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RESEARCHARTICLE

DETECTION OF HUMAN PAPILLOMA VIRUS IN ORAL SQUAMOUS CELL CARCINOMA

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ABSTRACT

Oral squamous cell carcinoma is the most common malignant neoplasm of oral mucosa, representing more than 90%. Tobacco and alcohol has been considered as the classical risk factors. Human papilloma Virus has been proposed as an etiological risk factor since 2007. Thirty five cases diagnosed with OC their ages and gender matched with controls were enrolled in this study. Fifty-five un-stimulated whole saliva samples (35 OC and 20 apparently health subjects) were collected. DNA was purified from exfoliate cells to amplify HPV-DNA using HPV-L1 gene sequence primers by polymerase chain reaction (PCR) method, the genotyping was performed using direct sequencing method. Mean age was 52.23±13.73 years in cases (range: 17-70 years) while in controls was 50.55±12.5 years (range: 24-74 years).Forty-six percent (16/35) of OC patients was positive for detection of HPV DNA (P<0.001). The most frequent type in patient group was HPV-18 type accounting for (31%) of cases (P<0.05). The prevalence rate of HPV was significantly higher among younger ages (<50 years) with P=0.042. In addition the prevalence of HPV was higher with other variables with no significant association: male, tongue tissue, and grade I differentiation, and squamous cell carcinoma (P=0.150, P=0.678, P=0.983, ad P=0.765 respectively).

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INTRODUCTION

Oral squamous cell carcinoma (OSCC) is the most common oral malignancy, representingmore than 90% of the malignant tumors, approximately 263,900 new cases and 128,000 deaths by cancer of the oral cavity are estimated to have occurred in the world in 2008 (Jemal *et al.,* 2011). The Established etiological factors of oral cancer (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 (Rosebush *et al.,* 2011). 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 (Syrjänen, 2003).

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International Agency for Research on Cancer (IARC) has acknowledged HPV as an independent risk factor since 2007 and that 30-50% of OSCC has been associated with HPV-16 (IARC, 2007).All HPVs are small DNA viruses with a genome of around 8 kb that consists of double-stranded circular DNA, and which is enclosed in a 52-55 nm viral capsid. The genome is divided into three regions; the early and late regions, and the non-coding control region (NCCR). The early region encodes the E1-E2, E4-E7 proteins responsible for gene regulation, replication, pathogenesis and transformation (ZurHausen, 2006). In HR HPVs, E6 binding and degradation of p53, and E7 binding and inhibition of the retinoblastoma protein (Rb) result in deregulation of cell cycle control, the late region encodes for L1 and L2, the major and minor viral capsid proteins respectively (ZurHausen, 2006). Incidence of HPV(+) OSCC varies greatly worldwide from 25-80% and it is predicted to increase in the near future. This rise in incidence is mostly occurring in individuals aged 40-55 years, without environmental risk factors, and is associated with persistent infection with HR-HPVs (Chaturvedi *et al.*, 2011). HPV(+) OSCC patients tend to be younger than HPV(-) ones (Lajer *et al.,* 2010). HPV-16 is the most common genotype found in almost 90% of the HPV(+) oropharyngeal cancers.

At present, HPV-16 remains the only HPV type that is classified as cancer-causing in the head and neck (Stransky *et al.,* 2011). The aim of the current study is to detect the HPV genotypes as an independent risk factor in oral cancer patients.

MATERIALS AND METHODS

Patients

This is a case-control design study was approved by the Committee of ethical standards in the Faculty of Medicine, Al-Nahrain University and under went to the terms of ethical considerations of the Iraqi Ministry of Health. Thirty five clinically newly diagnosed patients which were confirmed histopathologically by two independent pathologists with OC their ages and gender matched with 20 apparently healthy subjects 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.Fifty-five saliva samples were collected. 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; and c) no prior oncological therapy (surgery and/or radiation).

Saliva Samples

Up to 5 mL of unstimulated whole saliva samples from each subject was collected in a50 mL centrifuge tube which remains on ice while collecting saliva. The samples were centrifuged at 2,500 rpm for 15 min at 4^{O}C to spin down exfoliated cells, the saliva supernatant were discarded. Cell pellets were stored at -80^{O}C until further processing (Michael *et al*., 2010).

