Localization of Human Cytomegalovirus- Late Gene DNA, Expression of P53 Gene and CD8-Tumor Infiltrating Lymphocytes in Oral Squamous Cell Carcinoma.

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ABSTRACT:

BACKGROUND:

Molecular DNA hybridization has confirmed an association of CMV infection with a variety of oral cancers. While cell mediated immunity is most important in controlling primary or reactivated CMV infection, this virus has one of most effective strategies in oral carcinogenesis via impairment of structure and function of P53 protein by interaction with viraloncoproteins.

OBJECTIVE:

1.To determine the percentage of CMV-infected oral squamous cell carcinoma tissue specimens; 2. To evaluate the histopathological impact of the expression of mutated p53 tumor suppressor gene on CMV- related, as well as CMV-non-infected oral cancer; and 3.To through a light on some immunological microenvironment of OSCC,by assessing CD8-positive tumorinfiltrating cytotoxicT-lymphocytes.

METHODS:

This study was designed as retrospective research. A total number of seventy (70) formalin-fixed paraffin-embedded oral tissues were collected; 60 patients with oral squamous cell carcinoma, and 10 individuals with apparently-healthy oral tissues. The molecular methods for CMV DNA detection was performed by sensitive version of in situ hybridization, whereas the phenotype of cell surface antigen marker, namely CD8+ marker of tumor infiltrating lymphocytes and TP53 protein were detected via relevant immunohistochemical methods.

RESULTS:

Well differentiated grade constituted 81.7% of oral squamous cell carcinoma. Positive in situ hybridization reactions for CMV-DNA were observed in 43.3% of the total screened tissues. Thirty-three out of sixty (55%) oral squamous cell carcinoma showed positive immunohistochemical reactions indicating P53 over-expression, and 18.3% showed presence of CD8-positive tumor infiltrating lymphocytes. None of those control group showed positive reaction for CMV-DNA, p53, or CD8 marker.

CONCLUSION:

The detection of high percentage of cytomegalovirus-DNA in OSCC could mark for a parentral way of spreading of such important and well-known sexually transmitted infection among Iraqi general population.The obvious high percentage of mutated p53 over-expression indicates for an important role of such genetic events in the oral carcinogenesis.A little role for CD8-positive tumor infiltrating lymphocytes could be played in the immunological microenvironment of OSCC.

KEYWORDS: oral squamous cell carcinoma; human cytomegalovirus; in situ hybridization; P53; CD8 marker , tumor infiltrating lymphocytes.

INTRODUCTION:

Oral cancer refers to a neoplasm involving the oral cavity , from the lips to the ends of anterior pillar of the fauces[1]. Oral cancers account for only 2 % of all malignant tumors in In UK and USA, 40 % or more in India and Srilanka[2]and about 4.5 % in Iraq, where oral squamous cell carcinoma (OSCC) accounts for 91.5 % of all oral cancers and 37 % of the head and neck cancers[3].

The tongue and the floor of the mouth are most common sites for oral cancer in the United Kingdom. In contrast to India, buccal mucosa is most common part[4]. In Iraq, the most common site is lower lip , then tongue and lower gingival[3].

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Male to female ratio of OSCC is 3 : 1[5]and about 95% occur in people older than 40 years , with the average age approximately 60 years[4].Oral SCC is multi-factorial disease with both extrinsic and intrinsic factors may be at work such as tobacco smoke; alcohol; syphilis, some Candida strains; AIDS and those who are undergoing immunosuppressive therapy; and general malnutrition or iron deficiency anemia[4,5] .

One of the physiological roles of tumor suppressor gene p53 is to prevent malignant transformation and p53 protein has inhibitory effect on transformed foci by various oncogenes and it can suppress the growth of variety of cancer cell lines[6].Functional inactivation of p53 tumor suppressor gene is a key step in carcinogenesis or progression of many human malignancies[7]. The inactivation of p53 tumor suppressor activity can be caused either at the gene level by mutation , deletion and rearrangement[8], or at protein level by binding to oncogenic protein , such as Mdm – 2[9]. The inactivation of p53 function could also be involved in the process of cellular transformation[10].

