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ORIGINAL PAPER

A Survey of Human Papillomavirus and Epstein-Barr virus Immunohistochemical Status in Patients with Head and Neck Squamous Cell Carcinoma (HNSCC)

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Abstract

Background Head and neck cancers are among the most prevalent cancers in the body. The aim of this study was to evaluate the expression of P16 and Epstein-Barr virus/latent membrane protein (EBV/LMP1) markers by immunohistochemistry in patients with squamous cell carcinoma of the head and neck.

Methods In this study, all tissue samples of head and neck biopsies from 75 patients with confirmed diagnosis of squamous cell carcinoma (HNSCC) during 2016 to 2018 who admitted to the pathology laboratory of Imam Khomeini Hospital, Iran were selected. Paraffin blocks which prepared from these tissue samples were obtained. The slides were prepared from all samples for routine Hematoxylin-Eosin and immunohistochemical staining to evaluate the expression of EBV/LMP1 and P16 markers in cancer cells.

Results The mean age of patients was 63 years and most patients (85.3%) were male in 75 patients with HNSCC. There was a significant relationship between EBV/LMP1 biomarker expression and vascular invasion in patients ($p < 0.05$). There was no relationship between EBV/LMP1 biomarker expression and age, sex ,anatomical site of tumor and tumor differentiation of patients ($p > 0.05$). There was no relationship between P16 biomarker expression and age, sex, tumor differentiation, anatomical site of tumor and vascular invasion of patients ($p > 0.05$). There is a significant relationship between P16 biomarker and EBV/LMP1 biomarker staining ($p < 0.05$).

Conclusion The level of P16 positive biomarker was high in patients with HNSCC. However, the EBV/LMP1 positive biomarker was moderate in patients. There was a relationship between EBV/LMP1 biomarker expression and vascular invasion in HNSCC patients, as well as between P16 biomarker and EBV/LMP1 biomarker staining.

Keywords Head and neck squamous cell carcinoma · immunohistochemistry · P16 · EBV/LMP1

Introduction

Head and neck cancers are prevalent malignancies associated with high morbidity and mortality, and most cases of these cancers are squamous cell carcinomas of the head and neck (HNSCC) [1]. HNSCC accounts for approximately 5% of all cancers and is a serious public health problem worldwide [2]. Classification based on anatomical location and tumor stage may not indicate a high level of biological heterogeneity, and appropriate clinical diagnosis remains a major challenge [1]. In all anatomical subtypes, men with a 2:1 to 4:1 ratio are more likely to be diagnosed than women with HNSCC [3, 4]. The most common risk factors for HNSCC comprise smoking, alcohol consumption, and human papillomavirus (HPV) infection [5, 6]. According

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Table 1 Calculation of Allred score for EBV/LMP1 marker

Proportion of positive staining score		Staining intensity score	
PS	Range	IS	Type
0	0	0	No staining
1	< 1	1+	Weak positive staining
2	1–10	2+	Moderate positive staining
3	11–33	3+	Strong positive staining
4	34–66	Allred score = PS + IS	
5	67–100		

Abbreviations:

PS, Proportion Score; IS, Intensity Score

to epidemiological studies, 12.8 to 59.9% of HNSCCs are related to HPV infection [7].

Epstein-Barr virus (EBV) has long been associated with nasopharyngeal carcinoma [8] and a recent meta-analysis showed a significant association between EBV and HNSCC [9]. However, the possible role of EBV and other herpes viruses in HPV-related oral cancers is not yet well understood and most studies were based on HPV-DNA markers and failed to detect P16 expression, which is a substitute for oncogenic HPV infection [10]. Among EBV latent molecules, latent membrane protein 1 (LMP1) is the major oncogene of EBV because of its ability to utilize a set of cellular genes and inhibit apoptosis by increasing Bcl-2 levels [11, 12]. P16 overexpression by immunohistochemistry (IHC) is an outstanding surrogate marker of HPV association in oropharyngeal SCC [13] and is well established as a prognostic biomarker of favorable outcome in HNSCC [14]. Herein, we designed an immunohistochemical study to evaluate P16 and EBV/LMP1 expressions in patients with HNSCC.

