Detection of Human Herpesvirus Type-1 Antigen in Tissues of Oral Squamous Cell Carcinoma by Direct Immunofluorecent Assay

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Abstract

Background: Human herpesvirus is a large enveloped DNA virus and significant human pathogen. Many studies examined oral squamous cell carcinoma for herpes simplex virus and suggested an association with this virus; others have demonstrated human herpesvirus -1 DNA in different part of oral squamous cell carcinoma.

Objective: To detect the human herpes virus -1 antigen in tissues of oral squamous cell carcinoma.

Patients and methods: Fourty two formalin-fixed, paraffin embedded oral tissues blocks were collected from 30 patients with oral squamous cell and 12 individuals with apparently-healthy oral tissues from archives of histopathology laboratory of college of Dentistry - Baghdad University, during the period from 2010 till 2012. All samples were related to the period between 2004 to 2009. Human herpesvirus -1 antigen was detected by direct immunofluorecent assay (US biological, Cat. No. H2033-08E).

Results: Among oral squamous cell carcinoma group, 26 formalin-fixed, paraffin embedded oral tissues blocks were found to contain HHV-1 antigen, this result constituted 86.7% of the total oral squamous cell carcinoma screened for HHV-1 antigen and 75% within apparently-healthy oral tissues. The age of patients ranged from (25-70) years with mean of 53.26 ±12.1years. The highest percentage 60% was diagnosed in the age above 50 years. The percentage in males (61.68%) was more than in females (38.31%). On the other hand there was no significant difference between viral infection, age and gender distribution, while significant correlation noticed with tumor differentiation.

Conclusion: The detection of human herpesvirus -1 antigen in oral squamous cell carcinoma and apparently healthy control indicates virus with other factor such as chemicals and radiation, which play important role in the development of oral cancers

Key word: Oral squamous cell carcinoma, human herpesvirus type1, immunofluorescence.

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Introduction

Oral cancer is a significant cause of morbidity and mortality and consistently ranks as one of the top ten cancers worldwide, with broad differences in geographic distribution [1]. The majority of these cancers are squamous cell carcinoma. More than 274,000 new cases of oral squamous cell carcinoma (OSCC) are being diagnosed worldwide annually [2]. Usually is a neoplastic disorder in the oral cavity, which includes the following areas: lip, buccal mucosa, lower and upper alveolar ridges, retromolar gingiva, oropharynx, floor of the mouth, hard palate, and the anterior two thirds of the tongue [3].

Squamous cell carcinoma progresses from individual epithelial cell changes (atypia), to a generalized disturbance of the epithelium (dysplasia), then to carcinoma in situ, and finally to invasive SCC [4]. The first confirmations for associations of OSCC with infectious agents especially viruses have been proposed nearly two decades ago by some investigators [5, 6, 7].

Human herpesvirus type-1 (HHV-1) is a large, double-stranded DNA virus that predominantly infects the oral mucosa and causes oral 'cold' sores. The virus lives latently in the neurons of infected individuals and is thought to reactivate regularly and asymptomatically. The persistence of the virus in the oral cavity and its ability to stimulate host DNA synthesis and repair during reactivations suggest that it may contribute to OSCC development [8]. Human herpesvirus type has been associated with oral carcinomas, but much of the evidence is incidental. The DNA from human herpesvirus has been extracted from the tissues of some tumors but not from others, human herpesvirus type may aid carcinogenesis through the promotion of mutations, but the oncogenic role, if any, is uncertain [9].

Indeed, in vitro studies have clarified specific mechanisms through which human herpesvirus type-1 may induce the transformation of human cells; HHV1 infection of human cell cultures has been shown to be mutagenic, to induce DNA synthesis [10]. And to inhibit apoptosis, all of which may contribute to carcinogenesis [2]. Animal experiments also support the hypothesis that human herpesvirus contributes to tumor development [11].

Therefore, this study was designed to detect human herpesvirus type-1 glycoprotein C antigen in tissue sections of oral squamous cell carcinomas and apparently healthy control, and to find out the relationship between expressions of human herpesvirus type-1 glycoprotein C antigen and different parameters like; age, grade and tumor differentiation.