It was reported that 30– 70% of head and neck cancers showed inactivation of p53 proteinwhile the p53 gene is mutated in over one half of cases of oral cancer. Although the observed over-expression is usually a consequence of mutations in the gene , a small number of cases shows over-expression of the wild type(wt) p53 gene[11].

T- cell mediated immunity to tumor antigens is analogous to the body response to other T-dependent antigens.Tumor infiltrating lymphocytes are T lymphocytes , often CD8-cytotoxic T lymphocytes(CTLs) . They also include some CD4 T cells and NKT cells[12].

The CD8-CTLS recognize only antigen associated with class I MHC molecules, and since malignant transformation of cells is often associated with a reduction (or even a complete loss) of class I MHC molecules, a profound effect on the CTL-mediated immune response is exerted . Thus decrease in class I MHC expression is often accompanied by progressive tumor growth and a poor prognosis[13].

CMV genome is sufficiently large to encode over 200 proteins via 204 predicted open reading frames(ORF) . Of these , there was a direct evidence for a role of ORF 79 in transformation[14]. Human CMV infections havebeen implicated in etiology of several human malignancies, such as colon cancer, malignant glioma, adenocarcinoma of the prostate, EBV - negative Hodgkin's disease, Kaposi's sarcoma, Wilm's tumor and neuroblastoma, breast carcinoma, cervical carcinoma, and carcinoma of oral cavity[15,16].Many molecular methods are available for identification of nucleic acids of HCMV. Of these, in situ hybridization (ISH) can be used with frozen cells and tissues , cytological preparations and fixed tissues, using radioactive- labeled probes or probes with non-radioactive labels such as fluorescent moieties , biotin , digoxigenin or enzyme conjugated probe[17].

MATERIALS AND METHODS:

Study Groups:

This study was designed as a retrospective research. Therefore, subjects included in this study were represented by their oral tissue samples that were obtained as archival tissue blocks. During the period from October 2008 till April 2009, a collective number of (70) formalin-fixed, paraffin embedded oral tissue blocks enrolled in this study which comprised both patients and control samples. These retrospective paraffin-embedded samples were retrieved from the archives of the period 1998- 2008 belonging to Dental Clinics and Departments of Oral Diagnosis and Oral Histopathology /College of Dentistry/ BaghdadUniversity. These blocks included:

Agroup of (60) OSCC patients who had undergone surgical operation or biopsies .Their data were obtained from the attached histopathological reports including age, gender, provisional clinical and histopathological diagnosis.Following trimming process of these tissue blocks , a second confirmatory histopathological re – evaluation of each obtained tissue blocks was done in the College of Dentistry / Department of Oral Histopathology . Another group of [10] tissues with normal histological appearance (i .e. without any significant pathological changes that were taken from oral mucosal tissues of cheek and / or gingiva and/ or lip during surgical operations for oral lesions and from those around sound surgically extracted teeth for orthodontic purposes) were properly subjected to fixation as well as paraffin embedding and used for this research work as an age- and sex- matched healthy control group.

METHODOLOGY:

TISSUE SECTIONING AND SLIDE PREPARATION:

At the histopathological department of Teaching laboratories / Medical City, tissue sectioning was conducted. In order to prevent carry-over DNA contaminations from one tissue sample to another, only one disposable cutting knife, which was specified for each tissue block, was used and then each section was sticked on a single charged slide. The first tissue section was mounted on ordinary slide and specified to be used for Hematoxyline and Eosin staining. In addition , three subsequent 4 µm thick-paraffinized tissue sections were mounted on charged slides , one of them to be used for in situ hybridization for detecting DNA of CMV , whereas the second and third slides were specified for immunohistochemistry technique for detecting p53 protein and CD8 marker .

Procedures of cytomegalovirus in situ hybridization:

Molecular detection of CMV DNA in those tissue blocks was performed by recent high - sensitivitygeneration of in situ hybridization (ISH):(1) Using abiotinylated long- DNA probe for HCMV (Strain AD 169;Specific Gene Size : 400 bp; Sequence Alignment on database : Gene bank ; M 15120)(was purchased fromMaxim Biotech , USA ; Cat . Number : pB – 60409, 2009); (2)Using complete hybridization & immunodetection kit for CMV – DNA strain (AD 169) gene probe (was purchased from Maxim Biotech , USA ; Cat. No. IH -60001 ;IHD – 00S0).

