CLINICAL AND PATHOLOGICAL PRESENTATIONS OF PATIENTS WITH HPV POSITIVE OROPHARYNGEAL CARCINOMA – A SOUTH CROATIAN STUDY

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Objective: The objective of this study was to analyze the influence of human papilloma virus (HPV) in patients with oropharyngeal squamous cell carcinoma (OPSCC) from southern Croatia on survival, clinical outcomes, and pathological features. *Methods:* We analyzed HPV DNA presence and p16 immunohistochemistry staining in 68 formalin-fixed paraffin-embedded samples from patients diagnosed with OPSCC at the Split University Hospital Center between 2013 and 2017. Histologic features were analyzed using a light microscope. Clinical data were retrospectively collected from patient records and analyzed for HPV status. *Results:* In this study, 10.29% of patients were HPV positive (HPV+). Lymphocyte invasion was more prominent in p16 positive OPSCC. Overall survival (OS) was better in HPV+ and p16+ patients. HPV status is a significant prognostic variable for patients from south Croatia with OPSCC. *Conclusion:* HPV seems to have a minor influence on OPSCC in south Croatia in comparison to other Western European countries and the USA. Although the influence of HPV on survival was significant, traditional risk factors were more important in the carcinogenesis of OPSCC in our population.

Key words: Croatia, head and neck neoplasms, histology, human papilloma virus 16, oropharyngeal neoplasms, squamous cell carcinoma

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INTRODUCTION

Head and neck carcinoma is the sixth leading cancer by incidence worldwide, with 550,000 new cases and 300,000 new deaths emerging every year. In 90% of cases, it is classified as squamous cell carcinoma (HNSCC). Usually, this cancer affects men far more than it affects women (1,2). Traditionally, these cancers have been strongly associated with risk factors such as tobacco and alcohol exposure (3). Because of the harmful effect smoking has on carcinogenesis, many states have enforced actions against smoking. Because of this, there has been a steady decline in smoking incidence around the world. However, even though smoking rates are declining, the incidence of oropharyngeal cancers in the USA is rising (4-6). This is due to a shift in the etiology of oropharyngeal carcinoma, i.e., human papillomavirus (HPV). Recently, more cases are being attributed to certain HPV infections, with types HPV-16 and HPV-18 emphasized as the most commonly isolated (3,4). These high-risk oncogenic types encode oncoproteins E6 and E7 which dysregulate cell cycle control and induce cellular transformation of primary squamous epithelial cells by binding to tumor suppressor proteins p53 and pRB (7).

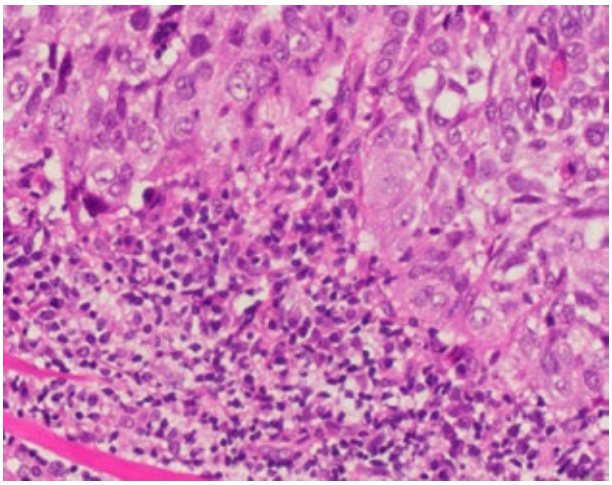

Figure 1. *Typical histologic presentation of HPV+ OPSCC. Syncytial clusters of atypical poorly differentiated squamous epithelial cells are surrounded by dense mononuclear infiltration.*

Clinical differences have been recorded among patients in regard to HPV status. HPV positive (HPV+) patients are known to have a more favorable survival rate than HPV negative (HPV-) patients, which is due to their better therapy response (8,9). These patients are typically characterized as younger white men of a higher socioeconomic status, without traditional risk factors such as smoking and alcohol exposure (10). Squamous cell carcinomas of the oropharynx, especially the palatine and lingual tonsils, make up most of the HPV+ head and neck carcinomas (11).

Not only are there differences in the clinical presentation of HPV+ oropharyngeal squamous cell carcinomas (OPSCC), but histologic differences have been clearly described. HPV+ OPSCC displays a basaloid appearance, lacking prominent keratinization. The tumor infiltrates surrounding tissue as expansive lobules. These lobules are usually surrounded by infiltrating lymphocytes (Figure 1). Usually, there is minimal to no desmoplastic reaction (12,13).

