijama. **Zaključak.** Rezultati ove pilot studije ukazuju na povezanost pušenja, konzumacije alkohola i visokoonkogenih tipova HPV sa razvojem karcinoma usne duplje i orofaringealne regije kod bolesnika u Srbiji.

**Ključne reči:** alkohol, pijenje; karcinom skvamoznih ćelija; papilomavirus, humani; usta, neoplazme; farinks, neoplazme; faktori rizika; srbija, pušenje.

## Introduction

Tumors of the oral cavity and oropharynx are among the most common tumors worldwide, with an estimated 529,500 incident cases and 292,300 deaths from oropharyngeal cancer in 2012, accounting for about 3.8% of all cancer cases and 3.6% of cancer deaths. In 2012, the estimated age-standardized rate of oral cavity cancer was relatively large (2.7 per 100,000 for both sexes combined; 3.7 in men and 1.8 in women), with substantial differences by sex, age, and region. The estimated age-standardized rate of oropharyngeal cancer was 1.4 per 100,000 for both sexes combined (2.3 for men and 0.5 for women), where the countries with high Human Development Index (HDI) scores had the highest proportional incidence of oropharyngeal cancer of both men and women <sup>1</sup>.

The estimated age-standardized rate of oral cavity and oropharyngeal cancer for both sexes in Serbia was 11.7 per 100,000 in 2012, with the higher incidence in men (18.8 for men and 5.2 for women) <sup>2</sup>.

Squamous cell carcinoma (SCC) is the most common epithelial malignancy in oral cavity and oropharynx. More than 90% of cases and over 50% of the cancers are often preceded by potentially malignant disorders such as leukoplakia, oral lichen planus and submucous fibrosis <sup>3</sup>.

Etiology of oral cavity and oropharyngeal cancer is considered to be a multifactorial process where environmental factors, viral infections and genetic alterations interact and induce malignant cell transformation <sup>3</sup>.

Numerous studies demonstrated that the major risk factors for the development of oral cavity and oropharyngeal cancers are tobacco smoking, alcohol consumption and human papilloma virus (HPV) infections, where tobacco smoking and alcohol consumption have synergistic effects with a nearly sevenfold increase of risk <sup>1,4,5</sup>.

Tobacco smoke condensate contains substances that act as both initiators and promoters of carcinogenesis. The risk of cancer development from smoking is significant up to approximately five years after quitting. Alcohol is well documented risk factor for oral and oropharyngeal cancers. Animal studies have shown that ethanol promotes 4-NQO-induced oral carcinogenesis <sup>4</sup>. The oncogenic potential of high-risk HPV genotypes is very well documented for anal-genital carcinomas. High-risk HPV genotypes (16, 18, 31, 33, etc.) transform the epithelial cells in the way that their proteins, E6 and E7 gene products, inhibit the function of tumor suppressor genes, thus inactivating the cellular proteins p53 and Rb <sup>6</sup>. Different studies suggest that HPV may be associated with the development of oral and oropharyn-

geal cancers with the similar molecular mechanisms. It is estimated that 25%–35% of oral and oropharyngeal cancers are infected with HPV. High-risk genotypes 16 and 18 seem to be the most important viruses responsible for carcinogenesis and can be found in premalignant and malignant lesions of the oral cavity in up to 80% of cases <sup>4</sup>.

The aim of this study was to determine contribution of tobacco smoking, alcohol consumption and prevalence and genotype distribution of HPV as tumor risk factors among patients with oral cavity and oropharyngeal tumors in Serbia.

## Methods

### Patients data

From January 2005 to January 2006, a total of 63 patients of both sexes with biopsy proven malignant or benign oral cavity or oropharyngeal lesions were treated at the Clinic of Otorhinolaryngology and Maxillofacial Surgery, Clinical Center of Serbia in Belgrade. The study group of patients with malignant lesions included 33 patients where the majority of patients had tonsils cancer (13 patients) or tongue cancer (9 patients). The control group of patients with benign lesions included 30 patients with tonsil hypertrophy and mucosa hypertrophy or fibroepithelial polyps localized in the oral cavity or oropharyngeal region, where the majority of patients (10 patients) were with miscellaneous benign lesions localized at buccal mucosa, retromolar trigonum and gingiva (Table 1).

