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# Tumor tissue *Helicobacter pylori* and human papillomavirus infection in head and neck squamous cell carcinoma patients and association with clinicopathological indices: A cross-sectional medical survey

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## ABSTRACT

**Background:** The associations between *Helicobacter pylori* and human papillomavirus (HPV) with head and neck squamous cell carcinomas (HNSCCs) are approved before. However, the association between demographic, clinicopathological, and histologic characteristics of HNSCC patients and molecular detection of HPV and *H. pylori* has not been enough investigated.

**Materials and Methods:** In this cross-sectional study, 62 patients with HNSCC from January 2016 to February 2020 were entered the study. For *H. pylori* detection 16S ribosomal RNA and glmM genes and HPV detection, MY09 and MY11 genes were used. $P < 0.05$ is considered as significant level.

**Results:** There were 34 patients with advanced-stage cancer (54.8%). Grade I patients (61.3%) had the highest frequency. There were 20 (32.25%) and 7 (11.29%) patients with positive *H. pylori* infection among tumor tissue and healthy tissue margins, respectively. Positive HPV infections were in 8 (12.90%) and 3 (4.83%) patients, respectively, in tumor tissue and healthy tissue margins ($P = 0.01$). There was a significant difference between histological grade and infection to HPV among HNSCC patients ($P = 0.01$), and most of the positive HPV cases had well-, moderate-, and poorly-differentiated tumors, respectively. Our study showed a significant increase in HPV infection in the advanced-stage group compared to the early-stage group ($P = 0.05$).

**Conclusion:** Our study findings concluded a significant relationship between HPV infection in HNSCC patients with age, stage, and grade. In summary, our findings based on polymerase chain reaction analysis concluded remarkably a potential role of HPV infection and to some extent *H. pylori* infection into the contribution of HNSCC malignancies.

**Key Words:** Grade, head and neck squamous cell carcinoma, *Helicobacter pylori*, human papillomavirus, stage

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## INTRODUCTION

One of the most common causes of death is head and neck squamous cell carcinoma (HNSCC) worldwide. Despite the aggressive treatment approaches, the prognosis of patients with HNSCC is exactly poor. Location of tumors in HNSCC patients is the oral

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cavity, lips, tongue, oropharynx, nasal cavity and paranasal sinuses, nasopharyngeal, laryngeal, and hypopharynx. Most of the HNSCC patients were in advanced stages (stages III and IV) and the survival rate of 70%–90% is related to the early stages of the disease (stages I and II).[1,2]

The accumulation of mutations or epigenetic changes in the proto-oncogene genes and tumor suppressor genes are the molecular basis of carcinogenesis. The change of proto-oncogenes to active oncogenes can be done by inherited changes or environmental factors (such as viruses, rays, and carcinogens).[3,4] Documents confirm the role of some bacteria in oral cancers. Different biological surfaces in the oral cavity have a great variety for the colonization of various bacterial species such as microbiota in the dorsal and lateral surface of the tongue.[5]

*Helicobacter pylori*, spiral Gram-negative bacterium, is a gastrointestinal tract, stomach, and oral cavity pathogen and considered one of the common infections in the world. However, its presence does not involve biofilm creation in the oral cavity.[6]

On the other hand, recently, the incidence of human papillomavirus (HPV) which is associated to oropharynx cancer has been increased.[7,8]

Among the different available diagnostic methods for the detection of *H. pylori* and HPV polymerase chain reaction (PCR) with the specific primers for the detection of region, 16S ribosomal RNA (16S rRNA) gene has the highest sensitivity rate.[9-11]

The association between pathological indices and the expression of some biomarkers in tumor tissue have been investigated in comparison with healthy tumor margin tissue in some studies.[12-14] In this study, the contamination of tumor tissue in SCC patients with *H. pylori* and HPV was compared with the healthy margin tissue of these patients.

