Corelation of Epstein Barr virus and CD3 T-Infiltrating lymphocyte with Oral Squamous cell carcinoma patients

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Abstract:

Oral cancers are still poses one of the top ten risk globally in most countries worldwide, there are some factors involved the survival and spread of cancerous tumors, we will determined some of them in this study, such as viral agent and some immunological aspects, like T-lymphocytes. The present study was designed to discuss the evolution of the relation of Epstein Barr virus, CD3 T- lymphocytes with oral squamous cell carcinoma patients, by using Insitu hybridization technique and Immunohistochemical method correlation with different aspects like, age, gender, stage, grade, site and smoking patients. Eighty biopsies were collected from oral squamous cell carcinoma patients during the period from july 2013 until September 2015. By using (ISH), Epstein barr virus was detected in 33.7%(27 out of 80), CD3 T-lymphocytes detected immunohistochemically in 61.2%(49 out of 80)patients. According to all of oral squamous cell carcinoma patients , with mean age 59 years, ranged between (27-76)years, male to female ratio 3:1 with 60 men and 20 women . histological grade involved 63 well , 6 moderate and 11 poor differentiated carcinoma with 60 tobacco smoker and 20 non smoking patients . Most of cases 38 out of 80 were tounge SCC and other 29 were gingival and 13 palate, 59 out of 80 (73.7%) falling in stage I and II, the rest 21 cases were falling in II and IV. The results of this study shows highly significant correlation between each of Epstein barr virus as well as CD3 T-lymphocytes with all aspects above like aspects above like age, sex, tumor grade and stage , smoking and the site of the tumor at $p < 0.01$.

Keywords: *Epstein Barr virus, CD3 T-Infiltrating lymphocyte, carcinoma patients*

Introduction:

In tumor histology, the oral squamous cell carcinoma ranks about 90% of all cases (1,2) which it coms from mucosal epithelium outgrowth (3). Oral squamous cell carcinoma(OSCC) affecting an older people for more than 90% of its occurrence with most occurring in tongue it accounts for about 25% to 40% (4), following by gingival lesions for approximately 10%(5).

Oral squamous cell carcinoma is more happen in male than in female(5). There are many risk factors have been recognized as associated with oral cancers, the most important one which increase the risk of OSCC is smoking (6,7).about more than 15% of human cancers worldwide, viral agent is contribute as a carcinogenic risk factor(8,9).

Epstein Barr virus classified as most common virus infected human causing co infection with multi human cancers and the oral cancers is one of top ten (10,11,12).its well known as associated with malignant and bengin tumors(13), previous studies indicates that more than 90-95% of humans are infected with EBV(14,15).it is persist and then replicates on oral mucosa in the epithelial cells. because of the EBV genome can be detected in lymphoid also in epithelial cell origin of malignancy(16).

Tumor infiltrating lymphocytes plays an important role in inhibiting the proliferation and metstasis of the tumors(17,18) and can use as a prognostic marker with good association for many type of tumors. It is reflect the immune response against the tumors when all the cells changing their functions according to different stimuli(2).

Some researchers reported that in oropharyngeal carcinoma CD3 high concentration, can be linked with positive results, whereas, others have been forecast that CD3 low concentration provide a shorter disease and stay a live in cervical and colorectal cancer (19,20).

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Patients and methods:

This study involved 80 patients with oral squamous cell carcinoma, age ranged 27-76 years with mean age 59 years. Male: female ratio 3:1 with 60 male and 20 female . the samples takes from different sites of oral cavity, 38 biopsies from tongue, 29 gingival biopsy and 13 from palate, whom already diagnosed by specialist in Baghdad medical city between the period from july 2013 to September 2015. Non of them had received anticancer therapy before surgery. The blocks were randomly collected from teaching laboratories/ medical city of Baghdad and compared with 20 apparently healthy control, whom there age and sex were matched to our studied patients.

Epstein barr virus and tumor infiltrating lymphocytes CD3 were determined , by using in situ hybridization and immunohistochemical method and performed as recommended in leaflet with kits.

Insitu hybridization method

- Prehybridization : tissue sections were cutting into 4μm , deparaffinized and dewaxed by xylene, (100,90,70%) ethanol, D.W. respectively, immersing in pre heated (98c˚) citrate buffer pH:6 , placing in proteinase K solution for deprotinization , dehydration were done by immersing slides into D.W. , 70, 90, 100% ethanol.

-        Hybridization by adding DNA probe by placing in oven 98c˚ , removed from oven and incubated over night at room temperature to allow hybridization of probe with target nucleic acid.