The probe was prepared as 8 % working dilution by mixing 8 µl from the concentrated probe with 92 µl of the hybridization solution. The 3 main procedures of in situ hybridization of CMV:(1) pre-hybridization,(2) hybridization, and (3) post-hybridization were carried out in accordance with the manufacturer's instructions that were listed in details in[18].

For proper quality control of achievement of in situ hybridization assay, different controls have been used. Positive control tissue slides were prepared from many malignant tissues that previously tested positive for CMV by in situ hybridization technique, were used as positive control. Positive probe control slides of oral squamous tissues that were processed in manner identical to the test sections , but were hybridized with biotinylated housekeeping gene probe. Negative probe control slides with oral squamous tissues were processed in an identical manner but adding 20 ul PBS instead of using probe.

Immunohistochemical preparation and staining procedures for detection of p53 protein:

Monoclonal Mouse Anti – Human p53 protein antibodies (Isotype : IgG 2a); Immunogen : Recombinant human wild type p53 protein expressed in E-coli (Clone No.: O.N.496), Code number : P1001 – 32 C (US Biological , USA). The procedures of immunohistochemistry were done according to the manufacturer instructions that were listed in details in[18].

In each immunohistochemistry(IHC) run , a positive and negative control sections were included along the work for quality control: .The IHC assay included a positive control tissue slides that were prepared from OSCC sections known to be immunoreactive for p53 antibody were used as positive control for p53. The negative control slides included sequential omission of the reactive components in the test , primary (monoclonal) antibody , the secondary antibody(the biotinylated link), the conjugate and the substrate .

Immunohistochemical staining procedures for immunophenotyping CD8 positive T – lymphocytes:

The kit contains: (A)monoclonal Mouse Anti – Human CD8 protein antibodies (Isotype : IgG , kappa) .;Immunogen : synthetic peptide corresponding to the 13 c – terminal amino acids of cytoplasmic domain of human CD8 α coupled to thyroglobulin(Clone No . : C8 / 144 B), Cat. code number: M 7103,(CA Institute , USA); (B) Detection System for CD8; Universal Dakocytomation Labelled streptavidin - Biotin 2 system , Horseradish peroxidase (LSAB- 2 system , HRP) ready to use detection system , Cat. Code number: K0673(Dako Corporation . Denmark). The IHC procedures were done according to the manufacturer instructions that were listed in details in [19].

STATISTICAL ANALYSIS:

T test, ANOVA test, and Chi square were applied for statistical examination of results obtained in our research. All these statistical analysis were done by using Pentium-4 computer through the SPSS program (version-10) and Excel application.

RESULTS:

1.Clinico – histopathological and demographic aspects of patients with oral squamous cell carcinoma : -

A total of sixty (60) Iraqi patients with oral squamous cell carcinoma(OSCC) were enrolled in this study. The mean age of these patients with OSCC was (56.4)years.Forty two (42) out of 60 (70%) of patients were above the age of 50 years.According to site distribution of OSCC ,

the tongue mucosa was the most affected site 28 .3 % (17 out of 60) of cases, then followed by other sites of oral cavity (Table 1). The sex distribution has showed that 61.7% (37out of 60) of OSCC patients were males while the rest 38.3 % (23 out of 60) of cases were females.In respect to OSCC grading, the well differentiated OSCC was the most predominant type (81.7 %)followed by poorly differentiated OSCC (comprised 11.7 %) then moderately differentiated OSCC(comprised 6.7 %) of the studied cases. However, no statistical differences were noticed regarding age, gender, site and grade factors (P values more than 0.05).

Table1: Distribution of oral squamous cell patients according to their age and the site at diagnosis.