Materials and Methods

Design and Study Sample

This cross-sectional study used tissue samples from 75 patients with HNSCC during 2016 to 2018. Patients admitted to the pathology laboratory of Imam Khomeini Hospital, Iran with confirmed HNSCC diagnosis were selected. All samples that met the inclusion criteria (diagnosis of HNSCC pathology and having sufficient information in the patient's hospital record) were included in the study. Clinicopathological features of the tumor (including age and sex of the patients, tumor differentiation, vascular invasion, P16 expression, EBV/LMP1 staining intensity, proportion of EBV/LMP1 staining score, Allred score for EBV/LMP1 marker in tumoral tissues (SCC) [15], EBV/LMP1 expression in lymphocytes and anatomical site of tumor were extracted and recorded. Allred score was calculated as presented in Table 1.

Method

We made 3 to 4 micron cuts on the paraffin block and placed it on the adhesive slide. Healthy tonsillar mucosa tissue was used in tonsillectomy specimens for P16 control and Hodgkin's lymphoma as LMP1 control. The slides were placed in an oven at 65 ° C for 24 h to remove paraffin and then watered in xylene, absolute ethanol, and 96% ethanol, respectively. Then, we washed the slides in running water and placed them in jars containing Tris with pH = 9 and put them in an autoclave for 15 min. The slides were completely cooled down to room temperature and rinse again under running water. We washed in Tris-buffered saline (TBS) buffer with pH = 7.6. We then placed it in jars containing oxygenated water and methanol in a dark room. We washed them again in theTBS buffer and left them in the TBS buffer at 4 ° C for 24 h. Then, we removed the extra buffer from the surface of the remaining slides and marked the texture range with a Daco pen. After that, we placed the tissues in a damp, dark room and covered the surface of the tissues with P16 and EBV/LMP1 antibodies for 60 min at room temperature.

The slides were then washed in the first jar of the first TBS buffer and placed in the second jar of the first TBS buffer for 10 min. We then placed the tissues in a damp, dark chamber and added the peroxide-labeled polymer for 30 to 35 min. Then, we washed the slides in the first round of the second TBS buffer and left them in the second round of the second TBS buffer for 10 min. It was placed the tissues in a damp chamber and surface the tissues was located in a solution of chromogen and a substrate (diamino benzidine) for 15 min. The tissues were rinsed in TBS buffer for 5 min and then in the running water. They were stained with hematoxylin for 1 min and washed again under running water and after that, immersed in lithium carbonate solution for 5 min and rinse again under running water. We put them in two containers of ethanol 96, two containers of absolute ethanol and one container of xylene each for 5 min and finally covered with lamel. After the above process, the slides were studied under a light microscope. The slides were read by an assistant and a pathologist. Some tissue blocks were not of good quality that either the blocks were melted and molded again or if the sample in the block was small, it was removed from the study and replaced with a new block. EBV/LMP1 and P16 expressions are shown in Fig. 1. We showed positive P16 staining as positive G-I (Faint or Focal), positive G-II (Moderate Diffuse), positive G-III (Intense and Diffuse). For EBV/LMP1 staining, we included intensity score that is equivalent to staining intensity (no staining = 0, weak positive staining = 1, moderate positive staining = 2, severe positive staining = 3) and proportion score represented the percentage of stained cells (no staining = 0, less than 1/100 cells = 1, 1/10 – 1/100 cells = 2, 1/10 – 1/3 cells = 3, 1/3 – 2/3