The geographic distribution of HPV+ OPSCC differs fairly among countries. Viral etiology outnumbers traditional risk factors in North America and northern Europe (4,14,15), while HPV- OPSCC still indicates the prevalent role of traditional risk factors in southern Europe (16). In Croatia, head and neck carcinomas make up 7% of all cases in men and 1% in women, with the trend for oropharyngeal carcinomas staying relatively consistent from 2010 to 2014 with about 100 new cases emerging *per* year (17). Recently, a study performed by Božinović *et al*. analyzed HPV status in OPSCC in northern Croatia. In this study, HPV RNA was found in 29.3% of cases (18).

In order to get a complete view on how HPV impacts patients with OPSCC in Croatia, we evaluated HPV status in patients from southern Croatia, analyzed the clinical and histologic features, and determined survival rates.

MATERIALS AND METHODS

Data collection

We collected 68 formalin-fixed paraffin-embedded (FFPE) tissue samples diagnosed with oropharyngeal carcinoma at the Split University Hospital Center between 2013 and 2017. Epidemiological data were collected from the computer database of the Department of Pathology, and narrow localization of tumors from the history of diseases at the Department of Ear, Nose and Throat Diseases with Head and Neck Surgery, Split University Hospital Center. The inclusion criterion was sufficient material in FFPE sample for real-time polymerase chain reaction (qPCR) analysis and hematoxylin-eosin (H&E) staining. Subjects who did not have enough tissue in the paraffin block for PCR analysis were excluded from the study. The study was approved by the Bioethical Board of the Split University Hospital Center (00-03/19-01/78).

Microscopy and qPCR

Every paraffin block was cut to a thickness of 5 mm for histologic analysis and to a thickness of 10 mm for molecular analysis. After fixation, histologic samples were dewaxed in xylene (3x5 minutes) and rehydrated in a gradient of ethanol (100% 1x5 minutes and 96% 1x5 min) to water. The samples were immersed in the hematoxylin solution for 5 minutes and stained by rinsing in running water. The samples were then immersed in eosin solution for 3 minutes, washed and dehydrated in an alcohol gradient (75% 1x5 min and 100% 1x5 min), clarified by brief immersion in xylene, and covered with mounting medium and a microscopic cover slip. The slides were analyzed by an Olympus BX51 (Olympus Corporation, Shinjuku, Tokyo, Japan) light microscope. The degree of cancer differentiation, inflammatory stroma reaction, keratinization, desmoplasia, and growth pattern were analyzed by two pathologists.

The tumor-nodes-metastases (TNM) classification was done according to the 8th edition American Joint Committee on Cancer and the Union for International Cancer Control (UICC) TNM classification of malignant tumors (19).

DNA isolation was performed by affinity chromatography according to Sigma-Aldrich protocol with GenElute™ FFPE DNA Purification Kit. The method begins with the process of dewaxing the paraffin sample in a series of washings with xylene and 96% ethanol. The sample was then dissolved with proteinase K, RNase and dissolution buffer A, after which the samples were incubated for one hour at 55 °C and for one hour at 90 °C. RL buffer and 96% ethanol were added to the obtained lysate and the solution was placed in spin columns. Nucleic acids bind to the spin column by an ionic gradient, and the contaminant passes smoothly through the column or is retained at the top of the column. To further remove impurities, bound DNA was washed with elution solution A. DNA was then eluted with elution buffer B. After DNA isolation, qPCR was performed with selected primers, GP5+/GP6+ primers. SYBR Green dye was used. The ready-made SYBR Green Master Mix (containing buffers, dNTPs and SYBR Green dye), the above primers, DNA/RNase-free water and an isolated DNA sample were mixed. The Applied Biosystems™ 7500 Real-Time PCR Systems were used to read the samples.

Fluorescence data on all 68 samples in 40 PCR cycles were extracted and modelled logistically and exponentially due to the biochemical course of the reaction. Since DNA is amplified 2n times (n=number of cycles) during qPCR, the primer is integrated into the positive DNA into the newly formed strands and emits fluorescence that can be measured spectrophotometrically in each cycle. As the amount of primers and dNTPs is limited, logistic or exponential growth in the samples is expected. Positive samples were considered to be those for which these two models were more likely than the linear model in both examples, i.e., the one that best described the negative control (20). The probability of the model was calculated based on the Akaike information criterion (AIC).

