



## Prevalence of human papillomavirus in oral and laryngeal squamous cell carcinoma: A comparative study by polymerase chain reaction

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Squamous cell carcinoma (SCC) is the most common malignant histological type in Oral cavity and Larynx. Human papillomavirus (HPV) is one of the most common causes of sexually transmitted disease in both men and women worldwide. It is associated with a variety of clinical conditions that range from innocuous lesions to cancer. Human papillomavirus (HPV) has been confirmed the primary etiological factor that transforms cervical epithelia into cancer. The presence of HPV in oral and laryngeal cancers suggests that HPV may play a similar role in transforming the oral epithelia. The study aimed to determine the prevalence of human papillomavirus infection in oral and laryngeal squamous cell carcinoma in Iraqi patients with high risk typing 16,18. Total of 80 cases oral and laryngeal squamous cell carcinoma are enrolled in this study, forty cases for each. The study sample analyzed by PCR to determine the HPV positive and negative cases using consensus probe. Genotyping of HPV was performed using a specific probe for high oncogenic-risk HPV genotypes 16, 18. Demographic data of the study sample showed that males were more than females (62.5 %, 37.5%) respectively. No statistical difference in age groups was found among the study groups, while there was a significant difference in grading among study them (P-value = 0.04). Polymerase chain reaction revealed positive HPV in 12 cases (15%). High-risk HPV16 was detected in 9 cases (2.5% laryngeal SCC and 20% were oral SCC). No significant relation was found between HPV in respect to the histopathological grade, gender and age group in the total sample. The high-risk HPV16 detection in 20% of oral squamous cell carcinoma cases it might play a role in transforming the oral epithelia. There was no significant association between HPV-16 DNA and the demographic data of OSCC.

### INTRODUCTION

Cancer is the common term for numerous distinct diseases characterized by the uncontrolled growth of abnormal cells as a result of mutations in specific genes (1). Specific infections represent other major cancer risk factors with an estimated 2.1 million (16.4%) of the 12.7 million new cases in 2008 attributable to infection. The most important infectious agents are Helicobacter pylori, Hepatitis B and C viruses and Human papillomaviruses (2). Furthermore, genetic changes have been linked to environmental factors such as physical carcinogens - ultraviolet (UV) and ionizing radiation chemical carcinogens: asbestos and tobacco smoke, biological carcinogens - viral infections (Hepatitis B or Human Papilloma Virus HPV), bacteria (Helicobacter pylori) (2). Human papillomavirus (HPV) is a non- enveloped double-stranded, circular DNA virus that has been implicated in a variety of anogenital and aerodigestive diseases, ranging from common warts to laryngeal papilloma to cervical cancer (3). The first isolation of these virus particles was performed in 1933 in rabbit papillomatosis (2). These viruses infect cells in the basal layer of squamous epithelium and the different types have