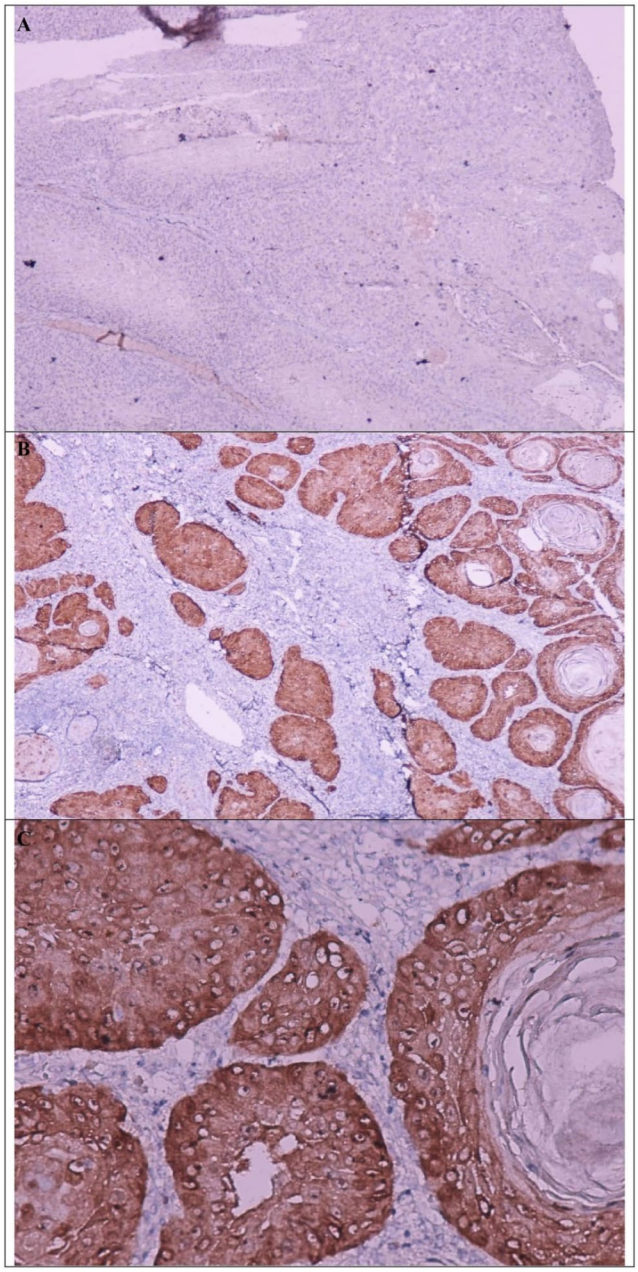

Fig. 1 P16 expression based on immunohistochemical staining: A) Negative (×50), B) Positive (×100), and C) Positive (×200)

cells = 4, more than 2/3 cells = 5). For EBV/LMP1 staining, we calculated "Allred Score = Proportion Score + Intensity Score" that Allred score is a score between 0 and 8. Allred scores are considered between 0 and 2 as negative and more or equal to 3 as positive.

Statistical Analysis

After collecting all the data, the data were analyzed using SPSS 22.0 software. Descriptive statistics (frequencies, percentages) were used to present the results. Chi-square test was used to determine the relationship between marker incidence and nominal qualitative variables.

Results

In this study, 75 patients with HNSCC were studied (Table 2). The age of patients was between 28 and 92 years with a mean of 63.2 years. According to the results of this study, most patients (85.3%) were male. Tumor was well-differentiated in 44% of patients. Vascular invasion was positive in 37.3% of patients. P16 biomarker was positive in 74.7% of patients and EBV/LMP1 biomarker in 57.3% of patients. On the basis of TNM8 system anatomical sites of tumor were divided into 6 groups; from the lip and oral cavity, the pharynx (including oropharynx, nasopharynx and hypopharynx), the larynx, the nasal cavity and paranasal sinuses (maxillary and ethmoid sinus), the unknown primary carcinoma (cervical nodes) and the major salivary glands.

Approximately, There were significant associations of EBV/LMP1 expression with vascular invasion ($p = 0.015$) and P16 expression ($p = 0.034$) that fewer patients with vascular invasion presented positive EBV/LMP1 expression (25.6%) than those no vascular invasion (74.4%) (Table 3) and also more patients with positive P16 expression presented positive EBV/LMP1 expression (65.1%) than those negative P16 expression (34.9%). Therefore, there was the association of vascular invasion and P16 expression with EBV/LMP1 expression ($p < 0.05$). There was no significant relationship between EBV/LMP1 expression and age, sex, tumor differentiation of patients and anatomical site of tumor. By anatomical location, 80.9% (17/21) of the oral cavity, 80% (4/5) of the pharyngeal ,68.4% (26/38) of laryngeal, 75% (3/4) of nasal cavity and paranasal sinuses, 100% (3/3) of major salivary glands and 75% (3/4) of unknown primary carcinoma (cervical nodes) were positive for P16 expression .In addition, there was no significant relationship between P16 expression and age, sex, tumor differentiation, vascular invasion of patients and anatomical site of tumor (Table 4).

