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Human Papilloma Virus Types 16 and 18 in a Sample of Iraqis Patients Presented with Oral Cancer

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Abstract

Background Oral squamous cell carcinoma is the most common malignant neoplasm of oral mucosa. Human papilloma (HPV) virus cause a broad scope of diseases from benign to invasive tumors, types 16 and 18 classified as carcinogenic to humans.

Objective To assess the occurrence rate of human papilloma virus genotypes in oral cancer patients and their association with various risk factors.

Methods Fifty five (55) unstimulated whole saliva samples were collected from 35 histopathologically confirmed patients with oral cancer and 20 apparently healthy subjects were enrolled in this study. Genomic DNA was extracted from exfoliate cells to amplify HPV-DNA using HPV-L1 gene sequence primers by polymerase chain reaction method, the viral genotyping was performed using direct sequencing method.

Results The mean age of patients group was 52.23±13.73 years, while in healthy subjects group was (50.55±12.5) years. Risk of smoking was highly significant with odds ratio 60.79 and a 95% confidence interval of 3.40-1086.71. However, the risk of alcoholism was significant with odds ratio 27.77 and a 95% confidence interval of 1.51-511.27. Forty-six percent (16/35) of oral cancer patients were positive for detection of HPV-DNA ($P < 0.0002$). The most frequent HPV genotypes in patients group was HPV-18 accounting for (31%) of cases ($P < 0.05$). The rate of HPV was significantly higher among younger ages (< 50 years) with $P = 0.042$. In addition, the rate of HPV was higher with other variables (male, tongue tissue, grade I differentiation, squamous cell carcinoma) with no significant association ($P = 0.273$, $P = 0.739$, $P = 0.173$, and $P = 0.700$ respectively).

Conclusion Human papilloma virus types 16 and 18 may be a risk factor for oral cancer independent of alcohol and tobacco.

Keywords OC, HPV, OSCC, PCR, Direct sequencing

List of abbreviation: OSCC= Oral squamous cell carcinoma, HPV= Human papilloma virus, OC= Oral cancer, DNA= Deoxyribonucleic acid, L1= late gene 1 (capsid), PCR= Polymerase chain reaction

Introduction

Oral cancer (OC) accounted for 300,000 cases (2.1% of the world total), with two thirds occurring in men. Worldwide, 145,000 deaths occurred (1.8% of the world total), of which 77% were in the less developed regions [(1)]. The established etiological factors of OC included cigarette smoking and heavy alcohol abuse; however, a growing group of patients, including young adults and women, have no known tobacco or alcohol exposure have been emerged, therefore; possible viral etiologic factors such as oncogenic human papilloma virus (HPV) have been proposed [(2)]. High-risk HPV-16 and 18, as etiological agents of anogenital carcinomas, have been firmly

established in the literatures and due to morphological similarities and epitheliotrophic nature of HPV as well as HPV's oncogenic potential, a link between OC and HPV seemed logical [3]. International Agency for Research on Cancer (IARC) has assigned HPV as an independent risk factor since 2007 and that 30%-50% of oral squamous cell carcinoma (OSCC) has been associated with HPV-16 [4]. Over 200 HPV types have been identified in many different human lesions, being categorized as low- and high risk HPVs, depending on their potential to lead the epithelium to carcinogenesis. In the oral cavity, low-risk HPV types 6 and 11 are the most prevalent in benign lesions, as the high-risk types 16 and 18 are respectively the most found in malignant ones. The viral genome of HPV can be sectioned into three parts: an early (E) region, which encodes proteins necessary for viral replication and transcription; a late (L) region, which encodes structural proteins of the viral capsid (L1 and L2); and a non-coding region segment, which contains elements that regulates the viral deoxyribonucleic acid (DNA) replication and transcription [5]. By definition, the nucleotide sequences of the E6, E7, and L1 of a new HPV type should be no more than 90% homologous to the corresponding sequences of known HPV types. HPVs have further been classified into subtypes, when they have 90% to 98% sequence similarity to the corresponding type; and variants, when they show more than 98% sequence homology to the prototype [6]. The estimates of overall HPV prevalence from a meta-analysis study by Petrick *et al.,* (2014), which reported an overall summary HPV prevalence of 29.0% (95% CI: 25.1–33.1%) [7]. The rise in incidence is mostly occurring in individuals aged 40-55 years, without environmental risk factors, and is associated with persistent infection with high-risk HPVs, HPV positive (+) OSCC patients tend to be younger than HPV negative (-) ones. HPV-16 is the most common genotype found in almost 90% of the HPV positive (+) oropharyngeal cancers [8].