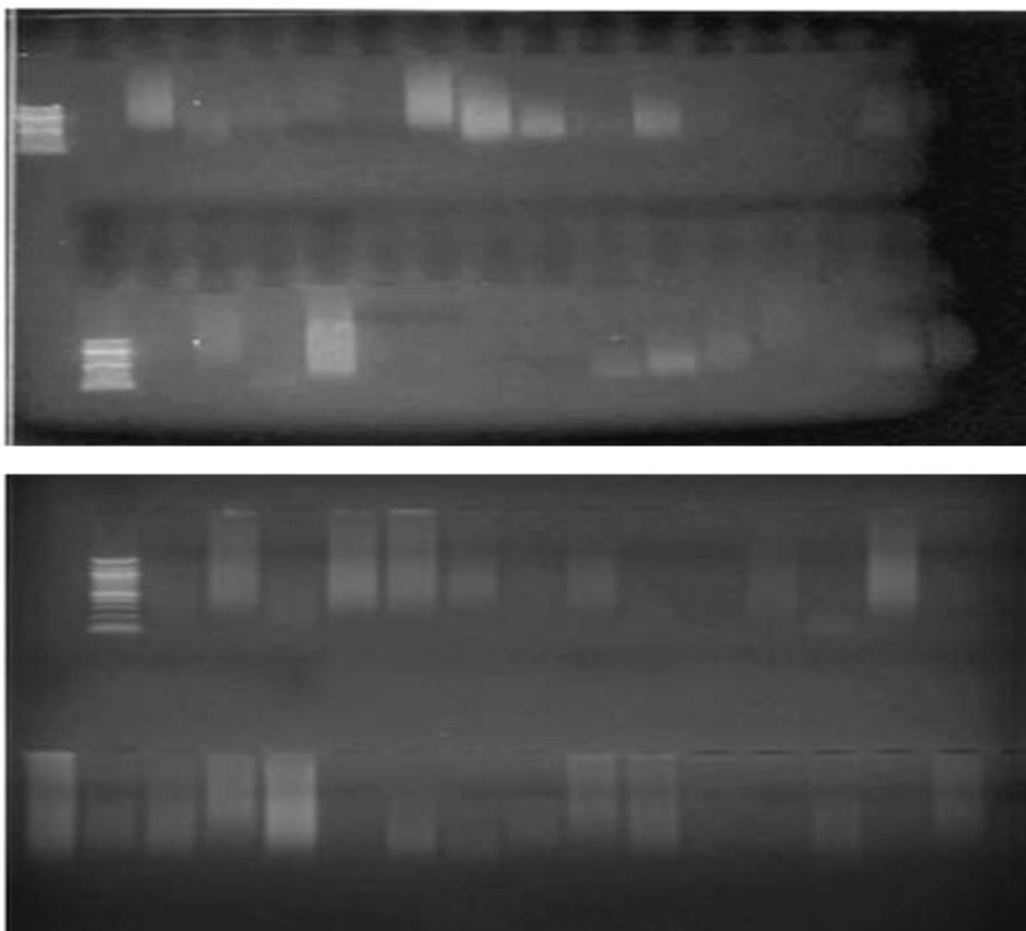
been traditionally separated based on tropism for cutaneous and mucosal sites, as well as high, intermediate, and low risk, depending on their association with malignancy (4). In 1950, the carcinogenic potential of the human papillomavirus (HPV) was discovered in patients with epidermodysplasia verruciformis (5). Although the first suggestion that HPV may play a role in the development of oral cancer was proposed only many years later a number of molecular and epidemiological studies have provided a strong correlation between high-risk HPV infection of the oral mucosa (specifically the oropharyngeal region) and the development of HNSCC (6). The International Agency for Research on Cancer (IARC) in 2003 determined that the infection of HPV is highly likely to play an etiologic role in HNSCC (7). Polymerase chain reaction is a method of amplifying target sequences from a DNA specimen, thus providing a higher degree of sensitivity and specificity than traditional hybridization methodologies. Polymerase chain reaction requires relatively small amounts of DNA (25–500 ng); consequently, the technique can be applied to DNA extracted from formalin-fixed paraffin-embedded tumor tissues (8).

### MATERIALS AND METHODS

The study sample included 80 Formalin-fixed, paraffin embedded (FFPE) tissue blocks which were diagnosed as oral and laryngeal squamous cell carcinoma (40 cases for each) obtained from archival

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**Figure 1** 1% agarose gel electrophoresis analysis of the total DNA extracted from paraffin embedded tissue. The DNA exhibited smearing pattern in almost all samples

histopathology laboratory of Ghazi Al-hariry hospital of specialized surgeries and oral pathology laboratory, Teaching Hospital/ College of Dentistry/ University of Baghdad during the period from 2014-2017.