Discussion

EBV/LMP1 expression was associated with vascular invasion and P16 expression among HNSCC patients while EBV/LMP1 expression was not associated with age, sex, and tumor differentiation of patients. In addition, P16 expression was also found not to be associated with age, sex, tumor differentiation, anatomical site of tumor and vascular invasion of patients. However, the highest P16 expression

Table 2 Characteristics of the patients

Variable	
Age, years	
Mean ± standard deviation	63.2 ± 13.66
<60	40 (53.3)
≥60	35 (46.7)
Sex	
Male	64 (85.3)
Female	11 (14.7)
Tumor differentiation	
Well-differentiated	33 (44)
Moderately differentiated	30 (40)
Poorly differentiated	12 (16)
Vascular invasion	
Positive	28 (37.3)
Negative	47 (62.7)
P16 expression	
Negative	19 (25.3)
Positive (Grade I)	17 (22.7)
Positive (Grade II)	17 (22.7)
Positive (Grade III)	22 (29.3)
EBV/LMP1 staining intensity	
Negative	26 (34.7)
Weak	22 (29.3)
Moderate	23 (30.7)
Severe	4 (5.3)
Proportion of EBV/LMP1 staining score	
Negative	26 (34.7)
Positive (< 1%)	13 (17.3)
Positive (1–10%)	15 (20)
Positive (11–33%)	13 (17.3)
Positive (34–66%)	7 (9.4)
Positive (67–100%)	1 (1.3)
Allred Score	
Negative (0–2)	32 (42.7)
Positive (≥ 3)	43 (57.3)
EBV/LMP1 expression in lymphocytes	
Negative	43 (57.3)
Positive (5%)	14 (18.7)
Positive (10%)	9 (12)
Positive (20%)	7 (9.3)
Positive (30%)	2 (2.7)

For P16 staining: Negative, No Staining; Positive G-I, Faint or Focal; Positive G-II, Moderate Diffuse; Positive G-III, Intense and Diffuse. For EBV/LMP1 staining: Intensity Score is equivalent to staining intensity (no staining = 0, weak positive staining = 1, moderate positive staining = 2, severe positive staining = 3) and proportion score represents the percentage of stained cells (no staining = 0, less than 1/100 cells = 1, 1/10 – 1/100 cells = 2, 1/10 – 1/3 cells = 3, 1/3 – 2/3 cells = 4, more than 2/3 cells = 5). Allred Score = Proportion Score + Intensity Score. Allred score is a score between 0 and 8. Allred scores are considered between 0–2 as negative and more or equal to 3 as positive

Table 3 Correlation between EBV/LMP1 expressions based on Allred score and the variables

Variable	EBV/LMP1 expression, N (%)		*p*-value
	Positive (43)	Negative (32)	
Age, years			0.618
<60	24 (55.8)	16 (50)	
≥60	19 (44.2)	16 (50)	
Sex			0.513
Male	5 (11.6)	6 (18.7)	
Female	38 (88.4)	26 (81.3)	
Tumor differentiation			0.107
Well-differentiated	21 (48.8)	12 (37.5)	
Moderately differentiated	13 (30.3)	17 (53.1)	
Poorly differentiated	9 (20.9)	3 (9.4)	
Vascular invasion			**0.015**
Yes	11 (25.6)	17 (53.1)	
No	32 (74.4)	15 (46.9)	
P16 expression			**0.034**
Positive	28 (65.1)	28 (87.5)	
Negative	15 (34.9)	4 (12.5)	
Anatomical sites			0.445
-Lip and Oral cavity	12(57.2)	9(42.8)	
-Pharynx	3(60)	2(40)	
-Larynx	18(47.4)	20(52.6)	
-Nasal cavity and paranasal sinuses	4(100)	0(0)	
-Unknown primary carcinoma (cervical nodes)	3(75)	1(25)	
-Major salivary glands	2(66.7)	1(33.3)	

was seen in the salivary glands ,oral cavity and pharyngeal cancers ,respectively, compared to the larynx and the nasal cavity.

Based on our knowledge, this is the first study to examine the concomitant association of EBV and HPV using tissue samples from patients with HNSCC. Although novel, lack of studies in this area limits the possibility of comparing our results with the existing literature. However, in some previous studies, EBV and P16 expressions have been investigated in patients with HNSCC [16–19]. Another study [18] evaluated 75 HNSCC tissue samples for the expression of P16 marker by IHC and they found P16 marker to be positive in 78.7% of the samples. HPV integration with viral oncoprotein transcription induces overexpression of P16, and therefore immunohistochemical expression of P16 could be used as an HPV marker. Turunen et al. [16] found the incidence of EBV in the tissue samples of HPV-positive HNSCC patients to be 21% in cancer cells, 41% in lymphocytes, and 15% in both groups of cells. The results showed that HPV/EBV synchronicity was associated with survival shortening. In our study, P16 biomarker was positive in 74.7% of patients and EBV/LMP1 biomarker in 57.3% of cancer cells and 42.7% in lymphocytes.