Immunohistochemistry

Tissue microarrays were assembled from the 68 samples. Each slide consisted of eight samples. The p16 was done using anti-p16ink (CINtec p16, Ventana, Roche Holding AG, Oro Valley, Arizona, United States) antibodies on Ventana Ultrabenchmark (Ventana, Roche Holding AG, Oro Valley, Arizona, United States) using Ventana Ultraview staining kit (Ventana, Roche Holding AG, Oro Valley, Arizona, United States). The p16 expression was assessed by two pathologists. Samples were considered positive if the overexpression of p16 was more than 70% of tumor cells (Figure 2).

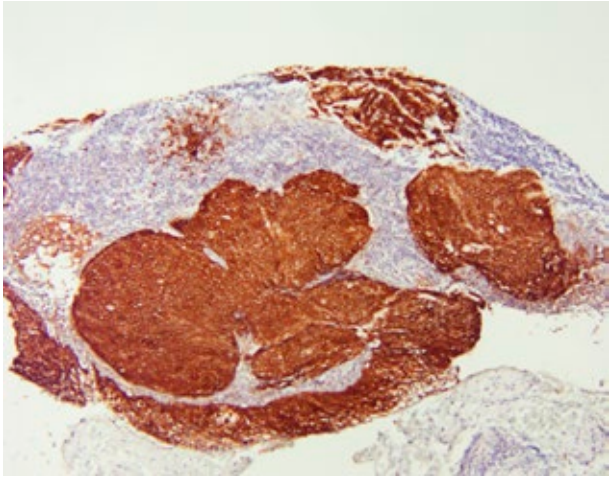

Figure 2. *p16 immunostaining on HPV+ OPSCC tissue.*

Statistics

Patient and sample data were entered into Microsoft Excel and analyzed with the GraphPad Prism 8.1 statistical program (GraphPad software, La Jolla, CA USA) and SPSS (version 26, IBM Corp., Armonk, New York, SAD). Differences between categorical variables were tested by the χ^2-test and Fisher exact test. Differences between continuous variables were tested with t-test for unpaired samples. The Shapiro-Wilk test was used to check the normality of the distribution. Missing data were categorized as unknown and removed from analysis. Samples with histologic features that could not be assessed were excluded from contingency calculations. Kaplan-Meier analysis was used to calculate survival data based on p16 status, HPV status, clinical stage, and therapy. Individual variable impact on survival was calculated *via* univariate Cox-proportional hazard regression models based on age, sex, HPV DNA presence, p16 status, T classification, N classification, distant metastasis presence, clinical stage, and type of therapy. A multivariate model was made considering the aforementioned variables, calculating AIC and removing insignificant variables. The level of statistical significance was set at $p<0.05$.

RESULTS

Out of the 68 samples collected that were diagnosed with OPSCC, 10.29% were HPV DNA positive, and 19.12% were p16 positive. Male sex was predominant in both HPV+ and HPV- groups, making up 71.43% and 81.97% of cases, respectively. There was no significant age difference between HPV positive and negative patients (p=0.6296, t-test), nor was there a difference regarding p16 status (p=0.1832, t-test). Anatomically, the most common location of HPV+ tu-