A four  $\mu\text{m}$  thickness sections were cut from each tissue block to be stained with H and E for histopathological reevaluation. Tumor tissues were classified with respect to the Broders grading system (conventional) into well, moderately and poorly differentiated SCC (9). Another  $8\mu\text{m}$  section was cut from each block for PCR procedure detection of high risk HPV16 and 18. DNA extraction of the cut sections has been performed using DNA MiniPrep extraction kit (ZYMO Research, USA) according to the manufacturer instructions. Briefly, paraffin was removed by treatment with xylene (three times or more depending on the sample). Xylene was subsequently removed by washing with absolute ethanol (three times). The deparaffinized tissue samples were then treated with proteinase k and incubated overnight until tissues were completely digested. DNA was then purified using Zymo-spin columns. The samples were stored at  $4^{\circ}\text{C}$  until further analysis. Determination of DNA content and quality was performed by measuring the optical density (OD) at 260nm wavelength and the ratio between OD260 and OD280. The DNA samples were analyzed by 1% agarose gel electrophoresis stained by ethidium bromide. A total of 10  $\mu\text{l}$  of each sample was loaded in the gel and run for 45 min with 25mA and 80 volts. Almost all samples showed abundant DNA quantity with low protein contamination. The samples that showed positive PCR results for HPV by consensus primers were further analyzed for detection of

genotype 16 and genotype 18 of HPV by PCR using type-specific primers. The amplified product was predicted to give a band of 180-200 and 250 bp in length for genotypes 16 and 18 respectively. Out of the 12 HPV positive samples, a total of 9 samples showed positive PCR product for genotype 16 in comparison to genotype 18 which could not be detected in any of the samples.

## RESULTS

### Clinicopathological data

Eighty paraffin-embedded tissue samples of oral and laryngeal SCC were collected. Table (1) showed the total numbers of studied samples distributed to the grading, well differentiated SCC was 35(43.75%), moderate differentiated SCC 32(40%) and poor differentiated SCC 13(16.25%), the females were 30(36.7) and males 50(63.3%) from the total number of samples.

### Association between HPV and type of SCC

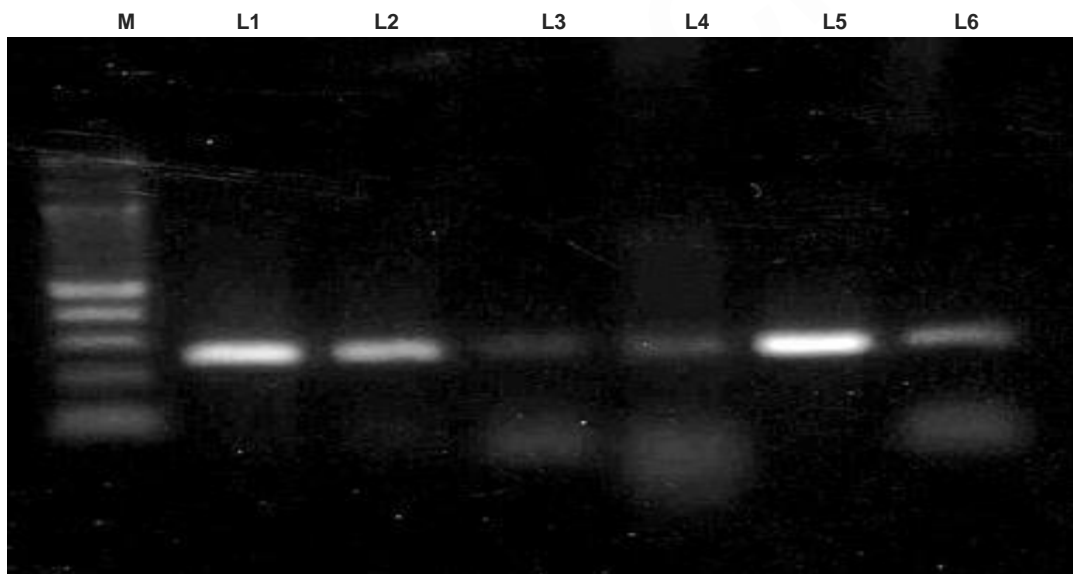
As showed in the table (2), the prevalence of positive HPV-16 genotype was significantly higher among Oral SCC group (20%) compared to laryngeal SCC (2.5%). OSCC showed significantly increased risk of positive testing for HPV-16 by 8 times compared to laryngeal SCC group (or testing positive for HPV-16 significantly increase the risk of having oral SCC in favor to laryngeal SCC by 8 times). The prevalence of positive HPV-unspecified genotype was significantly higher among oral SCC group (27.5%) compared to laryngeal SCC (2.5%). Oral SCC