-        Post hybridization done by using protein block buffer to fall off all coverslips, conjugate placed onto sections , using substrate, counterstained with nuclear fast red (NFR), dehydration 90,100% ethanol, xylene and mounted with DPX.

Immunohistochemical procedure

After cutting the sections into 4μm thikness , deparaffinized and dehydrated, dewaxed in xylene, (100,90,70%) ethanol, D.W. respectively, placing into endogenous peroxidase block for 25 min. adding CD3 primary antibody in each samples for 90 min., washing with PBS, adding secondary antibody and incubating for 1 hr. in humid chamber, adding streptavidin 30 min. counter stained by Mayers hematoxyline dehydration by 70,90,100 % ethanol and xylene.

Statistical analysis

The statistical analysis system-SAS(21) program was used to effect of difference factors in percentage study. Chi square test was used to measured the significantly in this study.

Results:

- Clinico – Histopathological aspects of patients with oral squamous cell carcinoma.

The series of resected blocks involved eighty(80) Iraqi patients with OSCC were enrolled in this study, by using Insitu hybridization(ISH) and Immunohistochemical (IHC)technique.

Patients age ranged between (27-76)years with mean age 59 years,forty nine out of 80(61.2%)of them were above the age of 50 years. With 60 smoker and 20 nonsmoker patients. Male: female ratio was 3:1 with 60 men and 20 women. According to the site distribution , tongue was the most affected site 38 out of 80(47.5%) followed by gingiva (36.2%) and palate(16.2%). According to the grading of OSCC the well differentiated was the predominant 63 out of 80 (78.7%) followed by poor differentiated (13.7%) and (7.5%) moderately differentiated respectively. In respect to OSCC staging , most cases were falling in the stage I,II (73.7%) and (26.2%) were falling in stage III,IV of the studied patients.

- Epstein Barr virus infected OSCC by using ISH.

Twenty seven (27)out of 80 (33.7%) of OSCC patients showed a positive results for EBV (figure 1) , According to their age, gender, grading, stage of the tumor , smoking and site distribution and as shown (table 1) there were highly significant association between EBV with OSCC patients (p = 0.0037) for age, and (p= 0.0001) for each of gender, grade , stage, smoking and site of the tumor at (p<0.01).

- CD3 T-lymphocytes with OSCC patients by using IHC

In this study (61.2%) 49 out of 80 OSCC patients detected positive results of CD3 TILs(figure2) , furthermore,according to their age, gender, site, grade, stage an d smoking, there were highly significant association with all aspect above (p=0.0048) for age , (p=0.0001) for each of gender, grade, stage and smoking tobacco , and(p=0.0038) for site at p<0.01, so as shown in(table2).

Table 1: correlation between Epstein Barr virus with oral squamous cell carcinoma patients, according to their age, gender, histological grade, staging, tobacco smoking and the site of tumor

The factor	EBV +ve 27	EBV –ve 53	p- value χ^2 test
Age			
≤ 50	10(37%)	21(39.6%)	**p** = 0.0037
> 50	17(62.9%)	32(60.3%)	χ^2 = 9.362**
Gender			
Male	17(62.9%)	43.(81.1%)	**p** = 0.0001
Female	10(37%)	10(18.8%)	χ^2 =13.402**
Grade			
Well	24(88.8%)	39(73.5%)	
Moderate	2(7.4%)	4(7.5%)	**p** = 0.0001 χ^2 =13.862**
Poor	1(3.7%)	10(18.8%)	
Stage			
I,II	21(77.7%)	38(71.6%)	**p** = 0.0001
III,IV	6(22.2%)	15(28.3%)	χ^2 =12.894**
Site			
Gingival	10(37%)	19(35.8%)	
Palate	4(14.8%)	9(16.9%)	**p** = 0.0001 χ^2 =11.073**
Tongue	13(48.1%)	25(47.1%)	
Smoker	20(74%)	40(75.4%)	**p** = 0.0001
Non smok.	7(25.9%)	13(24.5%)	χ^2 =13.028**

