original article

Association between human herpesviruses and head and neck squamous cell carcinoma: a molecular perspective

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BACKGROUND: Head and neck cancer (HNC) is the seventh most common malignant tumor. Herpesviruses are a significant risk factor in the multifactorial pathogenesis of HNC.

OBJECTIVES: This study aimed to investigate the association between herpesviruses and the development of head and neck squamous cell carcinoma (HN-SCC).

DESIGN: Experimental study

SETTING: A university hospital in Turkey

PATIENTS AND METHODS: Pathological archive tissue samples of 500 patients were included in the study. These samples were categorized into two groups: those diagnosed with HN-SCC (n=300, malignant group [MG]) and those diagnosed with benign head and neck lesions (n=200, benign group [BG]). The presence of herpesvirus in samples was detected using polymerase chain reaction.

MAIN OUTCOME MEASURES: Association of herpesviruses in the development of head and neck cancer.

SAMPLE SIZE: 500 patients

RESULTS: HHV-1, -2, -7, and -8 were not detected in any samples. In the malignant group (MG), EBV-DNA was detected in 1 patient (0.3%) and HHV-6 DNA in 2 patients (0.6%), while in the benign group (BG), VZV-DNA was detected in 1 patient (0.5%), EBV-DNA in 3 patients (1.5%), CMV-DNA in 5 patients (2.5%), and HHV-6 DNA in 3 patients (1.5%). While no significant difference was found between the groups for VZV, EBV, and HHV-6, a statistically significant difference was found in favor of the benign group for CMV.

CONCLUSION: Although herpesvirus seroprevalence is relatively high in the population, the lack of viral genome in tissue samples indicates that other factors might be prominent in developing HN-SCC.

LIMITATION: The storage conditions of the sample used (paraffinized sample) may have negatively affected the detection frequency of HHVs.

CONFLICT OF INTEREST: None.

Head and neck cancer (HNC) ranks as seventh most common malignant tumor worldwide.[1] HNC can affect various parts of the head and neck, such as the paranasal sinuses, oropharynx, lips, larynx, salivary glands, and thyroid gland. Over 90% of HNCs originate from squamous epithelium and are known as "squamous cell carcinomas of the head and neck"(HN-SCC).[2] Recently, there has been an increase in early-age onset HNC, linked to factors like tobacco and alcohol use, dietary changes, and viral infections.[2,3] Research has increasingly focused on the role of oncogenic viruses in HN-SCC development.[2,3] Human papillomavirus (HPV) and human herpesviruses (HHV) are the most studied viruses in relation to HNC.[3] HPV DNA integrates into host cells, leading to overexpression of early oncogenes E6 and E7, inactivating p53 and Rb tumor suppressor proteins, initiating carcinogenesis.[3] Studies have demonstrated a significant rise in oropharyngeal squamous cell carcinoma.[3,4] HHV's ability to escape the immune response increases the possibility of long-lasting latent infections.[2] Epstein-Barr virus (EBV) and HHV-8 are the most significant oncogenic viruses associated with various cancers.5,6 Other HHVs, like herpes simplex viruses -1 and -2 (HSV-1, HSV-2), varicella zoster virus (VZV), cytomegalovirus (CMV), HHV-6 and -7 have been linked to malignancy, yet their exact role in cancer development remains unclear.[7-9] This study aimed to investigate the presence of HHVs in archival tissue samples from 300 patients (malignant group, MG) with HN-SCC and 200 patients (benign group, BG) with benign oral cavity and oropharynx diseases and evaluate its potential association in HNC development.