The aim of the current study is to detection of the HPV genotypes as an independent risk factor in oral cancer patients.

Methods

Patients:

This case-control design study was approved by the Committee of Ethical Standards in the College of Medicine, Al-Nahrain University and underwent to the terms of Ethical Considerations of the Iraqi Ministry of Health. Thirty five (35) newly diagnosed patients (24 males and 11 females) were histopathologically confirmed with OC by two independent pathologists; these patients were attended to maxillofacial surgery clinic of Ghazi Al-Hariri for Specialized Surgery Hospital in Baghdad were enrolled in this study during the period from April 2014 till April 2015. The inclusion criteria for this study were a) presence of oral cavity cancer (including oral tongue, floor of mouth, gingival, lips, buccal mucosa); b) no previous head and neck cancer; c) no prior oncological therapy. Twenty (20) samples were taken from healthy volunteers whom attend to private clinic for routine dental were collected as control group. The samples processing and DNA extraction was done in Medical Legal Institute/Ministry of Health. Viral detection, PCR and genotyping were done in Central Public Health Laboratory/Public Health Directorate/Ministry of Health.

Saliva Samples:

Up to 5 mL of un-stimulated whole saliva samples taken from each subject and collected in a 50 mL centrifuge tube, which remains on ice while collecting them. The samples were centrifuged at 2,500 rpm for 15 min at 4 °C to spin down exfoliated cells, the saliva supernatant were discarded. Cell pellets were stored at -80 °C until further processing [9].

Genomic DNA extraction:

Viral DNA was extracted from frozen 200 µl of saliva samples (cell pellet) by using AccuPrep® Genomic DNA extraction kit

(Cat# K-3032) according to the manufacturer's guideline (Bioneer, Korea). The concentration and purity of the extracted DNA was quantified by the use of NanoDrop™ 2000 Spectrophotometer instrument following the manufacturer's instructions (Thermo Scientific, USA). The ratio of A260/A280 of wavelength absorbance is calculated, a ratio of ~1.8 was generally accepted as "pure" for DNA [10].

PCR analysis:

HPV-DNA was detected using conventional PCR for HPV-L1 primers (conserved L1 gene in HPV types). Alignments were obtained from the GenBank online BLAST server. HPV-DNA was amplified by PCR assay using primers were designed by using the complete sequence of HPV-L1 gene (GenBank: JX316023.1) as previously demonstrated by Agoston *et al.*, (2010) [11]. *Forward 5'-ACTGGAAAGGTGCTTGTACC-3'* and *Reverse 5'-ACAGGGTTCACAGCCAACAA-3'*, amplicon size 321bp. AccuPower® PCR PreMix Kit (Cat# K-2012) was used to prepare mastermix according to manufacturer's instructions (Bioneer, Korea) as follows: 5 µl of template DNA, 1.5 µl of (10 pmol from both primers), and 12 µl of PCR water. The 20 µl reactions were incubated in Thermocycler (MyGene, Korea). PCR thermocycler condition consisted of initial denaturation incubation at 95 °C for 5 minutes followed by 30 cycles at 95 °C incubations for 30 seconds (denaturation), 58°C incubations for 30 seconds (annealing), and at 72 °C incubation for 30 seconds (extension), finally incubation at 72 °C for 5 minutes for the final extension. Amplification products were analyzed in 2% polyacrylamide gel.

Sequencing analysis:

Amplification products were purified by EZ-10 Spin Column DNA Gel Extraction Kit (Cat# BS353) following the manufacturer's instructions (Biobasic, Canada). Genotyping of HPV was based on direct sequencing PCR fragments by AB DNA sequencing system performed by Bioneer Company in Korea.

Statistical analysis

Mean values were compared using independent samples t-test. Chi-square and Fischer Exact tests were used to study association between any two categorical variables. Correlation coefficient was used to evaluate correlation between numeric variables (e.g. age) and or ordinal nominal variables. Odds ratio statistic was used to assess risk.
P values of less than 0.0001 and less than 0.05 were considered highly significant and significant respectively.