Understanding the impact of *H. pylori* and HPV on the clinicopathological outcomes of HNSCC is critical for treatment approaches. There is no any relevant investigation for the detection of both *H. pylori* and HPV in HNSCC tumor and margin healthy tissues and its association with demographic and clinicopathological characteristics, including location, stage, and grade of tumors. The purpose of this study was to evaluate the presence of *H. pylori* and HPV with two gene of *H. pylori* (16S rRNA and glmM genes), as well as two genes of HPV (*MY09* and *MY11* genes) in fresh tumor tissue and fresh margin healthy tissue in HNSCC patients. We also aimed to investigate *H. pylori* and HPV presence with clinicopathological characteristics.[15,16]

## MATERIALS AND METHODS

### Patients

In this cross-sectional study, 62 patients with HNSCC who referred to the Oral and Maxillofacial Pathology Department and the Otorhinolaryngology Department of Mashhad University of Medical Sciences from January 2016 to February 2020 were included the study. These patients (47 men and 15 women) had a mean age 60.5 ± 13.4 years and ranged between 28 and 81 years old. SCCs in all patients were approved according to the standard histological diagnosis. They were not received any antibiotic treatments over the past 4 months. Patients who had undergone surgery, chemotherapy, and radiotherapy for their cancer were excluded.

Protocol of this study was approved by the Medical Ethics Committee of Mashhad University of Medical Sciences (IR.mums.sd.REC.1394.57). All patients were received informed consent before contributing present study.

### DNA extraction from tissue samples

Fresh tumor tissue and healthy tissue surgical margins from therapeutic surgery in transport solutions in the separate containers were collected. DNA was extracted from the healthy margin tissues and tumor tissues by QIAamp DNA Tissue Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Qualification of DNA in 260 nm wavelength was evaluated by NanoDrop instrument (Thermo Scientific 2000, Finland).

### Conventional polymerase chain reaction assay

To detect *H. pylori* strain in tissue samples from tumor tissues and margin healthy tissues patients, we used the specific primers targeting *16S rRNA* and glmM genes. PCR conditions, target gene, amplicon size, and primer sequence are presented in Table 1. The conditions for PCR amplification were as follows: initial denaturation at 95°C for 3 min followed by 35 cycles of denaturation at 95°C for 55 s, annealing for 55 s, polymerization at 74°C for 55 s, and final polymerization at 72°C for 5 min (ABI, USA). The PCR reaction products were electrophoresed on 2.0% Agarose gel at 100 v for 30 min.[17,18]

**Table 1: Primers set and conditions used for *Helicobacter pylori* and human papillomavirus detection by polymerase chain reaction method in this study**

| Genes | Nucleotide sequence (5'-3') | PCR product | PCR conditions | Reference |
|---|---|---|---|---|
| *16S rRNA* | 5'-GCGACCTGCTGGAACATTAC -3'<br>5'-CGTTAGCTGCATTACTGGAGA-3' | 138 bp | 95°C, 55 s; 59°C, 55s<br>74°C, 55 s (35 cycles) | [17] |
| *glmM* | 5'AAGCTTTTAGGGGTGTTAGGGGTTT -3'<br>5'-AAGCTTACTTTCTAACACTAACGC-3' | 294 bp | 95°C, 55s; 57°C, 55 s<br>74°C, 55 s (35 cycles) | [18] |
| *MY09*<br>*MY11* | 5'-CGTCCMARRGGAWACTGATC 3'<br>5'-GCMCAGGGWCATAAYAATGG 3' | 450 pb | 94°C, 45 s; 55°C, 45 s<br>72°C, 45 s (40 cycles) | [19] |

PCR: Polymerase chain reaction

For HPV detection, we performed PCR mixture containing 20 pmol of forward primers and reverse primers, 15 mM $MgCl_2$ (*buffer* ×1), 800 µM of dNTPs, 50 pmol/µl of each oligonucleotide primer, 1.25 U from Hot Star TaqDNA polymerase (Invitrogen) [Table 1].[19]

### Statistical analysis

Clinicopathological and demographic characteristics were analyzed by Chi-square test. For all statistical tests, $P < 0.05$ was considered as significant level. We performed SPSS software version 11.5 (Chicago, IL, USA) for the statistical analysis.

## RESULTS

### Patient characteristics

Patients' characteristics such as age, gender, clinical tumor stage, and tumor position are presented in Table 2. Information including the family history of cancer and history of gastric reflux and using smoke, alcohol, and opium was collected. The majority of patients, 34 patients (54.8%), were in advanced stage of disease. Grade I (61.3%) was the most frequent histological grade among patients. Frequency of SCC tumor positions was according to this order; larynx, tongue and oral cavity, lip, nasal cavity, and ear canal.[20] Family history of cancer and gastric reflux was negative dominantly in studied cases. Finally, most of the studied SCC patients had not consumed smoke, alcohol, and opium [Table 2].