		AgeGroups					
Sites		- < 49	50-59	60-69	70-79	80 +	Total
Buccal	Count	2	1	1	3	1	8
	% within Site	25.0%	12.5%	12.5%	37.5%	12.5%	100.0%
	% within AgeGp	11.1%	7.7%	7.7%	33.3%	14.3%	13.3%
Alveolar	Count	0	0	1	1	1	3
	% within Site	.0%	.0%	33.3%	33.3%	33.3%	100.0%
	% within AgeGp	.0%	.0%	7.7%	11.1%	14.3%	5.0%
Palate	Count	3	2	3	0	1	9
	% within Site	33.3%	22.2%	33.3%	.0%	11.1%	100.0%
	% within AgeGp	16.7%	15.4%	23.1%	.0%	14.3%	15.0%
Cheeck	Count	1	1	2	0	2	6
	% within Site	16.7%	16.7%	33.3%	.0%	33.3%	100.0%
	% within AgeGp	5.6%	7.7%	15.4%	.0%	28.6%	10.0%
Tongue	Count	7	2	5	1	2	17
	% within Site	41.2%	11.8%	29.4%	5.9%	11.8%	100.0%
	% within AgeGp	38.9%	15.4%	38.5%	11.1%	28.6%	28.3%
Maxilla	Count	3	2	1	0	0	6
	% within Site	50.0%	33.3%	16.7%	.0%	.0%	100.0%
	% within AgeGp	16.7%	15.4%	7.7%	.0%	.0%	10.0%
Lips	Count	1	2	0	3	0	6
	% within Site	16.7%	33.3%	.0%	50.0%	.0%	100.0%
	% within AgeGp	5.6%	15.4%	.0%	33.3%	.0%	10.0%
Other	Count	1	3	0	1	0	5
	% within Site	20.0%	60.0%	.0%	20.0%	.0%	100.0%
	% within AgeGp	5.6%	23.1%	.0%	11.1%	.0%	8.3%
Count	18	13	13	9	7	60	
% within Site	30.0%	21.7%	21.7%	15.0%	11.7%	100.0%	
% within AgeGp	100.0%	100.0%	100.0%	100.0%	100.0%	100.0%	

2.Cytomegaloviral – Infected Oral Squamous Cell Carcinoma :-

In this study, 43.3 % (26 out of 60) of oral SCC tissue blocks showed a positive signals for CMV- late gene DNA using ISH technique (figure 1). Statistical analysis showed significant difference in comparison to control group ($P<0.05$).