Table 1. *Patient clinical characteristics with respect to HPV and p16 status*

Variables	HPV + n (%)	HPV – (%)	p16 + (%)	p16 – (%)
Sex				
Men	5 (7.35)	50 (73.53)	11 (16.17)	44 (64.71)
Women	2 (2.94)	11 (16.17)	2 (2.94)	11 (16.17)
Tumour location				
Tonsils	5 (71.43)	17 (25)	5 (7.35)	17 (25)
Base of tongue	1 (1.47)	27 (39.71)	4 (5.88)	24 (35.29)
Soft palate	0	11 (16.17)	3 (4.41)	8 (11.76)
Palatine arch	0	3 (4.41)	1 (1.47)	3 (4.41)
Location unknown	1 (1.47)	3 (4.41)	0	3 (4.41)
Average age	65,14	63,12	59,85	64,15
Median age	64	62	62	62
Clinical stage				
Stage 1	1 (1.47)	13 (19.11)	2 (2.94)	12 (17.65)
Stage 2	1 (1.47)	0	1 (1.47)	0
Stage 3	1 (1.47)	15 (22.06)	3 (4.41)	13 (19.11)
Stage 4	3 (4.41)	10 (14.71)	5 (7.35)	8 (11.77)
T status				
T1	0	5 (7.35)	0	5 (7.35)
T2	1 (1.47)	9 (13.24)	2 (2.94)	8 (11.76)
T3	2 (2.94)	5 (7.35)	2 (2.94)	5 (7.35)
T4	1 (1.47)	17 (25)	6 (8.82)	12 (17.65)
N status				
N0	0	6 (8.82)	1 (1.47)	5 (7.35)
N1	3 (4.41)	11 (16.17)	5 (7.35)	9 (13.24)
N2	0	16 (23.53)	3 (4.41)	13 (19.11)
N3	2 (2.94)	5 (7.35)	1 (1.47)	6 (8.82)
M status				
M0	3 (4.41)	30 (44.12)	6 (8.82)	27 (39.71)
M1	3 (4.41)	9 (13.24)	5 (7.35)	7 (10.29)

mors were the tonsils (p=0.0157, Fisher exact test) (Table 1). Both patient groups presented more commonly in later clinical stages. Smoking data were collected on 13 patients only, out of which 92.37% were current or ex-smokers. Therapy data were found for 77.94% of patients. The majority of patients were treated with combined surgery and chemoradiotherapy (54.72%), while others underwent primary chemoradiotherapy (Table 2).

Patients with HPV+ OPSCC had a better survival rate than patients with HPV- OPSCC, although not statistically significant (p=0.7769) (Table 3, Figure 3). On the other hand, patients diagnosed with lower clinical stages had a significantly better outcome (p=0.0009). Patients that underwent combined surgical and chemoradiotherapy had a better outcome than those treated with primary chemoradiotherapy (p=0.0036).

Cox-proportional hazard regression models were made for univariate models and a multivariate model was formed. Both the univariate and multivariate models were calculated for 40 patients for which all of the clinical data were collected in order to reduce the risk of bias. Combined therapy confirmed to be beneficial in both the univariate model (p=0.002) and multivariate model (p=0.008) (Table 4). Presence of distant metastases worsened survival (p=0.021), given the univariate model (Table 5). The observed variables in our multivariate model were HPV status, sex, T status, N status, and therapy. In our multivariate model, several variables proved to be statistically significant. HPV+

Table 2. *Overall survival of patients with OPSCC based on HPV status, p16 status, clinical stage, and therapy*

Variables	Median survival	p	Hazard ratio	95% confidence interval	
				Lower	Upper
HPV status					
HPV +	1440	0.7769	1.156	0.4248	3.144
HPV -	930				
p16 status					
p16 +	1440	0.6277	1.218	0.5496	2.697
p16 -	930				
Clinical stage					
Stage 1	Undefined*	0.0009			
Stage 3	1740				
Stage 4	450				
Therapy					
Nonsurgical	450	0.0036	3.123	1.45	6.728
Combined therapy	1890				

Table 3. *Multivariate proportional hazard regression model for clinical and histologic features*

Variables	p	hazard ratio	95% confidence interval	
			Lower	Upper
HPV status				
Negative		1		
Positive	0.019	0.081	0.01	0.665
Sex				
Male		1		
Female	0.018	0.076	0.009	0.639
T status				
T1	0.407			
T2	0.339	4.450	0.209	94.858
T3	0.236	4.720	0.363	61.447
T4	0.352	0.524	0.135	2.041
N status				
N0	0.050			
N1	0.026	0.023	0.001	0.637
N2	0.006	0.037	0.004	0.389
N3	0.041	0.164	0.029	0.927
Therapy				
Nonsurgical		1		
Combined	0.008	0.123	0.026	0.572

patients, women and patients treated with combined surgery and chemoradiotherapy all had a more favorable outcome. The presence of metastases in lymph nodes had a better outcome, but it decreased for higher N stages.