Table 2: correlation between CD3 T-Lymphocyte with oral squamous cell carcinoma patients, according to their age, gender, histological grade, staging, tobacco smoking and the site of tumor

| The factor	CD3 +ve 49(61.2%)	CD3 –ve 31(38.7%)	p- value χ^2 test
Age			
≤ 50	19(38.7%)	12(38.7%)	**p** = 0.0048
> 50	30(61.2%)	19(61.2%)	χ^2=9.549**
Gender			
Male	34(69.3%)	26(83.8%)	**p** = 0.0001
Female	15(30.6%)	5(16.1%)	χ^2=13.061**
Grade			
Well	39(79.5%)	24(77.4%)	**p** = 0.0038
Moderate	2(4%)	4(12.9%)	χ^2=9.261**
Poor	8(16.3%)	3(9.6%)	
Stage			
I,II	35(71.4%)	24(77.4%)	**p** = 0.0001
III,IV	14(28.5%)	7(22.5%)	χ^2=13.873**
Site			
Gingival	17(34.6%)	12(38.7%)	**p** = 0.0001
Palate	5(10.2%)	8(25.8%)	χ^2=12.072**
Tongue	27(55.1%)	11(35.4%)	
Smoker	38(77.5%)	22(70.9%)	**p** = 0.0001
Non smok.	11(22.4%)	9(29%)	χ^2=12.825**
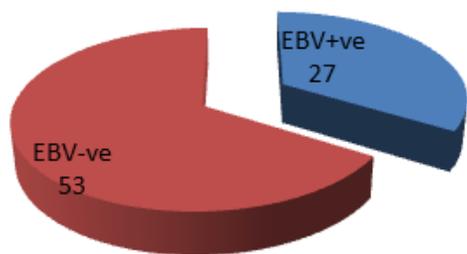
**Figure (1):** A/ Distribution of OSCC patients in relation with Epstein Barr virus
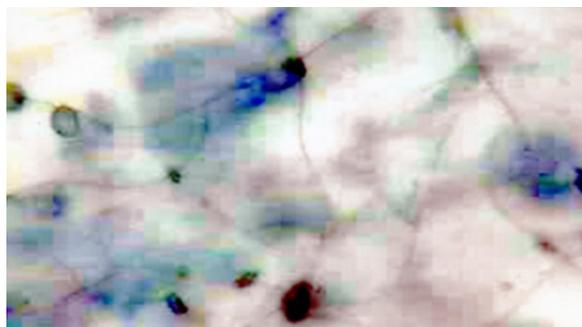
B/ oral sqaumous cell carcinoma with Epstein barr virus positive insitu hybridization reaction(400X) counter stained by nuclear fast red
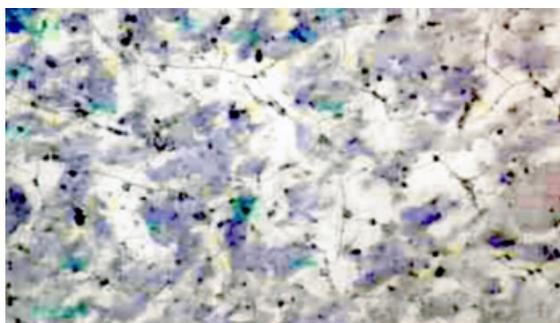

C/ oral sqaumous cell carcinoma with Epstein barr virus negative insitu hybridization reaction(200X) counter stained by nuclear fast red
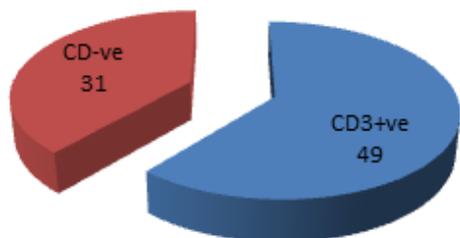
Figure (2): A/ Distribution of OSCC patients in relation with CD3 TILs
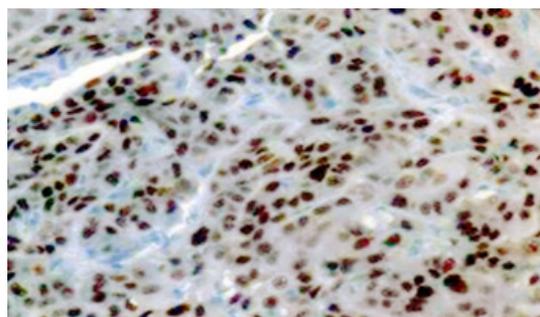
B/ oral sqaumous cell carcinoma with CD3 TILs positive immunohistochemical reaction(200X) counter stained by Mayer's hematoxyline.