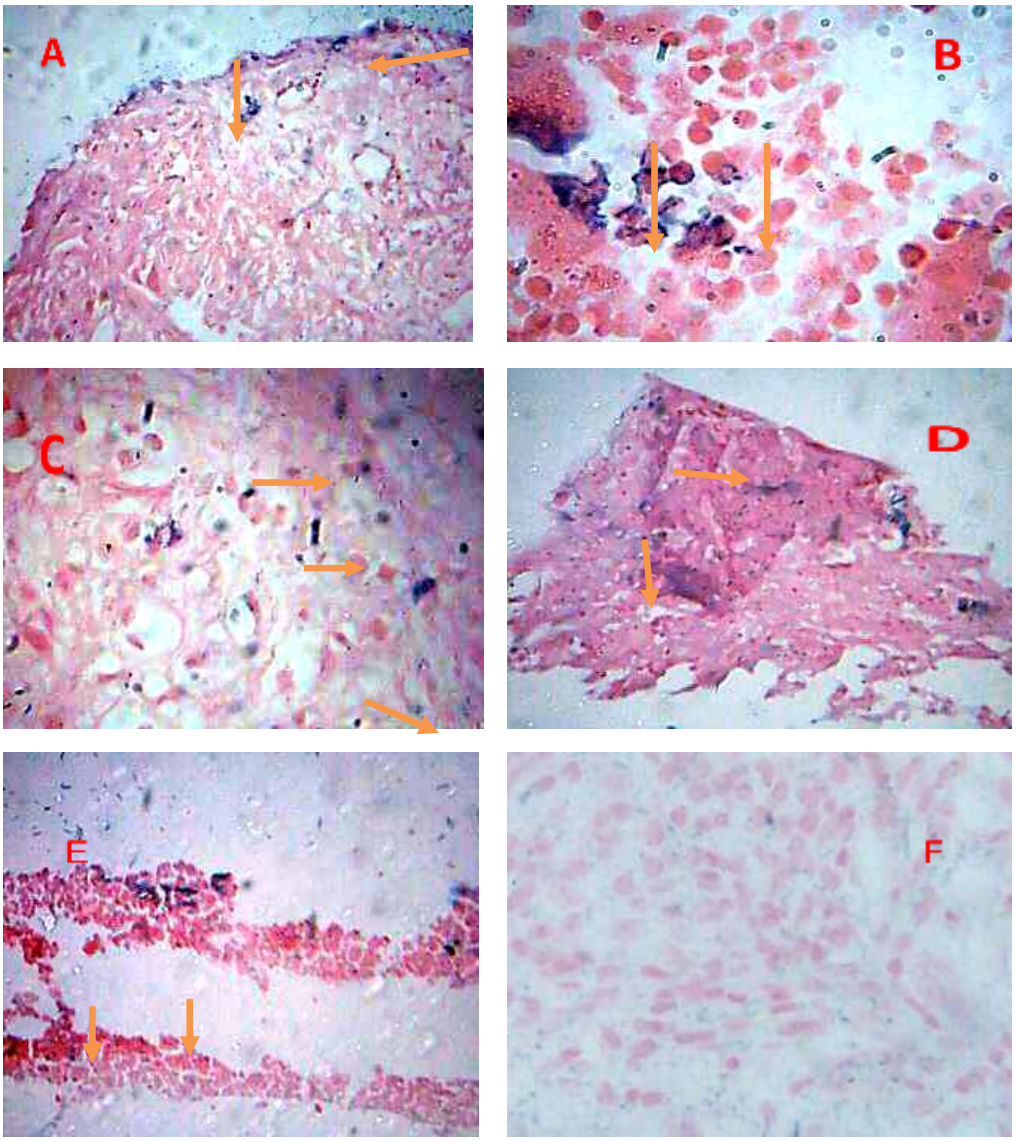

Figure 1:In situ hybridization results for cytomegalovirus- late gene DNA detection in oral squamous cell carcinoma (OSCC) tissues. The BCIP/NBT produced (dark blue) signals while the counter staining by nuclear fast red produced (pink) color ; (A): Poorly differentiated OSCC with positive ISH reaction (20x) . (B): Poorly differentiated OSCC with positive ISH reaction (100x). (C): Poorly differentiated OSCC with positive ISH reaction (40x).(D): Moderately differentiated OSCC with positive ISH reaction (20x).(E):Moderately differentiated OSCC with positive ISH reaction (20x) .(F): Poorly differentiated OSCC with negative ISH reaction (40x).

3. P53-Tumor Suppressor Gene Over Expression In OSCC Patients

In the present study, immunostaining for p53 protein was not detected in any tissue in the apparently healthy control group where as positive immunohistochemical (IHC) reactions related to mutated - p53 overexpression was detected in 55 % of the cases with OSCC (33 out of 60) as the rest 27 cases (45 %) were considered to have negative IHC reaction for p53 overexpression either because of the complete absence of any nuclear staining or having only ≤5% positive cells(figure 2).