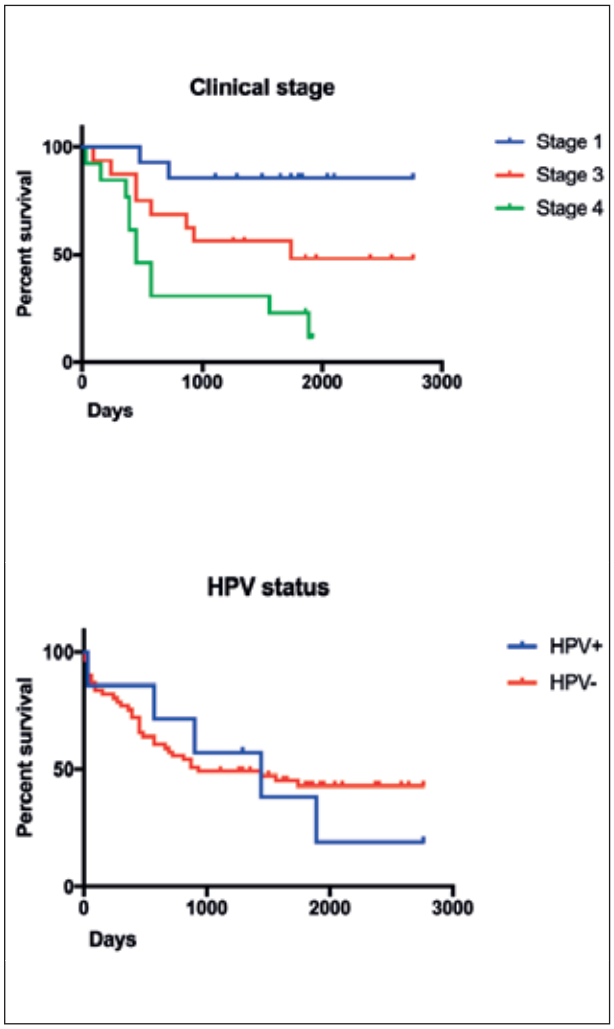

Figure 3a. *Overall survival of patients with OPSCC based on clinical stage and HPV status.*

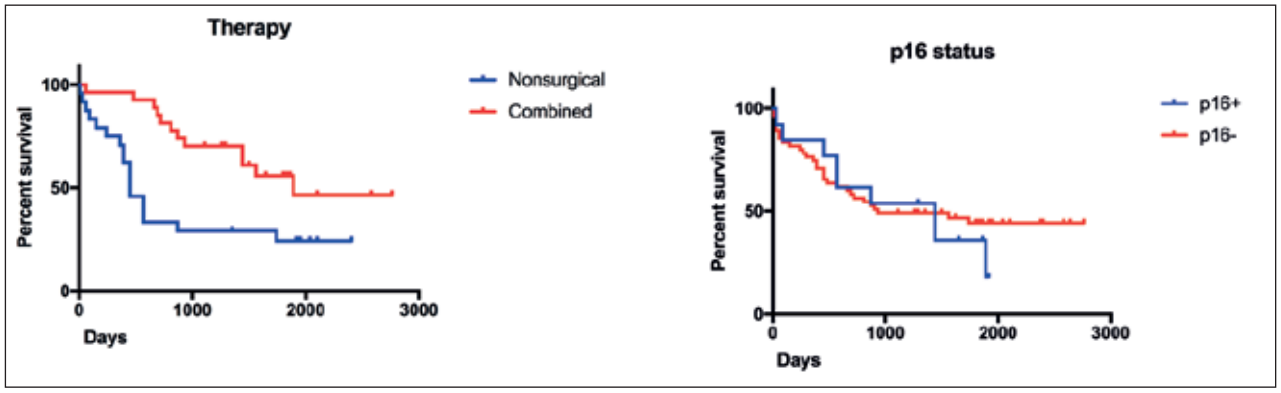

Figure 3b. *Overall survival of patients with OPSCC based on p16 status, and applied therapy presented in Kaplan-Meier curves.*

Table 4. *Univariate proportional hazard regression of patient survival for clinical and histologic features*

Variables	p	Hazard ratio	95.0% confidence interval		Overall significance
			Lower	Upper	
HPV status					0.230
Negative		1			
Positive	0.240	0.475	0.137	1.645	
p16 status					0.548
Negative		1			
Positive	0.550	0.746	0.286	1.948	
Sex					0.319
Male		1			
Female	0.329	2,070	0,480	8,926	
Age	0.103	1.035	0.993	1.079	0.100
T stage					0.038
T1		1			
T2	0.100	0.180	0.023	1.392	
T3	0.027	0.184	0.041	0.824	
T4	0.378	0.623	0.217	1.783	
N stage					0.039
N0		1			
N1	0.043	0.103	0.012	0.927	
N2	0.035	0.242	0.065	0.907	
N3	0.318	0.550	0.170	1.777	
M stage					
M0		1			0.016
M1	0.021	2.882	1.170	7.143	
Clinical stage					0.006
Stage 1		1			
Stage 2	0.003	0.096	0.021	0.447	
Stage 3	0.747	0.710	0.089	5.685	
Stage 4	0.148	0.493	0.189	1.285	
Therapy					0.001
Nonsurgical		1			
Combined	0.002	0.195	0.07	0.541	