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 wave length absorbance is calculated, a ratio of ~1.8was generally accepted as "pure" for DNA (Wilfinger *et al.,* 1997).

PCR analysis

HPV-DNA was detected using conventional PCR for HPV-L1 primers (conserved L 1 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 (accession No: JX316023.1) as previously demonstrated by Agoston *et al.*, (2010). *Forward* 5'-ACT GGA AAG GTG CTT GTA CC-3' and *Reverse* 5'-ACA GGG TTC ACA GCC AAC AA-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: 5µl of template DNA, 1.5µl of (10 pmol from forward and reverse primers), and 12µl of PCR water.

The 20µlreactions 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 $P<0.0001$ and $P<0.05$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 as in Table (1). Male to female ratio was 1.8:1.

Table 1. Demographic Criteria of the study group

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^{NS}
	Range	17-70	24-74	
Gender	Female	11 (31%)	7 (35%)	0.786^{NS}
	Male	24 (69%)	13 (65%)	
	Total	35 (100%)	20 (100%)	

*SD= Standard deviation; NS= Not significant

Sixteen patients out of 35 had positive HPV-DNA accounting for (46%) none of the control subjects were positive for HPV-DNAas in Figure (1).

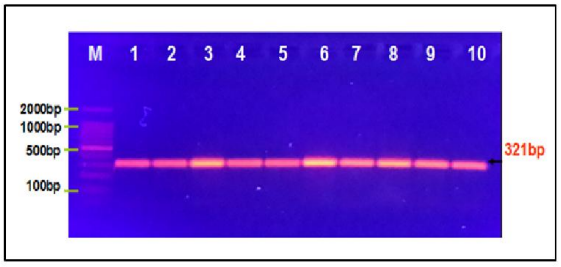

Figure 1. Agarose gel electrophoresis image that show the PCR product analysis of L1 gene in Human papillomavirus positive samples. Where M: marker (2000-100bp), lane (1-10) positive L1 gene at (321bp) PCR product

Table 2. Classification of patients according to HPV infection

HPV	Patients No (%)	Control No (%)	P-value	Odds ratio	95% CI
Positive	16 (46%)	0	<0.001*	34.69	(1.95-618.66)
Negative	19 (54%)	20 (100%)			
Total	35 (100%)	20 (100%)			

*Highly significant P< 0.001

This difference was statistically highly significant (P<0.001) the approximateodds ratio was 34.69 with a 95% confidence interval of 1.95 to 618.66as shown in Table (2).The predominant HPV genotype was HPV-18 accounting for (31.43%) of cases (11/35), HPV-16 and HPV-11 were seen in (11.43%) and (2.86%) of cases respectively as in Figure (2).

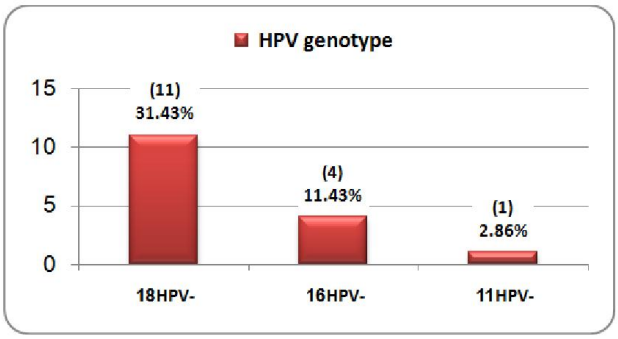

Figure 2. Human Papilloma Virus genotypes among patients group

Mean age of patients with positive HPV infection was significantly lower than that of patients with negative HPV infection, 47.13±13.01 years versus 56.53±13.13 years, the prevalence rate of HPV was significantly higher among younger ages (<50 years) with P=0.042as shown 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 prevalence with no significant association was found with HPV (P=0.150, P=0.678, P=0.983, and P=0.765 respectively) as in Table (3).