MATERIALS AND METHODS

Classification of malignant and benign groups

Tissue samples from a total of 500 patients at Ankara University Faculty of Medicine, Ibn-i Sina Hospital, Turkey, between 2000 and 2016 were archived and included in the study. These samples were categorized into two groups: those diagnosed with HN-SCC (n=300, MG) and those diagnosed with benign head and neck benign lesions (n=200, BG).Ethical approval was obtained from the Ethical Committee of Ankara University on June 17, 2021(no: İ6-404-21). The MG of HN-SCC consisted of 153 cases involving larynx, 122 cases of oral cavity, and 25 cases of oropharynx tumor samples. The BG consisted of 121 cases of chronic tonsillitis, 30 cases of squamous epithelial hyperplasia, 11 lichenoid mucositis, 10 chronic inflammatory mucosa, 8 squamous papilloma, and 20 cases with other diagnoses (benign lesions of various tissues such as oral mucosa, tongue, and gingiva). The hospital information management system provided the patients' demographic characteristics and clinical data.

Inclusion and exclusion criteria for the samples

Paraffin blocks of the oral cavity, oropharynx, and supraglottic larynx cancer cases excluding the lip, which were operated on in Ankara University Faculty of Medicine, Ibn-i Sina Hospital between 2000 and 2016, and paraffin blocks of mouth/oropharynx/supraglottic larynx biopsy/surgical specimens such as benign oral cavity tumors, tonsillectomy, etc. were included in the study. Lip cancer cases were excluded.

Obtaining DNA from tissue samples

The study used archival formalin-fixed paraffin-embedded (FFPE) tissues from surgical resection specimens. All tissue samples were deparaffinized with a deparaffinization solution, Cat. No/ID: 19093 (Qiagen,Germany) following the manufacturer's recommendations, DNA was then extracted from the samples using a commercial extraction kit (QIAamp DNA FFPE Tissue Kit, Qiagen,Germany). The bands of the total DNA extraction were visualized using gel electrophoresis and detected under U.V. transillumination (Vilber-Lourmat TFX–20.M [ETX–20 M],France).

Viral DNA detection

The presence of HSV-1, HSV-2, EBV, VZV, CMV, HHV-6, HHV-7,10 and HHV-8 was investigated using polymerase chain reaction (PCR) methods as described with minor modifications.[11] These modifications included the addition of 1% bovine serum albumin (BSA) to the PCR mixture and implementation of a "touchdown PCR" protocol for certain amplifications. A typical PCR process involves initial denaturation, denaturation-annealing-extension cycles, and a final extension step. Various PCR methods such as monoplex, multiplex, and nested PCR were utilized in this study.[12] Details including amplicon size, primer sequences, PCR mixture composition, and PCR conditions are provided in **Table 1**. Amplicons were analyzed through 1.5% agarose gel electrophoresis using Ultra Gel Red Nucleic Acid Stain (10000x) (Vazyme,China) for staining. They were then visualized under a UV transilluminator (Vilber-Lourmat TFX-20.M [ETX-20 M],France) at 312 nm.

Statistical analysis

Statistical analysis was conducted using SPSS version 22.0 (SPSS Inc.,Chicago, IL,USA). Descriptive analyses

Table 1. Primer sequences and amplicon sizes used in the study.