**Table 1** Comparison of oral SCC and laryngeal SCC frequencies regarding tumor grades and patients genders

Study group	Laryngeal SCC		Oral SCC	
	N	%	N	%
<b>Tumor Grade</b>				
Well-differentiated	12	30.0	23	57.5
Moderately differentiated	20	50.0	12	30.0
Poorly differentiated	8	20.0	5	12.5
Total	40	100.0	40	100.0
<b>Gender</b>				
Female	14	35.0	16	40.0
Male	26	65.0	24	60.0
Total	40	100.0	40	100.0

**Table 2** The relative frequency of positive HPV by the study groups

Study group	Total	Positive HPV-16 genotype		Positive HPV-unspecified genotype	
	N	N	%	N	%
Laryngeal SCC	40	1	2.5	1	2.5
Oral SCC	40	8	20.0	11	27.5
P (Chi-square)		0.03		0.002	

**Figure 2** PCR reaction of randomly selected samples for the detection of beta globin gene (internal control). The results showed that the tested samples produced positive signal with a band of 280 bp. M is 100 bp DNA ladder, L1-L6 extracted DNA samples from paraffin embedded tissues

had significantly increased the risk of testing positive for general HPV by 11 times compared to LSCC group (or testing positive for general HPV significantly increases the risk of having OSCC in favor of laryngeal SCC by 11 times).

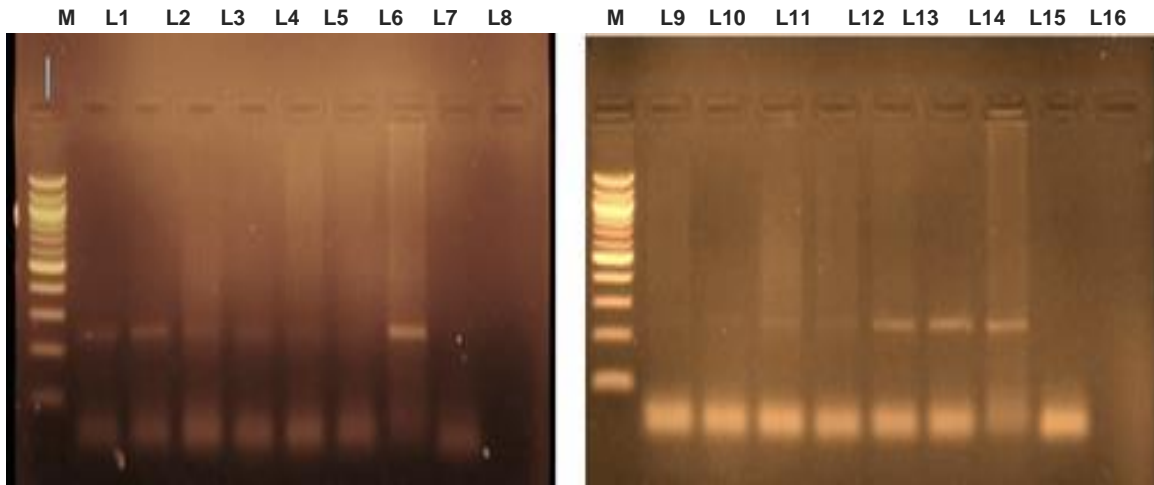
The association between having HPV and tumor grade, sex and age was assessed in only OSCC cases since there were very few HPV positive cases in the other group, which interferes with any possible (or valid) interpretation of tested associations.