Table 3 presents the relationship of clinicopathological and demographic characteristics with histopathological stage. This results in relation to stage for 62 patients showing that the relationship of histopathological grade and stage of tumors was significant. As most of the patients (82.1%) in early stage were in well-differentiated grade (I) ($P = 0.003$), there was not any significant relationship between consumption of smoke, alcohol, and opium with stage of tumors [$P > 0.05$, Table 3]. Besides, the relationship of tumor location (larynx, tongue and oral cavity, lip, nasal cavity, and ear canal) and histopathological stage of tumors was not significant [$P > 0.05$, Table 3].

**Table 2: Clinicopathological characteristics of 62 patients with head and neck squamous cell**

| Variable | *n* (%) |
|---|---|
| Gender | |
| Male | 47 (75.8) |
| Female | 15 (24.2) |
| Age (60.5±13.4 years) | |
| ≥60.5 | 31 (50.0) |
| <60.5 | 31 (50.0) |
| Clinical staging (TMN) | |
| Early | 28 (45.2) |
| Advanced | 34 (54.8) |
| Histopathological grade | |
| I | 38 (61.3) |
| II | 20 (32.2) |
| III | 4 (6.5) |
| Tumor position | |
| Lip | 13 (20.9) |
| Tongue and oral cavity | 17 (27.4) |
| Larynx | 26 (41.9) |
| Nasal cavity | 3 (4.8) |
| Ear canal | 3 (4.8) |
| Family history cancer | |
| Yes | 13 (20.9) |
| No | 49 (79.1) |
| History of gastric reflux | |
| Yes | 23 (37.1) |
| No | 39 (62.9) |
| Smoking | |
| Daily | 21 (33.9) |
| No | 41 (66.1) |
| Alcohol | |
| Daily | 5 (8.1) |
| No | 57 (91.9) |
| Opiate use | |
| Yes | 28 (45.2) |
| No | 34 (54.8) |

TMN: Tumor node metastasis

**Table 3: Relation of clinicopathological and demographic characteristics with histopathological stage**

| Variables | Overall | Participants, *n* (%) | | *P* |
|---|---|---|---|---|
| | | Early stage | Advanced stage | |
| Histopathological grade | | | | |
| Well (I) | 38 | 23 (82.1) | 15 (44.1) | 0.003* |
| Moderate (II) | 20 | 5 (17.9) | 15 (44.1) | |
| Poor (III) | 4 | 0 | 4 (11.8) | |
| Lymph node involvement | | | | |
| Yes | 29 | 9 (32.1) | 20 (58.8) | 0.03* |
| No | 33 | 19 (67.9) | 14 (41.2) | |
| Alcohol | | | | |
| Daily | 7 | 1 (3.6) | 6 (17.6) | 0.12 |
| No | 55 | 27 (96.4) | 28 (82.4) | |
| Opiate use | | | | |
| Yes | 28 | 12 (42.8) | 16 (47.1) | 0.8 |
| No | 34 | 16 (57.2) | 18 (52.9) | |
| Smoking | | | | |
| Daily | 21 | 9 (32.2) | 22 (64.7) | 0.79 |
| No | 41 | 19 (67.8) | 12 (35.3) | |
| Gender | | | | |
| Female | 15 | 7 (25.0) | 8 (23.5) | 0.56 |
| Male | 47 | 21 (75.0) | 26 (76.5) | |
| Age, years old | | | | |
| ≥60.5 | 31 | 15 (53.6) | 16 (47.1) | 0.79 |
| <60.5 | 31 | 13 (46.4) | 18 (52.9) | |
| Tumor position | | | | |
| Lip | 13 | 7 (73.8) | 6 (46.2) | 0.42 |
| Tongue and oral cavity | 17 | 6 (35.3) | 11 (64.7) | |
| Larynx | 26 | 13 (50.0) | 13 (50.0) | |
| Nasal cavity | 3 | 2 (66.7) | 1 (33.3) | |
| Ear canal | 3 | 0 (0.0) | 3 (100.0) | |

Numbers in the parenthesis showed percentage of studied patients in each related variable. *Analyses were performed under Chi-square test. *P*<0.05 considered as significant level

## Histologic characteristics and molecular detection of *Helicobacter pylori*

There were 20 (32.25%) and 7 (11.29%) patients with positive *H. pylori* infection among tumor tissue and healthy tissue margins, respectively. There was meaningful association between infection to *H. pylori* between tumor and margin tissue ($P$ = 0.001). In addition, there was 8 (12.90%) and 3 (4.83%) patients with positive HPV infection among tumor tissue and healthy tissue margins, respectively. The statistical analysis showed that there was significant association between infection to HPV between tumor and margin tissue ($P$ = 0.01).