Patients and Methods

Samples collection: A total number of 42 oral tissues were enrolled in this retrospective study, from the archives of histopathology laboratory of College of Dentistry - Baghdad University, 30 formalin-fixed, paraffin embedded oral tissues blocks were obtained from patients who had undergone surgical operation or biopsies from OSCC during the period from 2010 till 2012. All samples are related to the period between 2004 to 2009. They were 16 (males) and 14 (females) and their age ranged from 25 to 70 years. According to Broder's grading, 22 patients had well differentiated squamous cell carcinoma, 8 patients had moderately differentiated squamous cell carcinoma, while poorly differentiated squamous cell carcinoma not included [12]. In addition, 12 oral tissues blocks without any significant pathological changes (apparently healthy), they were 7 males and 5 female and the range of the age was the same as patients group were included as a control group for this study.

Tissue processing: Hematoxylin and eosin-stained slides were reviewed in all cases; unstained paraffin sections for detection of human herpesvirus-1 Glycoprotein C which target of HHV-1 Ag (United States biological, Cat. No. H2033-08E). By used direct immunofluorescence assay according to manufacturer's protocol.

Direct Immunofluorescence: As recommended by the manufacturer instruction (US biological), the slides were deparaffinized and rehydrated by xylene and serially graded alcohol for 5 minutes each and then distilled water. The slides were rinsed 3 times with cool phosphate buffer saline (PBS), and left to dry at room temperature, then blocked with blocking buffer (1-2% Bovine Serum Albumin) at room temperature for 2 hours. The slides were washed and left to dry at room temperature. All slides were treated with fluorescent-tagged primary antibody (dilution 1:10 with blocking buffer), then incubated overnight in refrigerator at 4˚C. The slides were washed, and then examined by used fluorescence microscopy. The slides were considered as a positive when the specimen contained one or more cells displaying HHV-1 specific fluorescence (apple-green fluorescence) and considered negative when there is no specific fluorescence. In each run used two types of controls, positive and negative controls. Positive control, Consisted of two patients having infection with herpes labialis, and a swab were taken from the site of infection and was put in a charged slide and the same procedure was done. Negative control: Two slides were prepared as the procedure of IF to the whole samples but one slide was prepared by putting sample without using the antigen, but instead of that we used the bovine serum albumin, while the other slide was prepared by using distilled water instead of the sample.

Statistical analysis

Fisher's exact test and student's t-test were used to obtain statistically significant differences between studied group with $p<0.05$ being considered statistically significant.

Results

As show in table (1), the mean age of patients with OSCC was 53.26 ±12.1years-old compared with control group 59.7 among patients minimum age was 25 years and maximum 70 years, there was no significant differences (P>0.05) noticed between both groups.

Table (1): Mean age distribution among the studied groups.

Studied groups	Number		Age/ years		(t-test)/ P-value*
		Mean	Mini	Maxi	
Healthy Control group	12	59.7±8.9	48	70	P>0.005
OSCC	30	53.26 ±12.1	25	70	
Total	42				

***Non- Significant**

Respect to age groups, majority of the samples was reported at age above 50 years and they are accounted 18 (60%) while less than 50 years was 12 (40%). No significant difference was noticed between them as shows in table (2).

Table (2): Distribution of patients according to their age strata.

Age stratum	Number	Percentage	Comparison of Significance	
			Chi^2-value	P-value
≤ 50	12	40%	0.27	Non-Sig.
> 50	18	60%		
Total	30	100%		

The results of current study demonstrated that percentage of OSCC within males 16 (53.33%) was more than females 14 (46.66%), but statistical analysis showed insignificant difference at P>0.05 as shows in table (3).

Table (3): Distribution of patients according to their gender**.**

Gender	Number	Percentage	Comparison of Significance	
			Chi^2-value	P-value
Male	16	53.33%	0.71	Non-Sig.
Female	14	46.66%		
Total	30	99.99%		

Relative to differentiation, results indicated that the highest percentage of the study sample was grade I (will-differentiation), and they are accounted for 22(73.33%). Followed by grade II (moderately differentiation) and they are accounted for 8(26.66%). Statistically significant difference at (P<0.05) was found as shows in table (4).