#### Association between HPV and tumor grade, gender and age in OSCC cases

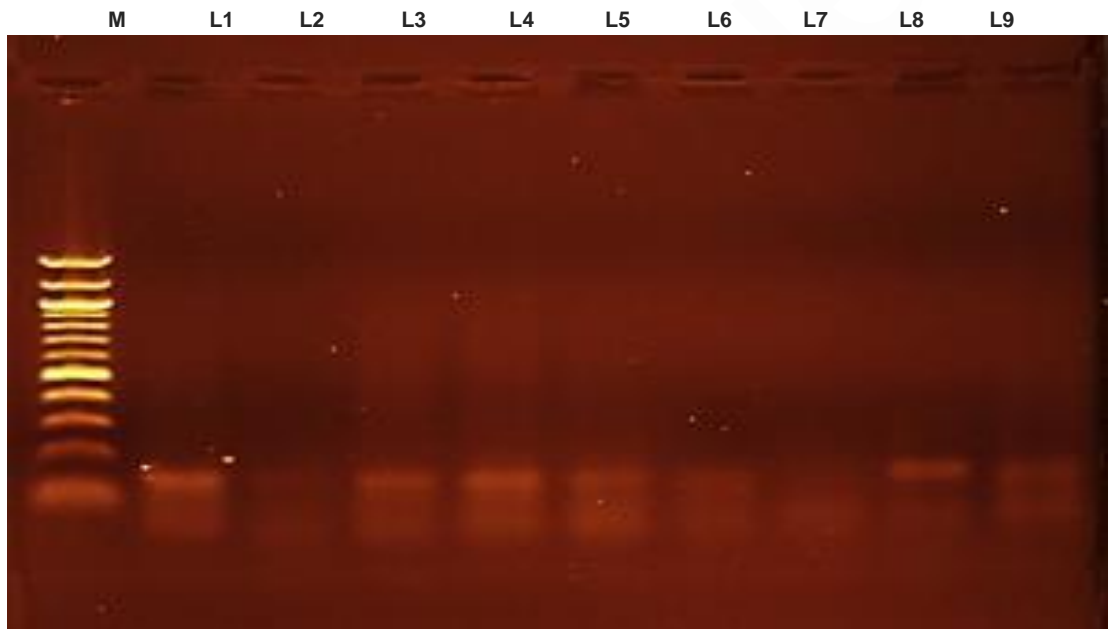
As showed in the table (3), there was no important association between tumor grade and the positivity rate of HPV-16 and general HPV. The prevalence of positive HPV-16 genotype was obviously higher among

females (31.3%) compared to males (12.5%), but the difference was not significant statistically. Being a female increase the risk of testing positive for HPV-16 by 2.5 times compared to males. The prevalence of positive general HPV was obviously higher among females (43.8%) compared to males (16.7%), but the difference failed to reach the level of statistical significance. Being a female increase the risk of testing positive for general HPV by 2.6 times compared to males.

The prevalence of positive HPV-16 genotype was obviously higher among younger age group (33.3% among <50 years of age) compared to older ages (13.3% and 18.8% among those with 50-60 and >60 years of age respectively), but the difference was not significant statistically. The prevalence of positive general HPV genotype was obviously higher among younger age group (44.4% among <50 years of age) compared to



**Figure 3** PCR reaction of positive samples using consensus primers of HPV. The results showed that the tested samples produced positive bands of 250 bp. M is 100 bp DNA ladder, L7 and L15; positive control, L8 and-L16; negative control, L2-L6 and L9-L14 are positive samples of HPV



**Figure 4** PCR reaction of positive samples for HPV genotype 16 using genotype specific primers. The results showed that the tested samples produced positive bands of 180 bp. M is 100 bp DNA ladder, L7; samples with positive consensus HPV PCR but with negative genotype 16. L1-L6, L8 and L9; samples with both positive consensus HPV PCR and positive genotype 16