The data about gender, age, smoking habits and alcohol consumption were obtained from the routine medical files. Patients, aged from 20 to 79 years (mean age  $54.7 \pm 4.6$ ), were classified into three groups in relation to age: patients from 20 to 39 years of age, 40–59 and from 60 to 79 years. In relation to alcohol consumption, patients were divided into the following groups: every day, occasionally and no consumption. According to tobacco smoking, patients were classified as smokers and nonsmokers.

### Samples for HPV detection and typing

The sample preparation and HPV detection and typing were carried out in the Virology Department, Institute of Microbiology and Immunology, Faculty of Medicine, University of Belgrade, Serbia. Paraffin embedded tissue samples of malignant or benign oral cavity or oropharyngeal lesions were cut into 4  $\mu\text{m}$ –6  $\mu\text{m}$  paraffin sections and collected on treated glass slides. The prepared samples were used for the HPV DNA detection and typing.

**Table 1**

**Localization of malignant and benign oral cavity and oropharyngeal tumors**

Lesions	Tumor localization						total
	tongue	floor of mouth	tonsil	soft palate	miscellaneous*		
Malignant	9	3	13	4	4		33
Benign	8	0	8	4	10		30
Total	17	3	21	8	14		63

\*localizations on buccal mucosa, retromolar trigonum and gingiva were classified as “miscellaneous”.

### HPV detection and typing

HPV detection was performed using HPV DNA Screening – REMBRANDT *in situ* hybridization kit (Kreatech Diagnostics, Amsterdam, Netherlands), according to the manufacturer's instructions. The presence of colored hybrids of HPV DNA and probes in the cells under the light microscope was considered to be positive for HPV.

After that, HPV positive samples were typed using HPV DNA typing – REMBRANDT *in situ* hybridization kit with 16/18, 31/33/35 and 6/11 HPV probes (Kreatech Diagnostics, Amsterdam, Netherlands), according to the manufacturer's instructions. The presence of colored hybrids of HPV DNA and type specific probes in the cells under the light microscope was considered to be positive for HPV types.

### Statistical analysis

Data were put in the spreadsheet package EXCEL for Windows XP and statistical analysis was performed with SPSS ver. 20.0 using Fisher's exact test. Differences being with  $p < 0.05$  were considered to be significant.

### Results

The majority of patients in groups with malignant and benign lesions were aged 40–59 years. Malignant lesions were more common in men than in women with statistically significant difference between patients with malignant and benign lesions (Table 2).

Regarding social habits, the majority of patients with malignant lesions were smokers, with statistically significant difference between examined groups. The alcohol consumption was more frequent in patients with malignant changes as compared to patients with benign lesions, with statistically significant difference (Table 2).

The prevalence of HPV infection was higher in the group of patients with malignant lesion as compared to patients with benign lesion, but there were no statistically significant difference between these two groups (Table 2). High-risk genotypes 16/18 were detected only in patients with malignant lesions, while low-risk types 6/11 were demonstrated in patients with benign lesions with a highly statistically significant difference. In one patient with malignant lesion, multiple infection with high-risk 16/18 and low-risk HPV genotypes 6/11 was detected (Table 2).

All HPV positive patients with malignant lesions were men, smokers, 40–59 years of age. Out of 5 HPV positive patients, 2 patients reported occasional alcohol consumption (Table 3). According to the localization of malignant lesions, 80% of positive HPV results were found in tonsil cancers and 20% in tongue cancers (Table 3). Out of all SCC specimens of tonsils, 30.76% were positive to HPV (4/13) and 11.11% samples collected from the tongue were positive to HPV infection (1/9). Multiple infections with high-risk and low-risk HPV genotypes were detected in patient with tonsil cancer.

In patients with benign lesions, HPV was detected in smokers, 40–59 years of age with occasional consumption of alcohol. One HPV positive result was detected in man and one in female. All HPV positive samples were obtained from benign lesions of tongue (Table 3).