Targeted viruses	Type of PCR	Primer	Primer Sequence (5'-3')	Base pair (bp)	PCR conditions	PCR mixture
HSV-1,2	Multiplex	HSVs-F	AAGCTGCTGGTGTCCGAG	272	Initial denaturation : 95°C 5 min ⬇ Denaturation: 94°C 30 sn Annealing: 60-55°C (touch-down) 60 sec Extension: 72°C 60 sec (38 cycles) ⬇ Final extension: 72°C 10 min	dH_2O: 5.8 µl Master mix (Qiagen, Germany): 12.5 µl HSVs-F: 1 µl of 10 Mm HSVs-R: 1 µl of 10 Mm EB-F: 1 µl of 10 Mm EB-R: 1 µl of 10 Mm BSA (1%): 0.2 µl DNA: 2.5 µl Total volume: 25 µl
		HSVs-R	ATTCGACGAGTTTCCTCCG			
HHV-4 (EBV)		EB-F	AAGACGTACTGGATGCGGTC	210		
		EB-R	TTTTGCCCGTCATTGACAT			
HHV-3 (VZV)	Monoplex	VZV-F	TCCGACATGCAGTCAATTTCAACGTC	161	Initial denaturation: 95°C 10 min ⬇ Denaturation: 94°C 30 sec Annealing: 70-60°C (touch-down) 30 sec Extension: 72°C 60 sec (45 cycles) ⬇ Final extension: 72°C 5 min	dH_2O: 8.8 µl Master mix (Qiagen, Germany): 12.5 µl VZV-F: 0.5 µl of 10 Mm VZV-R: 0.5 µl of 10 Mm BSA (1%): 0,2 µl DNA: 2,5 µl Total volume: 25 µl
		VZV-R	GGTCGGGTAGACGCTACCACTCGTTT			
HHV-5 (CMV)	Multiplex	CMV-F	GCGCGTACCGTTGAAAGAAAAGCATAA	131	Initial denaturation: 95°C 10 min ⬇ Denaturation: 95°C 10 sec Annealing: 65°C 10 sec Extension: 72°C 10 sec (35 cycles) ⬇ Final extension: 72°C 5 min	dH_2O: 3.8 µl Master mix (Qiagen, Germany): 12.5 µl CMV-F: 1 µl of 10 Mm CMV-R: 1 µl of 10 Mm HHV-6A/B-F: 1 µl of 10 Mm HHV-6A/B-R: 1 µl of 10 Mm HHV-7F: 1 µl of 10 Mm HHV-7R: 1 µl of 10 Mm BSA (1%): 0.2 µl DNA: 2.5 µl Total volume: 25 µl
		CMV-R	TGGGCACTCGGGTCTTCATCTCTTTAC			
HHV-6A,6B		HHV-6A/B-F	ATGCGCCATCATAATGCTCGGATACA	183		
		HHV-6A/B-R	CCCTGCATTCTTACGGAAGCAAAACG			
HHV-7		HHV-7F	GCCCGTTTTCGGAAATATTGGAGAGAT	347		
		HHV-7R	ACGCACGAGACGCACTTTTCTTAAACA			
HHV-8	Nested	Outer-F	AGCCGAAAGGATTCCACCAT	233	Outer PCR: Initial denaturation: 94°C 1 min ⬇ Denaturation: 94°C 1 min Annealing: 58°C 1 min Extension: 72°C 1 min (35 cycles) ⬇ Final extension: 72°C 10 min Inner PCR: Initial denaturation: 94°C 1 min ⬇ Denaturation: 94°C 1 min Annealing: 60°C 1 min Extension: 72°C 1 min (40 cycles) ⬇ Final extension: 72°C 10 min	Outer PCR: dH_2O: 7.8 µl Master mix (Qiagen, Germany): 12.5 µl HHV-8 Outer-F: 1 µl of 10 Mm HHV-8 Outer-R: 1 µl of 10 Mm BSA (1%): 0,2 µl DNA: 2,5 µl Total volume: 25 µl Inner PCR: dH_2O: 6.8 µl Master mix (Qiagen, Germany): 12.5 µl Outer PCR mixture: 1 µl HHV-8 Inner-F: 1 µl of 10 Mm HHV-8 Inner-R: 1 µl of 10 Mm BSA (1%): 0,2 µl DNA: 2,5 µl Total volume: 25 µl
		Outer-R	TCCGTGTTGTCTACGTCCAG			
		Inner-F	TTCCACCATTGTGCTCGAAT	211		
		Inner-R	TACGTCCAGACGATATGTGC			

were performed to demonstrate the distribution and homogeneity of the variables. Continuous variables were presented as median (min-max); while categorical variables were presented as frequencies (n) and percentages (%). Chi-square or Fisher-Exact tests were performed to compare groups. A *P* value of less than.05 was considered statistically significant.

RESULTS

Classification of the malignant and benign groups

The classification of malignant and benign groups, as well as the risk factors for the development of HNC, are provided in **Table 2**. In the MG, male gender (*P*<.001), advanced age (*P*<.001), and smoking (*P*<.001) were found to be significantly higher than BG. There was no difference between the groups in terms of alcohol use.