Figure 1a and b presents the relationship of tumor stage and *H. pylori* and HPV infection. Most of the cases with positive infection of *H. pylori* (60%) in tumor tissues were in advanced stage compared to early stage. There was significant relationship between stage of tumor and infection to *H. pylori* in tumor tissue ($P$ = 0.05). However, this was not significant for healthy margin tissue ($P$ = 0.69).

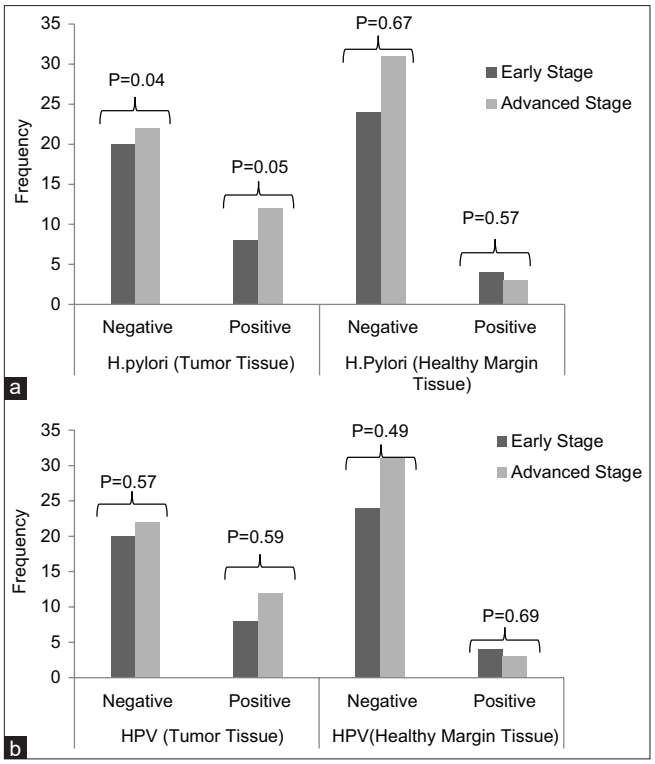


**Figure 1:** Relation of histopathological grade and molecular detection of *Helicobacter pylori* (a) and human papillomavirus (b) in tumor tissue and healthy margin tissue. Analyses were performed under Chi-square test.

## Histologic characteristics and molecular detection of human papillomavirus

Frequency of HPV infection cases into both tumor tissue and healthy margin tissue in advanced stages was 78.5% and 66.6%, respectively. In addition, there was not any significant relation between stage of tumor and infection to HPV in the tumor tissue ($P$ = 0.06) and healthy margin tissue ($P$ = 0.66).

The presence of *H. pylori* and HPV in the tumor tissues of HNSCC patients and its association with clinicopathological and histologic characteristics is presented in Table 4. Table 4 shows that there was not any significant difference between gender and infection to *H. pylori* or HPV among HNSCC patients. However, there was not any difference between age (more and less than 61 years) and *H. pylori* infection, but the statistical analysis showed that this relation was significant for HPV infection among HNSCC studied patients ($P$ = 0.05). In addition, infected *H. pylori* or HPV cases were similar about

**Table 4: Characteristics of head and neck squamous cell carcinoma tumor tissues and *Helicobacter pylori* and human papillomavirus status**

