



CARCINOMA OF THE LARYNX AND HUMAN PAPILLOMA VIRUS INFECTION

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ABSTRACT:

Background: Laryngeal carcinoma is one of the most common form of head and neck cancer. During the last two decades, it has been recognized that this cancer is causally related to human papillomavirus (HPV).

Objective: We presented a study on prevalence of human papilloma viruses (HPV) in patients with laryngeal carcinoma.

Methods: This study consists of 43 patients with laryngeal carcinoma who were diagnosed and treated with surgical techniques in Department of Otorhinolaryngology, University Hospital, Pleven, Bulgaria. Immunohistochemistry of p16INK4a and Ki-67 were used to prove the relationship between high-risk-HPV (HR-HPV) and carcinogenesis.

Results: Papilloma virus infection with high-risk oncogenic types of HPV was determined in more than 39.5% of surgically treated patients with histologically proven laryngeal cancer. HPV-induced carcinogenesis was assumed in 17 (13.9%) of all patients whose spouses were operated from cervical cancer. The patients with HPV-positive laryngeal carcinoma were younger than the others in the group (8 years on average). Risk factors for development of HPV-associated laryngeal carcinoma were related to higher number of sexual partners and the practice of oral sex. Frequently, in patients with HPV-associated laryngeal carcinoma we find data for so-called "family's carcinogenesis". The possibility of appearance (either preceding or following the treatment) of a second carcinoma and/or tumour recurrence is higher in HPV-positive laryngeal carcinomas.

Conclusion: It is recommended to extend the diagnostic methods for laryngeal and hypo pharyngeal cancer with a routine search for high-risk oncogenic HPV strains.

Key words: carcinoma, larynx, HPV, p16^{INK4a}, Ki-67

INTRODUCTION

About 200 various types of HPV have been identified. Due to their varied oncogenic potential the following types can be distinguished: high risk (HR) HPV -16, 18, 31, 33, 45 and low risks (LR) HPV - 6, 11 and others. Recently, many studies have focused on the relationship between HPV and laryngeal carcinoma [1, 2]. Cancer incidence in Bulgaria 2012 and distribution of the new cases show that there were registered 558 new cases with carcinoma of the larynx (incidence - 7.6 per 100 000 population, 1.7% from all cancers). Data show that men dominate - 521 (14.7 per 100 000 popu-

lation and 3.0% from all cancers). Aetiology of laryngeal carcinomas was multifactorial, and development consists of multistage cancer genesis preceded by dysplastic lesions of the mucous membrane. Classic risk factors for HPV-associated laryngeal carcinoma such as tobacco smoking and alcohol abuse were duly documented. Additional risk factors include sexual habits, a lowered age of sexual initiation, a high number of sexual partners, lack of condom use, oral sex. The contribution of HPV in the cancer genesis of cancers of the oral cavity and oropharynx was for the first time suggested by Zur Hausen in 1999 and confirmed by research groups later on. The HPV rate observed in cancers of the larynx is higher (up to 23%). HPV 16 sub type is the most frequently detected viral type [2, 3, 4].

Identification of HR HPV DNA as a diagnostic protocol includes molecular diagnostic tests for HPV. After a classic histological examination it is recommended to supplement with tests, confirming HPV infection. In practice there were applied *polymerase chain reaction* (PCR) and *reverse transcriptase PCR* (RT-PCR). Thanks to RT-PCR it is possible to determine the level of viral expression. *In situ hybridisation* (ISH) is additional method that allowing for analysis of viral transcripts directly in the examined tissue. An *immunohistochemical assay* for the p16^{INK4A} expression is a method of a direct analysis of malignant transformation associated with HPV in a cell. A number of authors have recommended for the evaluation of HPV in a tumour tissue the following: immunohistochemical (IH) detection of p16^{INK4A}, in situ hybridisation (ISH) and PCR [5, 6]. The objectives of this study were to investigate prevalence of human papilloma viruses and frequency of strains, causing laryngeal carcinoma.