Heiduschka et al. [17] found that the expression of the P16 marker occurred in 92% of all HNSCC patient samples. It was finally concluded that P16 positivity in patients with HNSCC who received radiotherapy and cetuximab was associated with a favorable prognosis. Another study [19] reported that the incidence of P16 in women was higher

Table 4 Correlation between P16 expression and the variables

Variable	P16 expression, N (%)		*p*-value
	Positive (56)	Negative (19)	
Age, years			0.943
<60	30 (53.6)	10 (52.6)	
≥60	26 (46.4)	9 (47.4)	
Sex			0.567
Male	8 (14.3)	3 (15.8)	
Female	48 (85.7)	16 (84.2)	
Tumor differentiation			0.360
Well-differentiated	22 (39.3)	11 (57.9)	
Moderately differentiated	24 (42.8)	6 (13.6)	
Poorly differentiated	10 (17.9)	2 (10.5)	
Vascular invasion			0.075
Yes	24 (42.9)	4 (21.1)	
No	32 (57.1)	15 (78.9)	
Anatomical sites			0.904
-Lip and Oral cavity	17(80.9)	4(19.1)	
-Pharynx	4(80)	1(20)	
-Larynx	26(68.4)	12(31.6)	
-Nasal cavity and paranasal sinuses	3(75)	1(25)	
-Unknown primary carcinoma (cervical nodes)	3(75)	1(25)	
-Major salivary glands	3(100)	0(0)	

than men (47.1% and 27.6%) in oropharyngeal SCC tissue samples. HPV-related HNSCC also showed better prognosis than non-HPV-related HNSCC.

A review [20] showed that EBV has a complex role in the pathogenesis of nasopharyngeal cancer. Viral proteins, particularly LMP1, LMP2, and EBNA1, are involved in modulating key factors involved in malignant conversion. LMP1 expression in EBV-related cancers is associated with the regulation of tumor cell proliferation, immortality, invasion, and angiogenesis [21, 22]. Chew et al. [23] showed the role of EBV/ LMP1 in the promotion of invasion and metastasis in nasopharyngeal cancer. In line with this result, our study showed a significant correlation between EBV/ LMP1 expression and vascular invasion in HNSCC cases.

A research [24] reported that overexpression of P16 is related to improved progression-free survival and locoregional control in EBV-positive nasopharyngeal cancer patients and P16 expression may complement EBV status in predicting treatment outcomes for nasopharyngeal cancer cases. Our study confirmed this result that there was a significant correlation between EBV and P16 expressions in HNSCC patients.

The most important limitations of this study were small sample size, not evaluating expression all types of tissues, no comparison with healthy tissues.

Conclusion

P16 positivity was 74.7% in patients with HNSCC while EBV/LMP1 positivity was 57.3% in the patients. EBV/ LMP1 biomarker expression was associated with vascular invasion and P16 expression in HNSCC patients. Therefore, P16 expression probably can complement EBV/LMP1 status in predicting treatment outcomes for HNSCC patients. Future prospective studies on larger sample sizes should evaluate the association of the expression of these markers with patient survival outcomes.

Authors' contributions Conceptualization: E.J.; Methodology: E.J.; Formal analysis and investigation: J.A. and A.M.Y.; Writing—original draft preparation: J.A.; Writing—review and editing: E.J., M.S., A.M.Y., R.C.L.; Funding acquisition: E.J.; Resources: A.M.Y.; Supervision: E.J. All authors have read and agreed to the published version of the manuscript.

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Data Availability The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Code Availability Not applicable.

Declarations

Competing Interests The other authors declare that they have no competing interest.

Ethics Approval The study was approved by the Ethical Committee of the Kermanshah University of Medical Sciences (registration code: IR.KUMS.REC.1398.089). All tissue samples were obtained based on ethical principles. Patient identity was kept confidential and data analysis was performed on all subjects. All methods were performed in accordance with the relevant guidelines and regulations.

Consent to Participate Written informed consent was obtained from patients or their guardian before starting the study.

Consent for Publication Not applicable.

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