Table (4): Distribution of patients according to tumor grade.

Tumor grade	Number	Percentage	Comparison of Significance	
			Chi^2-value	P-value
Grade I	22	73.33%	0.01	P<0.05
Grade II	8	26.66%		
Grade III	0	0		
Total	30	99.99%		

As shown in table 5 and Figure 1, positive expression of HHV-1 was detected in oral squamous cell carcinoma tissue in 26 out of 30 cases (86.7%). While 9 out of 12 cases (75%) cases were positive within healthy control group. No significant difference was noticed between patients and control group.

Table (5): HHV-1 positivity rate by DIF among study groups**.**

DIF results	Patient (n=30)	Control (n=12)	Comparison of Significance	
			Chi^2-value	P-value
Positive-HSV-1	26(86.7%)	9(75%)	0.35	Non- Sig.
Negative-HSV-1	4(13.3%)	3(25%		
Total	30(100%)	12(100%)		

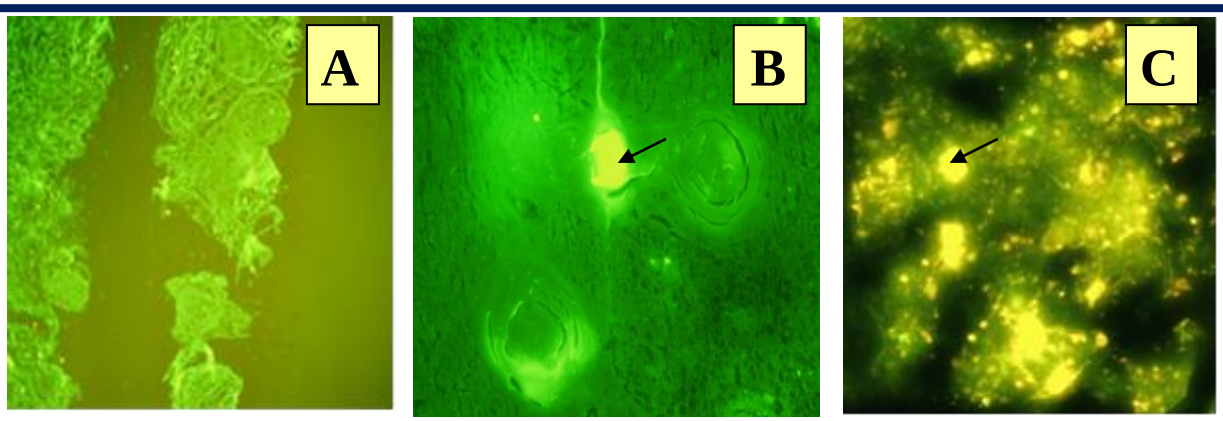

Figure (1): Direct Immunofluorescence in OSCC section, A- Negative expression B- positive expression, C- positive control.

Direct immunofluorecent results for HHV-1 are summarized in table 6. Showed that positive and negative expression of HHV-1 with different Parameters, no significant difference was noticed between expression of HHV-1 and different variable like age, gender and tumor grade.

Table (6): HHV-1 positivity rate by Direct Immunofluorescence assay according to age, gender and grade.

Variables	Positive	Negative	Comparison of Significance	
			Chi^2-value	P-value
Age			0.66	P>0.05
≤ 50	16(61.53%)	2(50%)		
>50	10(38.46%)	2(50%)		
Gender			1.30	P>0.05
Male	12(46.15%)	0		
Female	14(53.84%)	4(100%)		
Grade				
Grade I	20(76.92%)	2(50%)	0.28	P>0.05
Grade II	6(23.07%)	2(50%)		
Grade III	0	0		

Non – Sig. (P>0.01)

Discussion

The present study done in patient with OSCC. According to age group, majority of the patients was reported at the age above 50 years. These results are in agreement with the finding of Neville *et al*.,(2002) who reported that 90% of their patients with oral cancers were over 40 years of age [11].