**Table 2**  
Risk factors in patients with malignant and benign oral cavity and oropharyngeal tumor lesions

Risk factors	Lesions		<i>p</i>
	malignant	benign	
Age (years)			
20–39	0	4 (13.3)	
40–59	21 (63.6)	20 (66.7)	0.06
60–79	12 (36.4)	6 (20)	
Gender			
male	29 (87.87)	10 (33.33)	0.001
female	4 (12.13)	20 (66.66)	
Smoking			
yes	31 (93.9)	16 (53.3)	0.001
no	2 (6.1)	14 (46.7)	
Alcohol consumption			
every day	9 (27.7)	0	0.001
occasional	14 (42.4)	12 (40)	
no	10 (30.3)	18 (60)	
HPV detection			
positive	5 (15.15)	2 (6.66)	0.429
negative	28 (84.85)	28 (93.34)	
HPV types			
16/18	4 (80)	0	0.047
16/18 + 6/11	1 (20)	0	
6/11	0	2 (100)	

**Note: Results are given as number (%) of patients.**

Due to the small number of HPV positive patients with malignant and benign lesions, the statistical analysis for the association of HPV and social habits was not performed.

### Discussion

Oral cavity and oropharyngeal carcinomas are primarily diseases of older age, occurring most frequently in patients older than age 45. Epidemiological studies over last 20 years have shown a steady increase in the incidence of these cancers in younger adults (in age 18–45 years)<sup>7</sup>. This study cannot confirm this data since all of the patients with malignant lesions were over 40 years of age. However, many oral and oropharyngeal cancers present at a late stage of disease due to the delay in diagnosis. The delay in younger patients could be longer as cancer is not suspected in this age<sup>8</sup>.

Numerous epidemiological studies demonstrated higher incidence of oral cavity and oropharyngeal cancers in men compared to women<sup>1,9</sup>. This is consistent with the results of our study, where the majority of patients with malignant lesions were men (87.87%), with the statistically significant difference.

**Table 3**  
**The frequency of human papillomavirus (HPV) according to gender, age and social habits of patients with malignant and benign oral cavity and oropharyngeal tumor lesions**

Parameter	Malignant lesions		Benign lesions	
	HPV positive	HPV negative	HPV positive	HPV negative
Gender				
male	5 (15.15)	24 (72.73)	1 (3.33)	9 (30)
female	0 (0)	4 (12.12)	1 (3.33)	19 (63.33)
Total	5 (15.15)	28 (84.85)	2 (6.66)	28 (93.33)
Age				
20–39	0 (0)	0 (0)	0 (0)	4 (13.33)
40–59	5 (15.15)	16 (48.49)	2 (6.66)	18 (60)
60–79	0 (0)	12 (36.36)	0 (0)	6 (20)
Total	5 (15.15)	28 (84.85)	2 (6.66)	28 (93.33)
Alcohol consumption				
no	3 (9.09)	7 (21.21)	0 (0)	18 (60)
occasionally	2 (6.06)	12 (36.36)	2 (6.66)	10 (33.33)
every day	0 (0)	9 (27.27)	0 (0)	0 (0)
Total	5 (15.15)	28 (84.85)	2 (6.66)	28 (93.33)
Smoking				
yes	5 (15.15)	26 (78.79)	2 (6.66)	14 (46.66)
no	0 (0)	2 (6.06)	0 (0)	14 (46.66)
Total	5 (15.15)	28 (84.85)	2 (6.66)	28 (93.33)
Tumor site				
tonsils	4 (12.12)	9 (27.27)	0 (0)	8 (26.66)
tongue	1 (3.03)	8 (24.24)	2 (6.66)	6 (20)
soft palate	0 (0)	4 (12.12)	0 (0)	4 (13.33)
floor of mouth	0 (0)	3 (9.09)	–	–
miscellaneous	0 (0)	4 (12.12)	0 (0)	10 (33.33)
Total	5 (15.15)	28 (84.85)	0 (0)	28 (93.33)

**Note: Results are given as number (%) of patients.**

It is generally accepted that etiology of oral cavity and oropharyngeal carcinoma is multifactorial and the most common risk factors of these malignant diseases include tobacco smoking, alcohol consumption and HPV infection<sup>1,4</sup>. It has been demonstrated that tobacco and alcohol are traditional risk factors for oral and oropharyngeal cancers in adults, regardless of age. Individuals who smoke more than 20 cigarettes a day and consume more than 100 g of alcohol per day are believed to be at increased risk for oral and oropharyngeal epithelial dysplasia. In addition, alcohol has been found to be an independent risk factor for these cancers among non-smokers, as well as tobacco smoking in non-drinkers. Moreover, both factors together seem to enhance the carcinogenic effect<sup>4,7</sup>. This is supported in this study as almost all of the patients with malignant lesions were smokers (93.9%) and 69.1% of them consumed alcohol, which was significantly different from patients with benign lesions. The results of this study are similar to previous reports from our country<sup>10,11</sup>. The majority of these reports examined the correlation of genetic and epigenetic markers and above mentioned risk factors in oral and oropharyngeal cancers<sup>11–13</sup>.