MATERIAL AND METHODS

This study consisted of 43 (39 male/4 female) patients with laryngeal carcinoma who were diagnosed and treated with surgical techniques in Department of Otorhinolaryngology, University Hospital-Pleven, Bulgaria for the period 2012-2013. Patients were involved in the study after obtaining informed consent. Socio-demographic information about the life style of patients was collected by interview. Immunohistochemistry of p16^{INK4a} and Ki-67 were used to prove the relationship between HR-HPV and carcinogenesis. As a control group were used entities (n=15) with chronic pharyngitis. All resection specimens were fixed in 10% formalin and routinely processed for paraffin em-

bedding. Four micrometer thick tissue sections were done. They were deparaffinized and rehydrated by ethyl alcohol and treated by 3% hydrogen peroxide solution for 30 min at room temperature. Following buffering by citrate buffer (pH-6) was done for 5 min. Tissue sections were immunostained applying the antibody p16^{INK4a} (CIN tec Histology kit, DAKO) in a dilution 1:50. Vision Flex system was used. Ki-67 positivity was investigated with Flex Monoclonal Ki-67 Antigen, Clone MIB-1, DAKO. The Ki-67 antigen is a large nuclear protein (345, 395 kDa) preferentially expressed during all active phases of the cell cycle. Areas (unaffected by carcinoma tissue) were used as inner negative control. The expression of p16^{INK4a} was assessed in the cell nuclei and evaluated using a five-point grading system. Less than 10% tumor cells positive was scored 0, 10% to 25% scored as 1+, 25% to 50% scored as 2+, 50% to 75% scored as 3+, and more than 75% scored as 4+. At least 500 cells per high-power (objective lens×40) field and three fields were observed, and grades of 3+ and 4+ were regarded as over expression. There was performed a software package for the statistical analysis.

RESULTS

The socio-demographical characteristics of the popu-

lation participating in the study were summarized in Table 1. There were 39 men and 4 women, with average age of 56 years old (ranging within 27-79 years old). HPV-associated laryngeal carcinomas are proved to be predominately a male disease. The statistically significant difference was found to be - R = 0,39 (p = 0,009). The immunohistochemical findings are shown in Table 2. Papilloma virus infection with high-risk oncogenic types of HPV were determined in 17 (39.5%) of surgically treated patients with histologically proven laryngeal cancer, p16^{INK4a} being positive. 14 (82.4%) of them were male and 3 (17.6%) were female. HPV-induced carcinogenesis was assumed in 6 (13.9%) of all patients whose spouses were operated from cervical cancer. In the control group n=15 no patients with p16^{INK4a} positive immunohistochemical staining agent. Statistical analysis of the results reveals significant difference R= - 0,34 (δ=0,01). HPV-associated laryngeal carcinomas in the study group are the most common version of a well-differentiated carcinoma with keratinization. Secondly, is moderately differentiated squamous cell carcinoma (table 3). Patients with HPV associated laryngeal carcinoma are grouped mostly in the age group 50-59 and HPV negative in the age group 60-69 and 70-79 (figure 1).

Table1. Socio-demographical characteristics

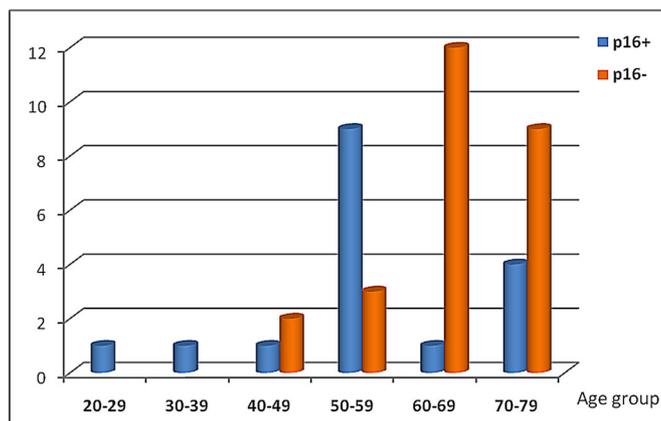
	Number, %		Number, %
Patient saverage age (in years)	43 56 (from 27 to 79)	Residence	
		Cities	24 / 56
		Villages	19 / 44
Gender		Smoking	
Males	39 / 9	Yes	33 / 76.7
Females	14 / 9	No	10 / 23.3
Marital Status		Alcohol abuse	
Single	18 / 58	Yes	36 / 83.7
Married	25 / 42	No	7 / 16.3
Family carcinogenesis		Sexual partners	
Husband / wife	6 / 13.9	To five	9 / 20.9
No	37 / 86.1	Over	34 / 79.1
Family carcinogenesis		Oral sex	
Mother, father, brothers and sisters	10 / 23.3	Yes	22 / 51.2
No	33 / 76.7	No	21 / 48.8