Detection of herpesviruses DNA

In the malignant group (MG), EBV-DNA was detected in 1 patient (0.3%) and HHV-6 DNA in 2 patients (0.6%). In the benign group (BG), VZV-DNA was detected in 1 patient (0.5%), EBV-DNA in 3 patients (1.5%), CMV-DNA in 5 patients (2.5%), and HHV-6 DNA in 3 patients (1.5%) (**Figures 1-4**). All samples were negative for HSV-1, HSV-2, HHV-7, and HHV-8. No significant difference was found between the groups for VZV, EBV, and HHV-6, a statistically significant difference was found in favor of the benign group for CMV (*P*=.01). The demographic characteristics and risk factors of the patients with HHV positivity are given in **Table 3**.

DISCUSSION

Although the roles of EBV and HHV-8 in cancer development have been revealed in previous studies,[5,6] the roles of other HHVs, such as HSV-1, HSV-2, VZV, CMV, HHV-6, and HHV-7 in cancer pathogenesis have not yet been sufficiently elucidated.[7-9]

HSV-1 and HSV-2 have been associated with the development of various malignant tumors, including HNC.[13] In a study evaluating the presence of HSV-1 and HSV-2 DNA in tissue samples of patients with oral cavity and larynx cancers, it was suggested that HSV plays a role in the carcinogenesis process.[14] On the other hand, some studies investigating the relationship between HSV 1/2 and the development of HNC found no relationship between the development of HNC and HSV infection.[15] HSV-1 and -2 DNA was also absent in any of the samples examined in this study, preventing any conclusions regarding their potential role in the development of HN-SCC in our population. Other risk factors may contribute to developing HN-SCC along with HSV-1 and/or HSV-2 infection. Whether HSV-1 and HSV-2 are the primary triggers of HN-SCC development, or act as cofactors that modify known risk factors must be clarified with further studies. Inconsistent results were also found in studies examining the relationship between herpes zoster caused by VZV and a concurrent cancer diagnosis.[16] Research on VZV's impact on the development of HNC is limited, and it is unclear whether the virus plays a role in the development of HNC.[2] In a study by Buntinx et al,[17] a significant increase in lung, prostate, breast, and colorectal cancer was observed following herpes zoster infection in patients over 65 years. However, focusing on a specific group

Table 2. Comparison of the classification of the groups and risk factors for the development of HNC.

Risk factor	Benign group (n=200)	Malignant group (n=300)	*P* value
Gender			
Female, n (%)	106 (53)	71 (23.66)	**<.001**
Male, n (%)	94 (47)	**229 (76.33)**	
Age, median, years (min-max)	40 (18-89)	**64 (23-91)**	**<.001**
Smoking, n (%)	89 (44.50)	**251 (83.66)**	**<.001**
Alcohol use, n (%)	35 (17.50)	52 (17.33)	.97
Herpesvirus prevelance			
VZV, n (%)	1 (0.50)	0 (0)	.40
EBV, n (%)	3 (1.50)	1 (0.33)	.30
CMV, n (%)	**5 (2.50)**	0 (0)	**.01**
HHV-6, n (%)	3 (1.50)	2 (0.66)	.39

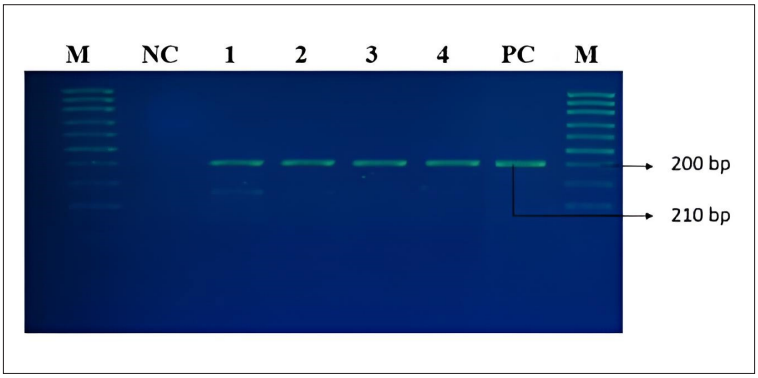

Figure 1. Agarose gel electrophoresis of PCR products with primer of the EBV DNA. Ultra GelRed Nucleic AcidStain (10000x) (GR501-01) (Vazyme, China) was used for staining agarose gel electrophoresis. M: Molecular size marker (Gene Ruler DNA Ladder, 50-500 bp, Thermo, Germany), NC: Negative control, 1-4: Patient isolates, PC: Positive control.