It is also demonstrated that benign lesions of oral and oropharyngeal regions are associated with tobacco smoking and alcohol consumption. However, the risk is lower com-

pared to malignant lesions<sup>14</sup>. This is supported by results of this study, where around 50% of patients with benign lesions were smokers with occasional consumption of alcohol.

Current literature data shows that at any given time approximately 7% of the population has a prevalent oral/oropharyngeal HPV infection. Most of these HPV infections do not progress to cancer and are usually cleared by the immune system. It has been suggested that the delayed clearance of oral/oropharyngeal HPV infection may be a risk factor for development of oral cavity and oropharyngeal cancers<sup>15</sup>.

A detection rate of HPV DNA in oral cavity and oropharyngeal cancers ranges from 25%–35%, with the dominance of high risk genotypes 16 and 18<sup>3</sup>. There have been numerous publications studying HPV presence in oral cavity and oropharyngeal tumors with the variability in detection rates of 0%–100%. This variability may be due to the multiple anatomical sites encompassed, the use of various detection techniques [*in situ* hybridization, southern blot hybridization, polymerase chain reaction (PCR)] and different sampling methods such as biopsies, scrapes, oral rinses, brushes<sup>16</sup>. Furthermore, numerous studies demonstrated that the locations of HPV positive oral cavity and oropharyngeal carcinoma are tonsils, the base of the tongue and oropharyngeal harbor<sup>17</sup>.

The results of this study showed higher detection rate of HPV using *in situ* hybridization method in the group of patients with malignant lesion compared to patients with a benign lesions (15.15% vs. 6.66%), but this was not statistically significant difference. *In situ* hybridization, which is used in this study, is a highly specific method that protects tissue morphology, but the method with lower sensitivity relating to PCR<sup>18</sup>. In the study of Kozomara et al.<sup>11</sup>, HPV detection rate using PCR was 64% in the tissues of tongue and floor of mouth cancers. The low percentage of HPV positivity in our pilot study may be due to the small cohort of patients, the analysis of multiple anatomic sites and limitations of *in situ* hybridization method. However in the study of Popovic et al.<sup>19</sup>, HPV detection rate using PCR was 10% in the tissues of oral cancers.

According to the localization, in this study HPV was detected only in patients with tonsils and base tongue carcinomas, which is consistent with the previous findings<sup>20</sup>. In addition, the detection rate of high-risk genotypes 16 and 18 in our study was consistent with previous studies<sup>21</sup>, where these types have been detected only in patients with malignant lesions (80%), while low risk types 6/11 were demonstrated in patients with benign lesions, with a highly statistically significant difference between these two groups.

Literature data favors the fact that negative HPV oral cavity and oropharyngeal carcinomas develop in senior patients (in the seventh decade of life), with predomination of males, whose main risk factors for cancer occurrence are smoking, alcohol consumption, bad oral hygiene and a vitamin-poor diet. HPV positive carcinomas are more likely to occur in men of younger age, which is explained by the

change of sexual habits<sup>9,22</sup>. This is consistent with the findings in our study, where all HPV positive patients with malignant and benign lesions were aged 40–59 years.

The majority of studies have shown that patients with HPV positive oral cavity and oropharyngeal cancers are less likely to have a history of tobacco exposure and alcohol consumption<sup>15,23</sup>. In this study, the consistence was found for alcohol consumption, but not for tobacco smoking because all HPV positive patients with malignant lesions were smokers. Similar results were found for all HPV positive patients with benign lesions.

To the best of our knowledge, this is the first study which examined the association of risk factors both in malignant and benign lesions of oral and oropharyngeal region.

### Conclusion

The obtained results of this study showed a significant association of tobacco smoking, alcohol consumption and HPV infection with oral cavity and oropharyngeal tumors in Serbian patients. These results as well as those of future studies with a larger cohort, would possibly provide more detailed information about the major risk factors for oral cavity and oropharyngeal tumors and may contribute to their prevention in Serbia.

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