Results

In the present study, the mean age of patients was 52.23±13.73 years with a range of (17-70) years, there was a male predominance among patients group with a proportion of (69%) in comparison to (31%) for female patients (Table 1). Male to female ratio was 1.8:1. Twenty one (21) patients out of 35 were smoker with a proportion of 60%. Meanwhile, alcoholism was reported in only 10 patients accounting for 28.5% where none in the control group were smokers or drinkers (Table 2). Sixteen (16) patients out of 35 had positive HPV-DNA accounting for (46%) while none of the apparently healthy subjects were positive for HPV-DNA. This difference was statistically highly significant ($P < 0.0002$). The approximate odds ratio was 34.69 with a 95% confidence interval of 1.95 to 618.66 as in (Figure 1). The predominant HPV genotype was HPV-18 accounting for (31.43%) and (11.43%) for HPV-16 (Figure 2). Mean age of patients with positive HPV infection was significantly lower than negative HPV infection, 47.13±13.01 years versus 56.53±13.13 years, the rate of HPV was significantly higher among younger ages (< 50 years) with $P = 0.042$ as in (Figure 3). Regarding association of HPV infection with other variables: male predominance, tongue tissue, grade I

differentiation, and squamous cell carcinoma subtype were higher in rates with no significant associations were found with HPV ($P > 0.05$). There were no significant association between tobacco smoking and/or alcoholism with HPV infection ($P > 0.05$) (Table 3).

Table 1. Demographic data of patients and control subjects

Characteristics		Patients group No=35	Control group No=20	P-value
Age (years)	Mean±SD*	52.23±13.73	50.55±12.5	0.654
	Range	17-70	24-74	
Gender	Female	11 (31%)	7 (35%)	1.000
	Male	24 (69%)	13 (65%)	
	Total	35 (100%)	20 (100%)	

*SD= Standard deviation; NS= Not significant

Table 2. Risk factors associated with oral cancer patients

Risk factor	Patients group		Control group		P-value	Odds ratio	95% confidence interval
Smoking	Smoker	21 (60%)	Smoker	0	< 0.0001*	60.79	3.40-1086.71
	Non-smoker	14 (40%)	Non-smoker	20			
	Total	35 (100%)	Total	20 (100)			
Alcoholism	Drinkers	10 (29%)	Drinkers	0	0.009**	27.77	1.51-511.27
	Non-drinkers	25 (71%)	Non-drinkers	20			
	Total	35 (100%)	Total	20 (100)			

**P* value highly significant (< 0.001); ***P* significant (< 0.05).

Discussion

The HPV average rate in OC was 25%, ranging from (<5-100%), in small studies and (1.4-48.8%) in larger ones based on ethno-geographic area, sample size, HPV DNA detections method in tissue and classification of head and neck subsites [12-14].

Current results revealed that (46%) of patients with OC had HPV-DNA which suggest a significant association between them, the results are consistence with similar case-control studies in Iran obtained by (Sahebjamee *et al.,* 2009; Kermani *et al.,* 2012; and Tabatabi *et al.,* 2015) they reported detection of HPV with rate of (40.9%, 42.8%, and 43.9%) respectively among OC cases [15-17]. PCR amplification is a method that consists of multiplying DNA sequences exponentially, making the detection of HPV DNA in human saliva samples easier and more sensitive than other methods, which may explains high rate of HPV-DNA in this study.

In regard to HPV genotypes, the predominant genotype was HPV-18, which is concurrent with a study by Kermani *et al*., (2012) who reported that rate of HPV-18 was (28.6%) followed by HPV-16 with (14.3%) of cases in

Iran [16]. According to many studies, HPV-16 is the most common high risk HPV in HPV related OC [13-15,17,18]. However, the results of few studies have indicated that other high risk HPV types can be the commonest [16,19]. It is difficult to determine the parameters, which make one genotype to be the most predominate due to variation in those parameters such as the type of samples, preparation of samples, sensitivity of the methods used, status of the disease, and geographical regional differences.