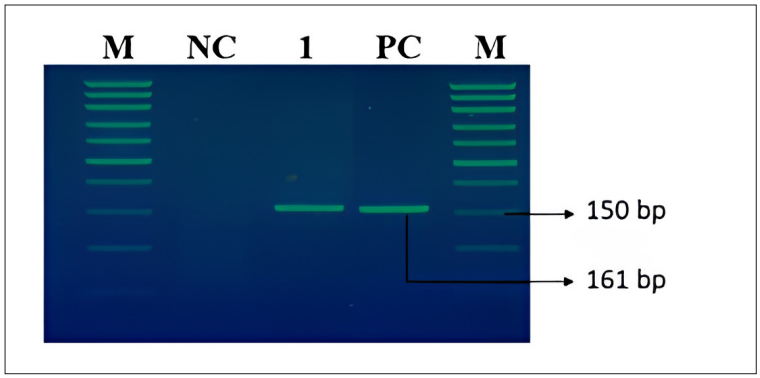

Figure 2. Agarose gel electrophoresis of PCR products with primer of the VZV DNA. Ultra GelRed Nucleic AcidStain (10000x) (GR501-01) (Vazyme, China) was used for staining agarose gel electrophoresis. M: Molecular size marker (Gene Ruler DNA Ladder, 50-500 bp, Thermo, Germany), NC: Negative control, 1: Patient isolates, PC: Positive control.

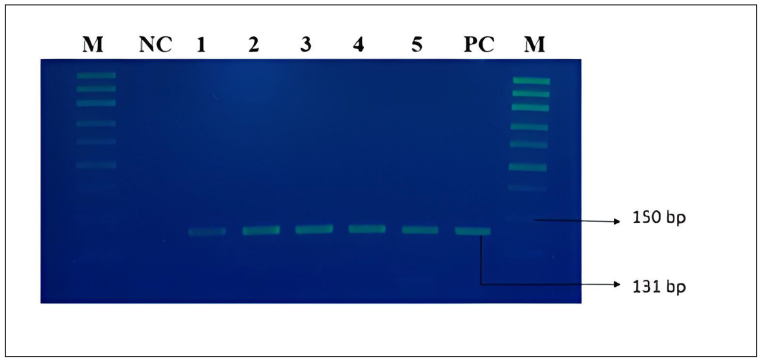

Figure 3. Agarose gel electrophoresis of PCR products with primer of the CMV DNA. Ultra GelRed Nucleic AcidStain (10000x) (GR501-01) (Vazyme, China) was used for staining agarose gel electrophoresis. M: Molecular size marker (Gene Ruler DNA Ladder, 50-500 bp, Thermo, Germany), NC: Negative control, 1-5: Patient isolates, PC: Positive control.

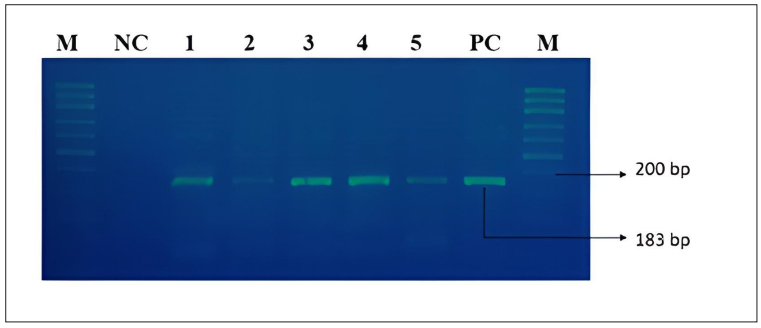

Figure 4. Agarose gel electrophoresis of PCR products with primer of the HHV-6 DNA. Ultra GelRed Nucleic AcidStain (10000x) (GR501-01) (Vazyme, China) was used for staining agarose gel electrophoresis. M: Molecular size marker (Gene Ruler DNA Ladder, 50-500 bp, Thermo, Germany), NC: Negative control, 1-5: Patient isolates, PC: Positive control.