| Variables | *n* (%) | *H. pylori* | | | HPV | | |
|---|---|---|---|---|---|---|---|
| | | *H. pylori*+ (*n*=20; 32), *n* (%) | *H. pylori*− (*n*=42; 68%), *n* (%) | *P* | HPV+ (*n*=8; 13%), *n* (%) | HPV− (*n*=54; 87%), *n* (%) | *P* |
| Gender | | | | | | | |
| Male | 47 (76) | 16 (80) | 31 (74) | 0.75 | 6 (75) | 41 (76) | 1.00 |
| Female | 15 (24) | 4 (20) | 11 (26) | | 2 (25) | 13 (24) | |
| Age, years old | | | | | | | |
| ≥60.5 | 31 (50) | 7 (35) | 24 (57) | 0.10 | 1 (12.55) | 30 (56) | 0.05* |
| <60.5 | 31 (50) | 13 (65) | 18 (43) | | 7 (87.5) | 24 (44) | |
| Tumor position | | | | | | | |
| Lip | 13 (20) | 5 (8.06) | 8 (12.90) | 0.57 | 1 (1.61) | 12 (19.35) | 0.91 |
| Tongue and oral cavity | 17 (27) | 7 (11.29) | 10 (16.12) | | 3 (4.83) | 14 (22.58) | |
| Larynx | 26 (41.93) | 8 (12.90) | 18 (29.03) | | 4 (6.45) | 22 (35.48) | |
| Nasal cavity | 3 (4.83) | 0 | 3 (4.83) | | 0 | 3 (4.83) | |
| Ear canal | 3 (4.83) | 0 | 3 (4.83) | | 0 | 3 (4.83) | |
| Grade | | | | | | | |
| Well (I) | 38 (61.29) | 13 (20) | 25 (40.32) | 0.55 | 1 (1.61) | 37 (59.67) | 0.001* |
| Moderate (II) | 20 (32.25) | 5 (8.06) | 15 (24.19) | | 3 (4.83) | 17 (27.41) | |
| Poor (III) | 4 (6.45) | 2 (3.22) | 2 (3.22) | | 4 (6.45) | 0 | |
| Stage | | | | | | | |
| Early | 28 (45.16) | 8 (12.9) | 20 (32.25) | 0.57 | 1 (1.61) | 27 (43.54) | 0.063 |
| Advanced | 34 (54.83) | 12 (19.35) | 22 (35.48) | | 7 (11.29) | 27 (43.54) | |
| Alcohol | | | | | | | |
| Daily | 7 (11.29) | 3 (4.83) | 4 (6.45) | 0.12 | 1 (1.61) | 6 (9.67) | |
| No | 55 (88.70) | 17 (27.41) | 38 (61.29) | | 7 (11.29) | 48 (77.41) | |
| Opiate use | | | | | | | |
| Yes | 28 (45.16) | 8 (12.90) | 20 (32.25) | 0.57 | 6 (9.67) | 22 (35.48) | 0.12 |
| No | 34 (54.83) | 12 (19.35) | 22 (35.48) | | 2 (3.22) | 32 (51.61) | |
| Smoking | | | | | | | |
| Daily | 21 (33.87) | 7 (11.29) | 14 (22.58) | 0.89 | 1 (1.61) | 20 (32.25) | 0.24 |
| No | 41 (66.12) | 13 (20) | 28 (45.16) | | 7 (11.29) | 34 (54.83) | |

Numbers in the parenthesis showed percentage of studied patients in each related variable. *Analyses were performed under Chi-square test. *P*<0.05 considered as significant level. *H. pylori*: *Helicobacter pylori*; HPV: Human papillomavirus

location of tumors [$P > 0.05$; Table 4]. There was significant difference between histological grade and infection to HPV among HNSCC patients [$P = 0.01$; Table 4]. This order was similar for *H. pylori*-positive and -negative cases, but this was not statistically significant [$P = 0.55$; Table 4]. Furthermore, there was not any significant difference between consumption of smoke, alcohol, and opium among HNSCC patients with infection of *H. pylori* or HPV [$P > 0.05$; Table 4].

## DISCUSSION

HPV cancer has been increasing in frequency in our study area (northeast of Iran).[21,22] The roles of bacterium infection might be into onset of cancer growth and maintenance of cancers.[6]

In gastric, *H. pylori* is a chronic infection that leads to chronic inflammation and so promotes tumorigenesis.[9] Previous studies showed that there was association between infection to *H. pylori* and the possibility of esophageal squamous cell carcinoma (ESCC) among East Asian populations.[23] Our results showed that among HNSCC studied cases, the positive *H. pylori* infection was 32%.

Only few studies have shown the connection between head and neck malignancy and *H. pylori* activity.[24-27] Some studies were examined the serology of oral cancer patients and control group and revealed the lack of absence of *H. pylori* frequency in patients with HNSCC cancer compared to control patients without oral cancers.[26]

In the study population in northeast of Iran, patients with HNSCC had significant difference between the frequency of *H. pylori* in the patient's tumor tissue and the patient's normal margin tissue, and most of

the positive *H. pylori* cases were in tumor tissues compared to normal tissues.