Table 5. *Tumour histology characteristics*

Variables	HPV +	HPV -	p	p16 +	p16 -	p
Dysplasia of surface epithelium			>0.9999			0.6867
Present	7 (10.29)	49 (72.06)		10 (14.71)	46 (67.65)	
Not present	0	12 (17.65)		3 (4.41)	9 (13.24)	
Tumour architecture			0.4066			>0.9999
Irregular cords and nests	1 (1.47)	22 (32.35)		4 (5.88)	19 (27.94)	
Expanding lobules	6 (8.82)	37 (54.41)		9 (13.24)	34 (50)	
Cannot be determined	0	2 (2.94)		0	2 (2.94)	
Desmoplasia			0.7029			0.7527
Prominent	3 (4.41)	21 (30.88)		4 (5.88)	20 (30.88)	
Often absent	4 (5.88)	37 (54.41)		9 (13.24)	32 (47.06)	
Cannot be determined	0	3 (4.41)		0	3 (4.41)	
Keratinization			0.4093			0.2045
Prominent	4 (5.88)	21 (30.88)		7 (10.29)	18 (26.47)	
Minimal or absent	3 (4.41)	40 (58.82)		6 (8.82)	37 (54.41)	
Differentiation			0.0861			>0.9999
Moderately differentiated	7 (10.29)	38 (55.88)		4 (5.88)	36 (52.94)	
Basaloid/poorly differentiated	0	23 (33.82)		9 (13.24)	19 (27.94)	
Inflammation reaction			0.1972			0.0257
Weak	2 (2.94)	39 (57.35)		4 (5.88)	37 (54.41)	
Intermediate	2 (2.94)	10 (14.71)		5 (7.35)	7 (10.29)	
Strong	3 (4.41)	12 (17.65)		4 (5.88)	11 (16.18)	

Out of the histologic features, inflammation was the only variable that showed a statistically significant difference regarding p16 status. p16+ samples had a stronger inflammatory reaction than p16- tumors (p=0.0257, Fisher exact test).

DISCUSSION

The aim of this study was to assess the impact of HPV on OPSCC in southern Croatia, and to describe clinical and histologic features of OPSCC. In this study, 10.29% of patients were HPV positive, which is less than in North America and West Europe (3,14,15). Although western countries seem to have a higher burden of HPV, South European countries seem to report lower prevalence (4,16,21-26). In a recent northern Croatian study, Božinović *et al.* report on 29.3% of HPV positive cancer cases, showcasing a difference in distribution within Croatia (18). Northern Croatia seems to follow a trend that befits western countries, while southern Croatia would be categorized with other South European regions.

Human papillomavirus proved to be an important prognostic factor in the survival of our patients. Our multivariate Cox regression-hazard model showed a more favorable outcome in patients with HPV+ OPSCC. It is widely described that HPV is a favorable prognostic factor, and that patients with HPV+ OPSCC have a better therapy response than those with HPV- OPSCC (27), and therefore better survival (28-30). The reason why HPV is a positive prognostic factor is unclear. This might be due to the lack of p53 mutation in patients with HPV+ OPSCC (31), making them more radiosensitive and more sensitive to chemotherapy, resulting in better outcome (32,33).

Patients with HPV+ OPSCC are usually described as white men of a higher socioeconomic status and of a younger age than patients with HPV- OPSCC (10). Although our patients were predominantly men, we did not find any age differences between the two patient groups. Similar findings have been reported in studies in Europe (18,22,23,34). This indicates the impact that classical risk factors such as cigarette smoking and alcohol consumption have in our population.

In Croatia, smoking is an important factor in carcinogenesis because more than 25% of adults smoke tobacco products every day (35). In 2011, Bergman Marković *et al.* announced that a higher proportion of smokers are in coastal cities than in inland Croatia

(36). Alcohol consumption is another important factor in carcinogenesis. In 2009, Bencević-Striehl *et al*. reported that coastal Croatia was second in the prevalence of alcohol consumption in both sexes, immediately after Slavonia in men and northern Croatia in women (37). It is possible, therefore, that the lower percentage of HPV+ OPSCCs in the sample of our patients is inversely related to the higher rate of smokers and alcohol consumption in southern Croatia, which contributes to the development of HPV- OPSCC.