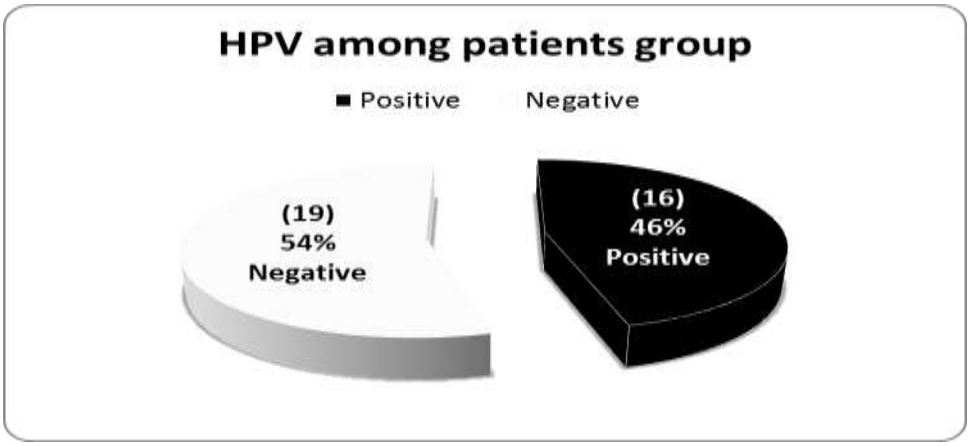

Figure 1. Human papilloma virus among patients group

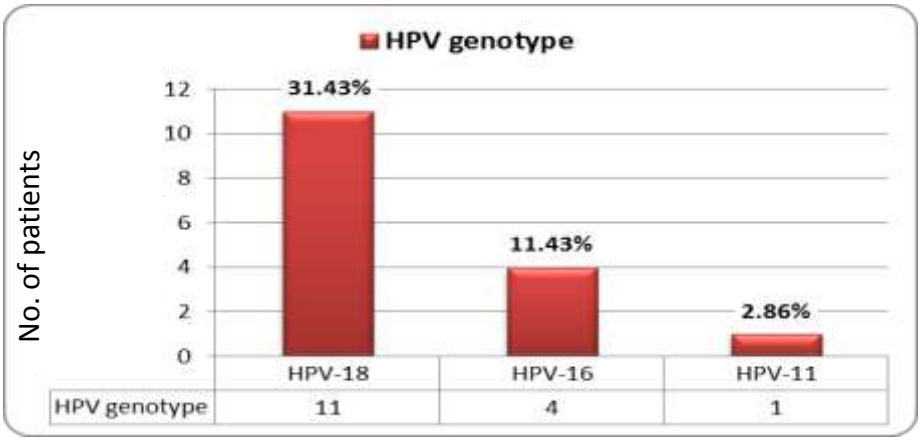

Figure 2: Human Papilloma virus genotypes among patients group

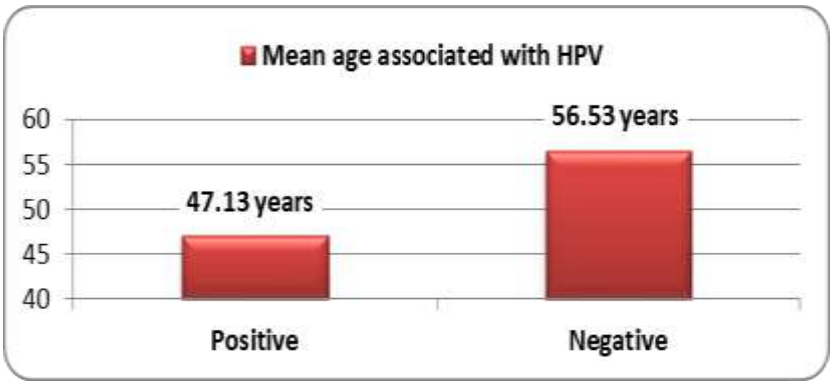

Figure 3. HPV positive cases association with mean age in patients group

Table 3. HPV association with demographic and clinico-pathological parameters

Variables		HPV-positive	HPV-negative	Total	*P* Value
Gender	Male	9 (56.25%)	15 (79 %)	24	0.273
	Female	7 (43.75%)	4 (21%)	11	
	Total	16	19	35	
Tumor location	Tongue	7 (43.75%)	7 (36.85%)	14	0.739
	Others	9 (56.25%)	12 (63.15%)	9	
	Total	16	19	35	
Tumor differentiation	Grade I	13 (81.25%)	11 (58%)	24	0.173
	Grade II	2 (12.5%)	2 (10.5 %)	4	
	Grade III	1 (6.25%)	6 (31.5%)	7	
	Total	16	19	35	
Histological subtypes	SCC	11 (68.75%)	15 (79%)	26	0.700
	Others	5 (31.25%)	4 (21%)	9	
	Total	16	19	35	
Smoking tobacco	Smokers	9 (56.25%)	12 (63%)	21	0.739
	Non-smokers	7 (43.25%)	7 (37%)	14	
	Total	16	19		
Alcohol drinking	Drinkers	6 (37.5%)	4 (21%)	10	0.454
	Non-drinkers	10 (62.5%)	15 (79%)	25	
	Total	16	19	35	

In the current study, patients ages with positive HPV was significantly lower than that of patients with negative HPV. This result was in agreement with Kermani *et al*., (2012) whom demonstrated that mean age of patients with positive high risk was 42.17±5.03 years with age range (35-50) years [16]. Results of the current study disagree with Sahebjamee *et al*. (2009) and Tabatabai *et al*. (2015) according to them, the rate of HPV in older people above 50 years was higher than those aged below 50 years [15,17]. Tonsil and oropharyngeal cancers increased in male predominance over the last 30 years, despite a decline in smoking, which may be linked to the increasing proportion of HPV positive cancers, may be due to changes in sexual activity [20].

In relation to gender, in this study there was male predominance with (69%). This finding was parallels with the results obtained by (Tabatabai *et al*., 2015; Kreimer *et al*., 2011) [17,18]. A possible reason for higher OC ratio in men could be due to higher consumption of tobacco and alcohol products.

Lateral border of tongue representing (43.75%) with HPV positivity was recorded in the present study, which comes in accordance with findings by Kermani *et al.*, (2012) who found that tongue with a proportion of (40%) than other sites [16]. Tongue is preferred topographical location for the OC, an observation in this study come is compatible with the results of previous Iraqi study conducted by Al-Sened *et al.*, (2009), which displayed that tongue was the predominant site in (42.4%) of cases [19]. In contrast to SahebJamee *et al*., (2009), in which, tongue with proportion of (45.45%) than other oral locations with HPV positivity [15]. A study by Kreimer *et al.,* (2011) found that there is site