On the other hand, previous investigations revealed that HPV infection might be associated with different cancers including carcinomas of the cervix, vagina, vulva, head and neck, anal, and penile.[28] Recently, the number of HNSCC and ESCC patients with positive HPV had been increased and is expected to surpass the incidence rate of cervical cancer.[7,29] Our study results showed that the frequency of positive HPV infection was 13% among HNSCC patients. Besides, there was significant difference between the frequencies of HPV in the patient's tumor tissue and in the patient's healthy margin tissue, and most of the positive HPV cases were in tumor tissue compared to normal tissue. Some studies showed that HNSCC in HPV-positive patients had considerably better prognosis and better results of treatment compared to HNSCC cases who were negative for HPV.[30,31] However, in the present study, we did not evaluate the prognosis and treatment outcomes among HNSCC cases with positive or negative *H. pylori* or HPV infection. Previous studies reported that the frequency of detection of *H. pylori* in the upper gastrointestinal tract varied between 0% and 100% according to the PCR method. However, different studies have shown that the results were between these two these values.[32-35] Recently, PCR-based methods for the detection of HPV and *H. pylori* infections have been recognized as the most approved valid test and used for the different clinical samples containing human saliva, stool, gastric juice, biopsies, and dental plaques.[36,37]

In addition of PCR tests, serology tests have been showed cases of *H. pylori* positive in patients with HNSCC compared laryngeal or hypopharyngeal cancer control patients.[38]

The differences in PCR results might be explain due to the differences in the HPV and *H. pylori* detection methods, kinds of samples for analysis, different selected specific target genes, and primers to identify *H. pylori* genome from similar species.[39] To avoid the similarity between the *H.pylori* gene and other species of Helicobacter, in the present study, primers selected completely specific for *H. pylori* detections and similar genomes of bacterial species were not detected.

In present research, analysis of relation between demographic variables and infection to *H. pylori* and HPV showed that most of *H. pylori* (80%) and HPV (75%) cases were male. However, there was no significant difference between gender and infection to *H. pylori* and HPV of HNSCC patients. Besides, our results revealed that age has an important role to infection of *H. pylori* and HPV among studied HNSCC patients. Although this association was not significant, 65% of positive *H. pylori* and 87.5% of positive HPV reported cases were in age more than 61 years old. Previous studied showed that the consumption of alcohol, smoke, and opium might be the direct role to infection, but our study showed that there was not any meaningful relation between consumption of alcohol, smoke, and opium and *H. pylori* or HPV infection.

However, previous studies as Morand *et al.* in Switzerland reported *H. pylori*-positive serology to be more prevalent among HNSCC patients compared normal controls to laryngeal cancerous patients.[37,40] They reported that *H. pylori*-positive serology test was presented almost four times greater in smoking patients than nonsmoking HNSCC patients.[37] Further, like our study results, analysis showed that increasing age is considered as a risk factor in positive serology tests.[37,38]

In our study, the only demographic variables were not significantly associated with a positive *H. pylori* or HPV cases. These differences between our study results and previous investigations about the relation between demographic variables and positivity of H.pylori test might be explained due to the study population in different countries, applied tests for the detection of infections, sample size, and selected targeted specific primers in PCR based tests. Our study results showed that most of the positive cases with *H. pylori* (60%) and HPV (87.5%) were in advanced stage compared to early stage of disease. However, this relation was not statistically meaningful. These results indicate that more participants in the east of Iran were presented in advanced stage of HNSCC as compared with other HNSCC patient populations.

## CONCLUSION

The impact of *H. pylori* and HPV in some cancers in different populations around the world has shown different results. Our study showed that in the northeast Iranian population, these two infection factors were significantly different in the tumor tissues of patients compared to healthy marginal tissue. In our study, there was a significant relationship between

HPV infection in HNSCC patients with age, stage, and grade. However, there was no significant relationship between these factors in patients with *H. pylori* virus. Our findings concluded remarkably a potential role of HPV infection and to some extent *H. pylori* infection into contribution of HNSCC malignancies.

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### Conflicts of interest

The authors of this manuscript declare that they have no conflicts of interest, real or perceived, financial or non-financial in this article.

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