Generally, HPV+ OPSCC is histologically described as poorly differentiated squamous epithelial cells that do not keratinize, without desmoplastic stroma and surrounding epithelial dysplasia (12). In this study, we did not find a statistically significant association of these parameters of a typical histologic picture with HPV tumor status. Both studies from Slovenia and northern Croatia did not manage to find significant difference in regards to histologic grade in HPV+ and HPV- OPSCC (18,21). In 2018, Liu *et al*. concluded that the structure of HPV+ OPSCC in patients who were smokers was histologically more similar to HPV- OPSCC (38). This confirms that traditional risk factors were prevalent in both groups.

As expected, we found that HPV+ OPSCC was most commonly found on the palatine tonsils. It is believed that HPV+ OPSCC originates from the epithelium of tonsillar crypts, while HPV- OPSCC emerges from the surface epithelium (39). Similar to cervical transformation zones, the tonsils are characterized by deep invaginations of mucosal surface called crypts. These tonsillar crypts are lined by monolayered epithelium, showing similarities to mucosal basal keratinocytes (40). Deep tonsillar crypts facilitate the transfer of external antigens to lymphoid tissue and the presentation to antigen-presenting cells, which stimulates lymphocytic infiltration (41,42). Stronger lymphocyte infiltration we found in HPV+ OPSCC, which most often occurs in the tonsils, can be partly explained by the reaction of lymphatic cells to viral antigens.

CONCLUSION

In conclusion, traditional risk factors are the cause of OPSCC, as well as of many other cancers in our environment. Prophylactic vaccination against HPV is still not mandatory in Croatia, so in the future we can expect a natural increase in the number of HPV+ OPSCC in today's generation of young people, who are slowly adopting lifestyle behaviors as seen in western countries. Our study implies that histologic features of HPV+ OPSCC change towards that of HPV- OPSCC in populations where traditional risk factors are still the main cause of OPSCC. Although there are no significant differences between most of the histologic features, HPV+ status still has a more favorable outcome.

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S A Ž E T A K

KLINIČKI I PATOFIZIOLOŠKI PRIKAZ BOLESNIKA S OROFARINGEALNIM RAKOM PLOČASTIH STANICA POZITIVNIH NA HUMANI PAPILOMAVIRUS – ISTRAŽIVANJE U JUŽNOJ HRVATSKOJ

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Cilj: Svrha ovog istraživanja bila e analizirati utjecaj humanog papilomavirusa (HPV) na preživljavanje, kliničke pokazatelje i patohistološke značajke u ispitanika oboljelih od orofaringealnog raka pločastih stanica (OPSCC) u južnoj Hrvatskoj. ***Metode:*** Istražili smo prisutnost HPV DNK i imunohistokemijsko bojanje na p16 u 68 u parafinske blokove uklopljenih uzoraka tkiva ispitanika oboljelih od OPSCC-a i liječenih u Kliničkom bolničkom centru Split u razdoblju od 2013. do 2017. godine. Svjetlosnim mikroskopom utvrđene su histološke značajke tkiva. Retrospektivno smo prikupili kliničke podatke ispitanika i proučili ih s obzirom na HPV status. ***Rezultati:*** U ovom je istraživanju 10,29% pacijenata pozitivno na HPV (HPV+). Invazija limfocita značajnija je u ispitanika s p16 pozitivnim (p16+) OPSCC-om. Ukupno preživljavanje (OS) bolje je u HPV+ i p16+ ispitanika. HPV je značajan prognostički čimbenik u ispitanika koji boluju od OPSCC-a iz južne Hrvatske. ***Zaključak:*** Čini se da je HPV manje utjecajan uzročni čimbenik nastanka OPSCC-a u južnoj Hrvatskoj u usporedbi sa zapadnoeuropskim zemljama i SAD-om. Iako je HPV značajan čimbenik preživljavanja, tradicionalni čimbenici rizika pokazali su se važnijim karcinogenima za nastanak OPSCC-a u našoj populaciji.

Ključne riječi: Hrvatska, neoplazme glave i vrata, histologija, humani papilomavirus 16, orofaringealne neoplazme, planocelularni karcinom