**Table 3** The relative frequency of positive HPV by tumor grade, sex, and age in Oral SCC group

	Total		Positive HPV-16 genotype		Positive HPV-unspecified genotype	
	N		N	%	N	%
<b>Tumor Grade</b>						
Well-differentiated	23		4	17.4	7	30.4
Moderately differentiated	12		3	25.0	3	25.0
Poorly differentiated	5		1	20.0	1	20.0
P (Chi-square)			0.87[NS]		0.87[NS]	
<b>Gender</b>						
Female	16		5	31.3	7	43.8
Male	24		3	12.5	4	16.7
P (Chi-square)			0.23[NS]		0.08[NS]	
<b>Age group (years)</b>						
<50	9		3	33.3	4	44.4

50-60	15	2	13.3	4	26.7
>60	16	3	18.8	3	18.8
P (Chi-square)		0.49[NS]		0.38[NS]	

older ages (26.7% and 18.8% among those with 50-60 and >60 years of age respectively), but the difference was not significant statistically.

## DISCUSSION

In the last 30 years or so, considerable evidence has been found about the role of several infectious agents, particularly viruses, in human cancer (4). Worldwide an estimated 1.9 million cancer cases were attributable to infectious agents in 2002, representing 17.8% of all cancers (6). Most of this burden was related to viral infections (12.1%) and a very small proportion (about 0.1%) to the parasitic infections (8). On the basis of mounting epidemiologic and molecular evidence, in 1995, the International Agency for Research on Cancer recognized that high-risk HPV types 16 and 18 were carcinogenic in humans (8). Together, these 2 HPV types are responsible for approximately 70% of cervical cancer cases. HPV 16 is the predominant (90% to 95%) genotype detected in head and neck tumors, with different prevalence between anatomic sites (10).

Concerning sex distribution the present study showed that 50/80 (62.5%) of the cases were males; 30/80 (37.5%) were females with no statistical difference among the studied groups. Previous Iraqi studies, as well as foreign studies, showed males predominance (11, 12). However, regarding OSCC the disparity in the male to female ratio has become less pronounced over the past half-century, probably because women have been more equally exposing themselves to known oral carcinogens such as tobacco and alcohol (9).

Regarding histopathological grading, in the present study 35 (43.75%) cases were WD, 32 (40%) MD and 13 (16.25%) PD; there was the statistically significant difference among the studied groups. These results are in agreement with the results of (13, 14), regarding OSCC but disagree with some other studies which showed that moderately differentiated OSCC were more predominant (11, 12), while other studies showed the high frequency of poorly differentiated OSCC (15). The overall variation in histopathological grading mainly depends on the site of a tumor. The differences could be attributed to the different prevalence of the risk factors worldwide, the intensity of exposure to these factors or both.

Concerning the age, the study found no obvious significant difference among the study groups, the mean age was 59, 58.2 for laryngeal and oral respectively. The results are in accordance with other studies (14, 16).

The study investigated the occurrence of HPV, particularly high-risk HPV 16 and HPV 18 genotypes among Iraqi patients with OSCC and LSCC. The finding showed that HPV infection in the studied cases is quite uncommon (12.5%). Generally, similar findings were obtained from studies applied standard PCR technique in the head and neck region particularly those conducted in Asia and Africa in comparison to higher prevalence in North America and Europe due to geographical variations (17).