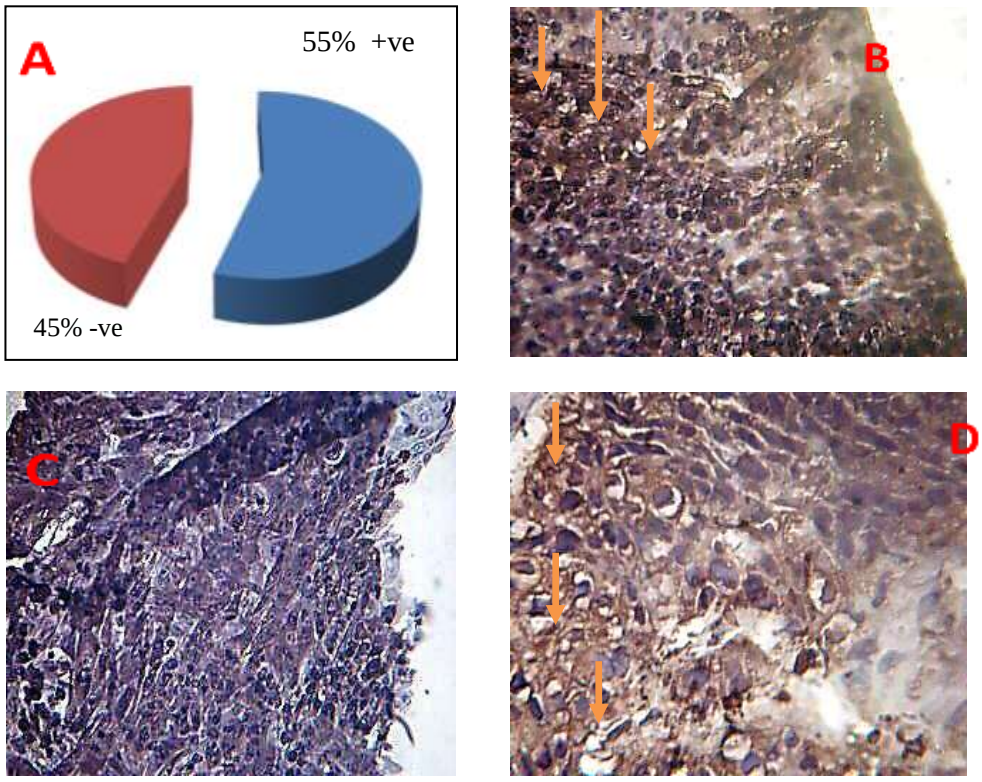

Figure 2: Immunohistochemical results for p53 gene overexpression detection in oral squamous cell carcinoma tissues ; DAB chrormogen stained(brown signals) and counter stained with Mayer's hematoxyline (blue color); (A): The percentage of IHC reactions for p53 protein. (B): Moderately differentiated OSCC with positive IHC reaction (40x). (C): Moderately differentiated OSCC with negative IHC reaction (20x).(D): Poorly differentiated OSCC with positive IHC reaction (40x) .

In this study, the percentage of mutated p53 over-expression in tissues that were simultaneously infected with HCMV was found to decrease with the proceeding of the grading of cancer. Their counterparts OSCC tissues that, neither shared mutated p53 over-expression, nor CMV-DNA infection, were also found to have a similar trend with decreasing of grades of OSCC. Statistical analysis showed no significant differences among these groups (P>0.05) (Table 2).

Table 2: The co-existence of CMV-DNA & P53 over-expression in relation to grade of oral squamous cell carcinoma .

	Grade Differentiation	Co-existence of CMV+P53		
		Positive	Negative	Total
Poor	Count	3	4	7
	% within Grade	42.9%	57.1%	100.0%
	% within CMVP53	27.3%	8.2%	11.7%
Moderate	Count	1	3	4
	% within Grade	25.0%	75.0%	100.0%
	% within CMVP53	9.1%	6.1%	6.7%
Well	Count	7	42	49
	% within Grade	14.3%	85.7%	100.0%
	% within CMVP53	63.6%	85.7%	81.7%
Total	Count	11	49	60
	% within Grade	18.3%	81.7%	100.0%
	% within CMVP53	100.0%	100.0%	100.0%

4.CD8+ Cytotoxic T-lymphocytes in oral squamous cell carcinoma :

In this study, the total positive IHC reactions related to CD8 (cytotoxic T-lymphocyte) was detected in 18.3% (11 out of 60) cases with OSCC(figure 3). Only one case of OSCC has showed positive signal result of both ISH reaction for CMV and IHC reaction for CD8+ ; whereas , 35 out of 60 cases have shown either CD8 or CMV infection, and the rest 25 cases did not show any ISH or IHC positive reactions for detection CMV and CD8. Statistically, no significant differences were observed(P> 0.05).