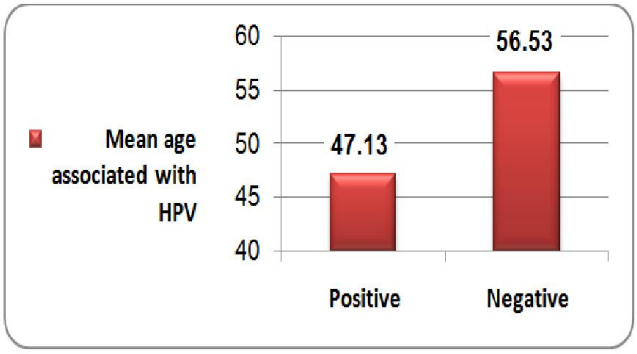

Figure 3. HPV positive cases association with mean age in patients group

DISCUSSION

In world map of cancer prevalence, Iraq stands alongside with countries such as Iran, India, Pakistan, Bangladesh, in southern region of Asia with a prevalence of 20 to 36.3 in each 100 thousand people (Petersen, 2003).

The results of current study suggest a strong association between HPV and OSCC which are in accordance with similar case-control studies obtained by (Saheb Jamee *et al.*, 2009; Kermani *et al.*, 2012; and Tabatabai *et al.*, 2015) they reported detection of HPV with prevalence of (40.9%, 42.8%, and 43.9%) respectively among OC cases in Iran. Possible explanation could be due to the often ulcerating cancer in the oral cavity that might serve as an entry site for the virus when there is exposure. Prevalence of HPV in this study is also in agreement with meta-analysis study carried out by Dayanni *et al.* (2010) in USA; the frequency of HPV related oropharyngeal SCC tumor was (41%) in 5681 patient.

Considerations about viral detection should be kept in mind, the screening of a limited number of genotypes in some studies, is an important factor that may lead to false negative results, and consequently, to an underestimate of HPV role in OSCC. A remarkable example is the study by Kojima *et al.* (2002) in which ISH-tyramide exposed an elevated prevalence of HPV-38 (n=35/53) accounting for 66%, a genotype rarely investigated in other studies. Meanwhile, Pannone *et al.* (2012), who detected genotypes (44, 53, and 70); by DNA sequencing following PCR, but were not successful with the ISH method, since the available commercial kits do not include the corresponding probes: they are primarily designed to detect high-risk genotypes associated with anogenital lesions. It is may be still too early to confirm whether the eight most common HR-HPV types in cervical cancer (16, 18, 31, 33, 35, 45, 53, and 58) are also the most prevalent types in oral, oropharyngeal and laryngeal cancers, because of the lack of studies using HPV testing methods covering most of the mucosal types such as Luminex-based multiplex genotyping, which can detect 100 different HPV genotypes simultaneously.

To the best of our knowledge, the current study is first in Iraq concerning detection of HPV from saliva exfoliate cells by identification of viral DNA with sets of primers based on HPV partial capsid protein (L1) gene sequence, then genotyping was performed using direct sequencing method. Mean age of patients with positive HPV was significantly lower than that of patients with negative HPV, the HPV(+) OSCC rises in incidence is mostly occurring in individuals aged 40-55 years, without environmental risk factors, and is associated with persistent infection with HR-HPV (Chaturvedi *et al.,* 2011), these HPV(+) OSCC patients tend to be younger in age than HPV(-) ones (Lajer *et al.,* 2010). Kermani *et al.*, (2012) in his study demonstrated that mean age of patients with positive HR-HPV was 42.17±5.03 years with age range (35-50) years.

Western studies in Australia, Sweden, Finland and Czech Republic revealed an increasing incidence of OSCC had been observed during the last decade, they declared that, younger age group affected with HPV positive tumors occur frequently in the oropharynx (Syrjanen, 2004; Tachezy *et al.,* 2005; Näsman *et al*., 2009; and Hong *et al*., 2010). Recent studies have revealed high risk sexual behavior including oral sex and multiple sexual partners to be the risk factors for HPV related OC in young ages (Martín-Hernán *et al*., 2013; and D'Souza *et al*., 2014). Meanwhile others reported prevalence of HPV in older people above 50 years was higher than those aged below 50 years (SahebJamee *et al*., 2009; Tabatabai *et al*., 2015).