According to data collected from the majority of international studies, HPV16 is the most common HPV genotype found in many types of SCC including HNSCCs and cervical SCCs (18). Similarly, in this study HPV 16 was the most common genotype in the OSCC, it was accounted for the overwhelming majority of HPV-positive cases (72.7%), whereas it was not in laryngeal SCC. The statistical association was found between HPV genotype 16 and OSCCs however; it could be established; unfortunately the explanation of this reason is still not well

understood since we were not able to have clear description regarding the other risk factors including tobacco use, alcohol and probably changing sexual behavior, among the study patients. On the other hand, HPV genotype 18 was not detected in any HPV-positive cancers in this study. The extreme rarity of HPV18 in oral and laryngeal SCCs is confirmed in most of the largest studies worldwide and could be explained by the special tropism of virus for the glandular tissue found mostly in adenocarcinomas, which in turn, are rare in the head and neck (19, 20) and occur mainly in salivary gland tumors and cervix (21) which were not included in this study.

Many studies around the world have been reported the presence of HPV in OSCC; its frequency varies greatly, depending on the number of patients and tumor site, as well as detection methods applied (14). The link between OSCC and HPV seems logical, given the viral propensity for epithelial cell involvement (5, 8). An Iraqi study (19), which screened 41 OSCC cases for HPV using a modern generation of in situ hybridization (ISH), the genotyping results revealed that HPV DNA of genotype 16 was detected in 43.7% and HPV 18 (68.7%) of HPV +ve cases. These results disagreed with this study since among (27.5%) of OSCC HPV +ve cases genotype 16 was the predominant (72.7%).

Moreover, using nested PCR, recorded slightly higher genotype 18 than 16, thus study was inconsistent with the current study. In addition, higher HPV prevalence was reported by several studies which applied more advanced nested PCR and real-time PCR techniques since the sensitivities of these methods were much higher than the conventional PCR protocol applied in our study (8).

In India and South East Asia OSCCs is the most common malignancy amounting up to 50% of all malignant tumors. Although most of the OSCC is attributed to tobacco and alcohol consumption, a significant proportion of oral cancers have been demonstrated to contain HPV infection (5). HPV infection was reported in (27%) of oral cancer (23). These results are in accordance with our results. Additionally changing patterns of sexual behavior might represent the most widely accepted explanation for the increase in HPV-positive OSCC (5).

Finally, the IARC (2005) evaluated the carcinogenicity of HPV in humans concluded that there is sufficient evidence for the carcinogenicity of HPV-16 in the oral cavity and oropharynx and limited evidence for HPV-18 in the oral cavity (22).

In this study, HPV18 was not detected; while HPV16 was detected in OSCC cases a finding compatible with the finding of IARC. The present study added weak or no evidence to support the correlation of HPV 16/18 with LSCC as only 1/40 case was positive (2.5%). In European and Asian countries, most squamous cell carcinomas of the larynx result from an exposure to carcinogens, such as tobacco and alcohol, which cause diffuse mucosal changes, but HPV has been found in a good proportion of laryngeal cancers (24%) in a meta-analysis of HNSCC cases (5, 8, 21). In India, the prevalence of HPV infection was found in 34% of invasive LSCC (21); these results of the study suggested that HPV may act as a synergistic factor cooperating with chemical factors (smoking, alcohol, etc) in the multistep of carcinogenesis of laryngeal carcinomas.

## CONCLUSION

The presence of HPV in OSCC suggests that it may play a role in transforming the oral epithelia. The data supported many studies in different geographic areas regarding the increased incidence of HPV

among young age's groups. The current results showed the presence of high frequency of HPV16 in Oral cancer, but there may be the possibility of an involvement of low- risk HPV as a cofactor in the malignant process. There was no significant association between HPV-16 DNA and the demographic data of OSCC (Histopathological grade, sex and age groups).

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## Informed Consent

All authors declare that written informed consent was obtained from the patient (or other approved parties) for publication of this article.

## Competing Interests

We (authors) declare that we have no conflict of interest.

## Disclosure Statement

There is no special financial support for this research work from the funding agency.

## Ethical approval

All authors hereby declare that all experiments have been examined and approved by the appropriate ethics committee from Iraqi Ministry of Health (code: 280000235) and Faculty of Medicine / Misan University (code:88) and have therefore been performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki.

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