rather than the general population may have influenced the outcomes of this study. In the current study, only one patient (0.5%) with chronic tonsillitis in BG tested positive for VZV.

EBV is the first virus demonstrated to be directly associated with carcinogenesis.[18] Many studies have shown a direct relationship between the development of HNC and the presence of EBV.[7,19] Nasopharyngeal carcinoma, one of the most frequent HNCs, has a proven involvement of EBV in its etiology.[20] In a study investigating the presence of EBV in various squamous cell proliferative lesions in the oral cavity, EBV contributed to the transformation to a malignant phenotype in the oral squamous epithelium.[21] In contrast, some studies exhibited higher EBV DNA positivity in the control group.[22] EBV-DNA was detected in only one sample (0.3%) from the MG and three samples (1.5%) from the BG in our study, with no significant difference between groups (*P*=.30). On the other hand, a meta-analysis evaluating 13 case-control studies found a higher EBV positivity rate in fresh/frozen tissues than in paraffin-embedded tissues, suggesting that different sample types and storage conditions may contribute to the heterogeneity of results.[5] Given the high seropositivity rate for EBV in the Turkish population,[23] the detection of too few EBV-DNA targets in both groups may not reflect the true prevalence, and the discrepancy may be due to the investigation of paraffin-embedded archive tissues in our study. While studies finding a low prevalence of EBV in HNC patients have suggested an absent or negligible etiological role[24] of EBV in HNC development, studies showing a relatively high prevalence of EBV often support its potential etiological involvement.[21] Although CMV is not considered an oncogenic virus, its presence in cancer tissues has been interpreted as either a companion to cancer development or as an opportunist that infects cancer tissues in various studies.[25] In a study where samples from patients with nasopharyngeal cancer and examined for CMV DNA, attention was drawn to the possible relationship between CMV and nasopharyngeal carcinoma.[26] In our study, CMV DNA was only detected in five samples (2.5%) in the BG, and a statistically significant difference was found between groups in favor of the BG (*P*=.01). Patients in the BG whose samples were positive for CMV-DNA had benign tongue lesions and buccal polyps, prompting an evaluation of the relationship between CMV and these lesions. Similarly, in a study investigating the presence of CMV-DNA in benign and malignant tumors of the orofacial region, CMV-DNA was not detected in malignant tumor tissue but was found in 10% of benign tumor samples.[27]

Table 3. Classification of patients with human herpes virus DNA detected in tissue samples.

Sex	Age	Clinical diagnosis	Group	HHV positivity	Other risk factor	
					Smoking	Alcohol use
Female	59	Chronic tonsillitis	Benign	VZV	-	-
Male	66	Tongue SCC	Malignant	EBV	+	-
Female	65	Tonsillar lymphoid hyperplasia	Benign	EBV	-	-
Female	18	Tonsillar lymphoid hyperplasia	Benign	EBV	-	-
Male	18	Chronic tonsillitis	Benign	EBV	-	-
Male	66	Benign lesion of the tongue	Benign	CMV	+	-
Female	59	Fibrous hyperplasia of the tongue	Benign	CMV	+	-
Female	49	Polyp of the buccal mucosa	Benign	CMV	-	-
Male	64	Granulation tissue of the tongue	Benign	CMV	+	-
Male	40	Chronic tonsillitis	Benign	CMV	-	-
Female	56	Benign lesion of the buccal mucosa	Benign	HHV-6	+	+
Female	27	Chronic tonsillitis	Benign	HHV-6	-	-
Female	30	Chronic tonsillitis	Benign	HHV-6	-	+
Male	73	Larynx SCC	Malignant	HHV-6	+	-
Male	58	Larynx SCC	Malignant	HHV-6	+	+

There are also studies suggestingthat CMV may be associated with polyp formation in various regions of the gastrointestinal and respiratory systems.[28,29] The low incidence of CMV infection in oral SCC supports the claim that the virus can infect epithelial cells but likely does not have a direct oncogenic role.