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

Variables		HPV-positive	HPV-negative	Total	P-Value
Gender	Male	9 (37.5%)	15 (62.5%)	24	P=0.150^{NS}
	Female	7 (64%)	4 (36%)	11	
	Total	16	19	35	
Tumor location	Tongue	7 (50%)	7 (50%)	14	P=0.678^{NS}
	Mouth floor	2 (40%)	3 (60%)	5	P=1.000
	Cheek	1 (33%)	2 (66%)	3	P=1.000
	Maxilla	1 (25%)	3 (75%)	4	P=0.726
	Others	5 (56%)	4 (44%)	9	-
	Total	16	19	35	
Tumor differentiation	Grade I	13 (54%)	11 (46%)	24	P=0.983^{NS}
	Grade II	2 (50%)	2 (50%)	4	P=1.000
	Grade III	1 (14%)	6 (86%)	7	P=0.136
	Total	16	19	35	-
Histological subtypes	SCC	11 (42%)	15 (58%)	26	P=0.765^{NS}
	Others	5 (56%)	4 (44%)	9	P=0.930
	Total	16	19	35	-

NS= non-significant

In relation to HPV genotype, in this study HPV-18 was recorded in men with OSCC slightly higher than female (55% versus 45%). This finding was parallels with the results obtained by (Kreimer *et al.*, 2011; Tabatabai *et al.*, 2015), however; the results disagree with both regarding the HPV subtype, in which HPV-16 was the most predominant (Kreimer *et al*., 2011; Tabatabai *et al*., 2015). The results are contrary to another study in Iran carried out by Kermani *et al*. (2012) in that prevalence of HPV in OSCC in females is higher than males. Therefore, gender cannot be considered as a risk factor, which affecting the HPV prevalence in this study.

Lateral border of tongue representing (43.75%) with predominance of HPV-18 was recorded in the present study, which comes in accordance with findings by Kermani *et al*. (2012) HPV-18 was the most frequent subtype had seen in tongue with a proportion of (40%) than other sites but in contrast to SahebJamee *et al*. (2009) in which the most frequent subtype was HPV-16 has been seen in tongue with proportion of (45.45%) than other oral locations. However; results comes contrary to Tabatabai *et al.* (2015) who reported that the most frequent site of HPV-16 prevalence was the buccal mucosa with proportion of (42.8%). Few investigators found that there is site specific predilection of HR-HPV toward non-keratinized tongue tissue (Ringström *et al*., 2002; Hansson *et al*., 2005).

Concerning grade of tumors differentiation in relation to HPV positivity, the vast majority of the patient group has well differentiated grade I tumors, 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. Meanwhile, the findings of this study are contrary to Kermani *et al*. (2012) in which majority of patients had advanced stage of the disease grade III with proportion of 64%. Some studies have shown the relevance of HPV positive OSCC and more advanced grade of tumors and nodal metastases (Syrjänen *et al.,* 2007; Chaturvedi *et al.,* 2008). 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 (Johnson *et al.,* 2011).

When the histological subtype of the tumors is taken into consideration, 26 out 35 of cases are squamous cell carcinoma (SCC), which was seen in (74.29%), of patients. This finding go with that obtained by (Delavaran *et al*., 2009; Aghbali *et al*., 2011; and Maleki *et al*., 2015) the SCC accounting for (73%, 71.3, 79% and 70%) respectively was the most common among all types of OC in Iran. A study by Johnson *et al*., (2011) have emphasized that OSCC represent 80 to 90% of all oral malignant in developed countries. The SCC presents no difficulty in diagnosis for the experienced pathologist and to an extent the judgement about early invasion is subjective and it can be important for the pathologist to communicate the difficulty in interpretation to the clinician. Some pathologists will indicate that while no unequivocal evidence of invasion is demonstrated, they nevertheless feel that the lesion should be regarded as early invasive carcinoma. In conclusion the present study suggests that HPV 16/18 may be as an independent risk factor in creating OSCC.

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