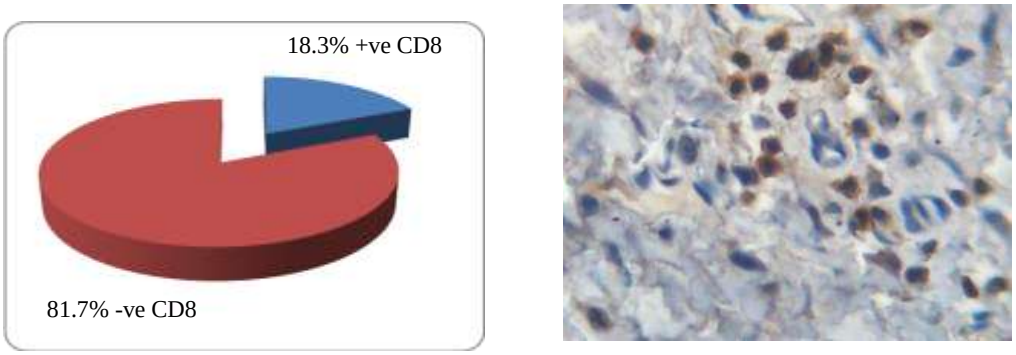

Figure 3:(A).Percentage of Immunohistochemical reactions forCD8- immune surface marker in oral squamous cell carcinoma.(B).Immunohistochemistry for CD8-immune surface marker detection in moderately differentiated oral squamous cell carcinoma tissue with positive IHC; DAB chromogen stained (brown signals) and counter stained with hematoxyline (blue) (40x) .

DISCUSSION:

Clinico – histopathological and demographic aspects of Iraqi patients with oral squamous cell carcinoma : -

The percentage of oral squamous cell carcinoma (OSCC) was noticedto increase with age. About 70 % (42 out of 60) of the studied patients were above the age of 50 years. This finding is in agreement withprevious Iraqi and Western countries studies that foundtheir studied oral cancer patientswere aged over (40-50) years[5,20,21].

The association of oral cancer development with age could be explained by the relatively prolonged exposure to many environmental carcinogens , such as chemicals , radiations and viruses which are important promoting factors

in the development of oral cancer[4]. In addition , the observed impairment in the immune system in such ages where the senescent decline in the immune surveillance might led to accumulation of cellular DNA mutations that could be a significant factor in the development of malignancies[22].

Regarding sex distribution of this study, 61.7% (37out of 60) of OSCC were males, These findings are in agreement with previous study(21,23). However , the disparity in the male to female ratio has become less pronounced over the past half century since women have been more equally exposing themselves to the known oral carcinogens, such as cigarette smoking and alcohol consumption .Moreover, stress and increasing number of female working in factories exposing themselves to carcinogens can be considered as another factors[5].

The well differentiated OSCC was the most predominant type (81.7 %). This is in agreement with previous Iraqi studies[21,23] .However, some studies showed tendency for a decreasing frequencies from well to poorly differentiated OSCC grade[24]. While others either showed higher frequency for moderately differentiated OSCC grade[25]or equal frequencies for poorly and well differentiated grades(1). Undoubtedly , such discrepancies might be related to variations in the methods and data collected , the size of samples used in the different studies and to the factors related to the individual differences in the visual judgment of those pathologists .

Cytomegaloviral – Infected Iraqi patients with Oral Squamous Cell Carcinoma :-

Up to our best knowledge , this is the first work in Iraq with a molecular design that used a sensitive version of in situ hybridization technique to demonstrate the DNA of the late gene of HCMV in Iraqi patients with different grades and different anatomical sites of OSCC. Hereabout, the result of this study (43.3 %) for CMV- late gene DNA detection in OSCC patients is less than the result (91.5 %) of Sugiura et al., 2006[26] who reported strongest positive ISH reactions of CMV in OSCC tissues. On other hand, the present result is very higher thanthe result of (Yu-Yen *et al* .,2004)[27]who used PCR technique and did not show any relation between CMV infection and OSCC paraffin embedded biopsyso as to suggest that the most determinative factor for oral cancer may be chemical in nature rather than viral infection.However , little is currently known about the mechanism of how HCMV be able to infect and replicate in oral tissues. Equally elusive is to identify viral determinants responsible for oral infection . Specifically , it is unknown whether HCMV encodes specific genes that are responsible for infectivity in the lesions of oral cavity[28]. In this study, CMV might not directly be involved in the oncogenic processes , but in addition to viral induction of tumor , it may enhance the possibility of oncogenesis, too .