Table 2. p16^{INK4a} expression and presence of Ki-67

	Male (number, %)	Female (number, %)	Total	Control group
p16^{INK4a} expression				
Positive	14 / 82.4	3 / 17.6	17 / 39.5	
Negative	25 / 96.2	1 / 3.8	26 / 60.5	15 / 100
presence of Ki-67				
Positive	15 / 83.3	3 / 16.6	18 / 41.9	
Negative	24 / 96.0	1 / 3.8	25 / 58.1	15 / 100

Table 3. Histological finding and p16^{INK4a} expression

	p16 ^{INK4a} positive n, %	p16 ^{INK4a} negative n, %
Well differentiated keratinizing squamous cell carcinoma n=28	14	14
Poorly differentiated squamous carcinoma n=12	3	9
Moderately differentiated n=2	0	2
Verrucous n=1	0	1

Fig. 1. Age distribution of patients depending on the p16^{INK4a} expression

DISCUSSION

Recently, many studies have focused on the relationship between HPV and laryngeal carcinoma. In the present study, we found that HPV infection is more frequent in younger patients than elderly patients (8 years averagely). Cases of laryngeal cancer were registered in a wide age range. The literature is increasingly reported that those affected are young people of working age, no smoking and no alcohol abusers [7]. Gender ratio was 10/1 in favor of men. A predominance is established among urban residents. Patients with laryngeal carcinoma associated virus 11 (25.6%) were urban residents ($p > 0.05$). 25 (42%) of the respondents were married. It is established that there is not dependency between marital status and HPV positivity ($p > 0.05$). In the studied group of patients smokers are prevailing - 40 (93%) and most consuming alcohol. No statistically significant difference of HPV-associated laryngeal carcinoma with alcohol and smoking ($p > 0.05$). Important factors for development of HPV-associated laryngeal carcinoma were the higher number of sexual partners and the practice of oral sex. Frequently in patients with HPV-associated laryngeal carcinoma we find data for so-called "family's carcinogenesis". The results showed that HPV infection was correlated with increased expression of p16^{INK4a}. The study of p16^{INK4a} biomarker was useful in the diagnosis of HPV associated laryngeal cancers. The authors postulate the so-called "Gold standard" in research of carcinomas of

the head and neck. It must present prominently p16^{INK4a} study [8]. The purpose of that investigation tool is to demonstrate transcriptional activity of oncogenes virus. Moreover, although PCR is more sensitive than other approaches, as total DNA are extracted from tissue samples, so the detected HPV DNA can be derived from non-cancerous cells, a tumor surface contamination or just only few cancer cells. Compared with PCR method, ISH can identify the HPV infection in the tumor cell nuclei, which could provide more reliable results [9].

HPV infection is considered as an indicator of better prognosis in patients with laryngeal carcinoma in many studies. Number of authors reported that HPV positive cases have higher survival rates than HPV negative cases. However, there are still controversial and contradictory results [6, 10, 11].

CONCLUSION

HPV-associated cancers have different biological parameters which are associated with a profile of the p16 expression. They are well differentiated in a histological examination. The average age of patients with HPV-associated cancers is significantly lower and the contribution of addictions that are considered to be classical risk factors, namely smoking and alcohol abuse is significantly lower or negative; and sexual habits are different: a higher number of sexual partners and oral sex.

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