specific predilection of high risk HPV toward non-keratinized tongue tissue [18].

HPV positivity was among the vast majority of well differentiated grade I tumors accounting for (81.25%; 13 out 16 patients) these results are in agreement with a recent study by Patil *et al.*, (2014) who investigate the correlation of HPV in histological grades of OSCC; well differentiated tumors were the most prevalent in tongue tissue followed by buccal mucosa [21]. The results disagree with Kermani *et al*., (2012) in which majority of patients had advanced stage of the disease grade III with proportion of 64% [16]. Some studies have shown the relevance of HPV positive OSCC and more advanced grade of tumors and nodal metastases. Possible explanation about the current results is the small size of studied group and the bias toward grade I tumors come with fact the tongue site are histologically diagnosed as grade I tumors mostly.

There was no correlation between cigarette smoking and/or alcohol consumption with HPV positive tumors had been found in the current study. These results can be interpreted as that both smoking and/or alcohol consumption association with HPV-related OC are independent risk factor for the OC, however; Gillison *et al*., (2008) discover a weak correlation with the use of marijuana [22]. There is some controversy over the impact of smoking and alcohol consumption on HPV infection in OSCC; it had suggested that additive effects of smoking on HPV positivity in oral cavity and oropharyngeal SCC, and that HPV seropositive smokers have a higher risk for developing OSCC [23,24].

In conclusion, Human papilloma virus types 16 and 18 may be a risk factor for oral cancer independent of alcohol and tobacco.

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Author contributions

Al-Malkey collected the samples, conducted the experimental aspects of the study, and writes the manuscript; Abbas put the concepts of the study design, revised and approved the final version of the manuscript; Yassen did general consultation.

Conflict of interest

Authors declare no conflict of interest

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