Immunohistochemical Expression of p53-Tumor Suppressor Gene in Oral Squamous Cell Carcinoma :

To detect mutations of p53 , DNA sequencing is the most accurate method, but it is time consuming and can not detect all (more than 1000) kindsof mutations in p53 gene that have been identified in human neoplasms[29].However , the present study has used immunohistochemical (IHC) method for detection of p53 protein product so as to utilize the advantages of this IHC method in being achieved in a short time without any specialized equipments , as well as its application for relatively small pieces of tissues and its ability for exact localization of p53 proteins in tissues or cells[30].

The protein encoded by the wild type of p53 gene is a 53-KD nuclear phosphoprotein with a half - life of 20-30 minutes . Normally , this p53 protein is not demonstrable immunohistochemically in normal tissue because of its short half life . In contrast , the product of the mutated gene that has detected in this study has a half - life of 6-8 hours and can be readily visualized by immunohistochemistry[29].Regarding non detectable TP53 protein in the present study, however, could be related to a nonsense mutations , detections and splice site mutations that are not associated with a longer half-life of p53 protein. It should also be related to stabilization of p53 protein without mutations in p53 gene where it can also be detected by theseimmunohistochemicalmethods [31].

Overexpression of p53 gene in CMV-associated OSCC :

Many viruses, such as HPV, EBV and CMV, encode proteins that interfere with p53 function to ensure an optimal environment for viral replication, either by releasing cells from cell cycle checkpoints, or by protecting cells from p53-dependent apoptosis [27].These various viruses are considered among the risk factors for oral squamous cell carcinoma (OSCC)[26].

In this study, the percentage of mutated p53 over-expression in tissues that were simultaneously

infected with HCMV was found to decrease with the proceeding of the grade / differentiation of cancer (Table 2). Their counterparts OSCC tissues that, neither shared mutated p53 over-expression, nor CMV-DNA infection, were also found to have a similar trend of decreased grading of OSCC. In addition, the percentages of well, moderate and poor differentiations of OSCC were irrelevant to the presence or absence of co-existence of mutated p53 and CMV positive reactions of the present study. Although there is no other study describing the relationship between OSCC patients with CMV and p53 co-expression, these findings could be related to an earlier oral surgical diagnosis of such cases before proceeding to an elapse of a long time for occurrence of such OSCC lesions. More over, oral lesions and tumors, especially those occurring in the tongue, are curiously dealt with by the patients since they have interference with speech , mastication and appearance that lead to earlier awareness and in turn lead for following up their lesions so that could be found at better grades before their proceeding into an aggressive histological appearance of OSCC .

CD8+ Cytotoxic T-lymphocyte in CMV-associated OSCC oral squamous cell carcinoma:

The current study is a pioneer attempt that addresses IHC expression of CD8+ in Iraqi patients with OSCC. In this study,the total positive IHC reactions related to CD8 (cytotoxic T-lymphocyte) was detected in 18.3% (11 out of 60) cases with OSCC . This finding is in an agreement with [32]who reported a decrease in CD8 T-cells, resulting in a marked difference in the CD4+:CD8+ ratio in the OSCC patients. Thepresent findings are supporting the findings of [32]that a successful immune response to OSCC may require the reversal of the inherently low intra-tumoral CD4+:CD8+ ratio. The lower rate of CD8+ cells with OSCC might indicate that the malignant lesion is less attractive for CD8+ cells,or that patients with HNSCC were more immunocompromised than patients with cancers in other anatomical sites.

In addition, the CD8-positive CTLs were detected in only 1.7% of HCMV-positive OSCC which is statistically found not significant. A similar finding was reported by Richard and his associates in 2007 where no association between the level of HCMV-specific CD8 T-cellular immunity and HCMV was found, too. This finding might indicate that no association exists between the level of HCMV specific CD8 T-cells and HCMV infection in those patients.On the other hand, these findings could be explained by different mechanisms of immune evasion and viral- induced immune tolerance as well as viral persistence [13].

CONCLUSION:

The detection of high percentage of cytomegalovirus-DNA in OSCC could mark for an additional parentral mode of transmission of such important infection among Iraqi general population. The obvious high percentage of mutated p53 over-expression indicates for an important role of such genetic events in the oral carcinogenesis.A little role for CD8-positive tumor infiltrating lymphocytes could be played in the immunological microenvironment of OSCC.

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