The relationship of oral cancer development with age could be explained by the long chance of exposure to environmental carcinogens such as chemicals, radiation and viruses, which were regarded as an important stimulating factors in the development of oral cancers [13].

In addition, the observed weakening in the immune system in such ages, due to the senescent decline in the immune surveillance, might lead to accumulation of cellular DNA mutations that could be regarded as an additional significant factor in the development of such malignancies [14].

Regarding gender distribution, the results revealed that the OSCC was higher in males than females, but statistical analysis didn't show significant differences. These results

were in agreement with study of Khashman *et al*., (2008) who indicated that, no significant difference between males and females. Also was in agreement with other study [16]. However, the difference in the male to female ratio has become less noticeable over the past half century, probably because women have relatively been more equally exposed to the known oral carcinogens such as cigarette smoking and alcohol drinking. Moreover, the impact of stress and increasing numbers of females who are working in factories are exposing themselves to carcinogen can be considered as another factors [11, 17].

According to tumor grade, the present study demonstrated that most OSCC cases occurred within Grade I (will differentiation). These results were in agreement with study of Khashman *et al*.,(2008) who indicated the high percent within will differentiation OSCC [15]. These results are disagreement with the findings of a number of investigators had showed a higher frequency for moderately differentiated OSCC grade [18, 19], while not record any cases within poorly differentiated, this disagreement with study done by Helliwell (2003) who had described an equal frequency for poorly and well differentiated grades [20].

Recent study revealed that HHV-1 was found in (86.7%) of OSCC and (80%) in healthy control, This result in agreement with other researchers, who found no differences in HHV-1 seropositivity between OSCC cases and matched healthy controls [21,22]. And disagree with early epidemiological studies that showed OSCC cases have higher levels of HHV-1 antibodies than healthy controls [23, 24].

Statistically the results of HHV-1 express in both OSCC cases and apparently healthy cases show no significance with P-value >0.05, which means the HHV-1 does not increase the risk of OSCC in the absence of other risk factors, this is compatible with Parker *et al.*, (2006), who suggested that patients with head and neck cancer who reported oro-genital contact were compared with those who did not [25]. Other study done by Murrah *et al.* (1996), who indicated that HHV1 and smokeless tobacco may have synergic effect in oral carcinogenesis [26].

The oncolytic effects of herpes simplex virus-1 are limited, possibly because of premature death of infected cells by apoptosis, which limits the amount of progeny virus that is produced. It has been proposed that inhibition of apoptosis in infected tumor cells would allow increased viral persistence, replication and therapeutic effect [27].

Few investigators have reported an interaction between HHV-1 and non-viral risk factors, such as cigarette smoking and alcohol use, or between HHV-1 and other suspected viral risk factors such as human papilloma virus (HPV), laboratory studies show that HPV may also depend on the action of cofactors to transform cells to a malignant phenotype. Human herpesvirus-1 could potentiate the HPV-induced transformation of cells [28]. The basic science literature suggests, however, that HHV1 may particularly influence OSCC development when other risk factors are present [29, 30].

The presence of high percentage of HHV1 in normal control can be explained by the fact that in all cases HHV infection is not removed from the body by the immune system. Following a primary infection, the virus enters the nerves at the site of primary infection, migrates to the cell body of the neuron, and becomes latent in the ganglion [31]. Moreover, at least 70% of the population shed HHV-1 asymptomatically at least once a month, and many individuals appear to shed HHV-1 more than 6 times per month. Shedding of HHV-1 is present at many intraoral sites, for brief periods, at copy

numbers sufficient to be transmitted, and even in seronegative individuals [32].

In conclusion, the detection of human herpesvirus -1 antigen in oral squamous cell carcinoma and apparently healthy control indicates virus with other factor such as chemicals and radiation, which play important role in the development of oral cancers, unrelatedly of age and gender distribution, our study revealed that there is no significance of the expression of HHV1 antigen and both parameters. Further studies with large sample size are needed. Studying the role of other viruses in tumergenesis of OSCC

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