HHV-6 and HHV-7 have been linked to the development of different malignancies. While salivary glands serve as reservoirs for these viruses, it is debated whether they play a role in the development of salivary gland cancer.[8] A study showed a high occurrence of HHV-6 in both malignant and benign oral cavity lesions, suggesting that the virus may be involved in the development of oral lesions, and have tumorigenic potential in vitro.[30] In our study, HHV-6 DNA was found in two samples (0.6%) from the MG and three samples (1.5%) from the BG, with no significant difference between groups (P=.39). HHV-6 can persist throughout life by integrating into the chromosome. Before determining its role in developing HNCs, various samples, including the patient's plasma, should be evaluated simultaneously. In our study, HHV-7 was not detected in any samples. Based on this data, although it is speculated that HHV-6 and HHV-7 may contribute to carcinoma development through different mechanisms, further research is needed to establish their potential role in HNC.

HHV-8, first discovered as a causative agent of Kaposi's Sarcoma, is associated with approximately 1% of all human malignancies.[31] In a study investigating the role of HHV-8 in the development of HNC, no association was found.[32] Another study examining the presence of HHV-8 DNA in laryngeal SCC reported that HHV-8 may be involved in the process of laryngeal carcinogenesis.[33] HHV-8 was not found in either groups in the present study.

Existing studies on herpesvirus seroprevalence in the Turkish population show that the prevalence rates for HSV-1, VZV, EBV, and CMV are around 90%.[23,34-36] However, the rate of viral genome detection in the tissue samples did not match the seroprevalence rates. This discrepancy may be due to patient characteristics,

sample types, region, methods, quality, and infection stage. In our previous study, HPV-positivity was assessed in the same groups, with no statistically significant difference found for HPV-positivity (53 patients [17.67%] in the MG and 29 patients [14.50%] in the BG, *P*=.27).[37]

In the present study, we evaluated the association of risk factors other than HHV, such as smoking, alcohol consumption, and age, with the development of HNC. Similar to the findings of the meta-analysis by Zhang et al,[38] smoking was identified as a stronger significant risk factor for HNC than alcohol consumption in our study. According to the Global Cancer Statistics 2020,[39] HN-SCC is more frequently diagnosed in male patients over 50. Similarly, our study found that advanced age and male gender were significantly more prevalent in the malignant group compared to the benign group. The limitations of our study include the storage conditions of the samples used (paraffinized samples) and the fact that it is a single-center study. Although the paraffinized sample negatively affects the frequency of HHV detection, the fact that the study was conducted in a single center indicates that multicenter studies will be needed to better understand the relationship between viruses and the development of HN-SCC in the Turkish population.

CONCLUSION

Considering the multifactorial nature of cancer etiology, the contribution of herpesviruses alone to cancer development may be relatively small. Therefore, it may be helpful to investigate the role of viruses as cofactors, in cancer development. Evaluating the role of viruses not examined in this study, either alone or in combination with other viruses and the relationship between various risk factors would provide more direct results. To obtain more reliable data, increasing the number of patient groups or conducting long-term prospective studies will help to clarify this issue. To the best of our knowledge, this study is the first to determine the connection between the presence of herpes virus and other risk factors such as smoking and alcohol consumption with the development of HN-SCC in the Turkish population. Despite the relatively high prevalence of herpesvirus in the Turkish population, no viral genome was found in tissue samples. This suggests that factors other than herpes viruses may play a more significant role in the development of HN-SCC in this population.

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