C / oral sqaumous cell carcinoma with CD3 TILs negative immunohistochemical reaction(100X) counter stained by Mayer's hematoxyline

Discussion:

In oral cavity, the most common cancer is squamous cell carcinoma, it ranks about more than 90% of all cancers(2), this study involved 80 oral squamous cell carcinoma (OSCC) patients with m ean age 59 years, many previous Iraqi and other studies are in agreement with us (22,23,24,25)which their studied patients aged over 50 years. Oral cancers increasing with age could be explained by exposure frequently to some carcinogenic agent like viruses and chemicals which can promote developing cancers(26). Male, female ratio is 3:1 approach to polandian study(22,27) which report that oral cancers constitute 3.8% in men and 1.3% in women, because of men have been smoker and alcohol abuse generally more than women and the predisposing agent causing oral cancer is smoking(1). The most predominant type was well differentiated (78.7%), many Iraqi studies (24,28) were in agreement with this finding, it is related to data collected, samples size and the methods used, also the diagnostic of specialests . In united kingdom, tongue is the most common site affecting for oral cavity cancer, so as in Iraq the tongue is most common followed by gingival cancer(29)these studies are compatible with current results, I,II were the predominant stages in this study (73.7%)whereas, the rest 26.2 falling in the III,IV stages, that finding depends on the samples collected and pathologist diagnosis.

Epstein Barr virus infected Iraqi patients with oral squamous cell carcinoma In this study we can highlight its one of the rare studies in Iraq that are interested with molecular design by used a highly sensitive version of insitu hybridization method for DNA demonstration of EBV in Iraqi patients with different sites, grades, and the stages of OSCC. The results of current study (33.7%)for EBV/DNA detection in OSCC patients is less than (30)results who reported strong positive of EBV in oral SCC by using RNA insitu hybridization and approach to (31) EBV positive results (45.5%)

by using PCR in OSCC , so as agreement with(13,25) who reported that 37.9%, 27.1% respectively were EBV positive. According to the general aspects of patients age, gender, site distribution , grading , stage and smoking in this study there were highly significant association between EBV infection with OSCC, it was disagree with (13) who found that significantly there were no correlation between EBV infection with age, and tobacco smoking.also disagree with (25)who reported there were no significant correlation of EBV with age and histological grade, gender and location of OSCC patients, but in agreement with(32) who found the statistically significant of prevalence of EBV with OSCC histological grade, alsi in agree with (33)who reported that highly significant association with gender and (34)which reported highly significant relation with site of OSCC.The low detection of EBV/DNA in some researchers have been described by hit and run theory in which the DNA only required in the induction of cancer, and apsent in uncontrolled cell cycles of the host(35).

CD3 T-Infitrating lymphocyte associated OSCC patients. The present study is one of the rare studies that addresses IHC expression in CD3 with OSCC patients in iraq .the total positive reactions between IHC and CD3 ILs was detected in (61.2%) 49 out of 80 OSCC patients . these results reinforce a study of (36) who recorded that the heavy infiltration of OSCC patients by TIL CD3 boost the good prognostic in OSCC cases , in contrast with (37) who refers that prognostic attempt of CD3 TILs differs according to the site of tumor, so, the highest level occurring near the area of malignant cells. According to the general aspects of patients age, gender, site, grade, stage and smoking , the current study were highly significant association between CD3 TILs and OSCC patients. It was agreement with(38) who reported that higher grade in well differentiated followed by moderately and poor, also it was disagree with (39) who found there is no significant association between CD3 and OSCC patients grade, stage of tumor and site distribution.

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علاقة فايرس الابشتاين بار والمرشح اللمفاوي مع المرضى المصابين بسرطانات الخلايا الحرشفيه للفم

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الخلاصه:

سرطانات الفم لاتزال تحتل احدى المستويات العشره الاكثر خطورة عالميا في معظم البلدان. هناك بعض العوامل التي تساعد على بقاء وانتشار الاورام السرطانيه, اذ في هذه الدراسه سنقوم بتحديد جانبا منها مثل العامل الفايروسي وبعض الجوانب المناعية مثل المرشحات اللمفاوية.صممت هذه الدراسه لبحث العلاقه بين فايروس الابشتاين بار والمرشح اللمفاوي CD3 في المرضى المصابين بسرطانات الخلايا الحرشفيه الفمويه. باستخدام طريقة التهجين بالموقع والتصبيغ المناعي. وللتحري عن وجود علاقة مع مختلف العوامل المتعلقة بالمرضى مثل العمر والجنس والحاله الورمية ودرجة التمايز الورمي وموقع الخلايا الحرشفيه المصابه. مع العلاقه بكون الشخص مدخن ام لا. جمعت ثمانين خزعة نسيجيه من مواقع مختلفه للخلايا الحرشفيه الفمويه خلال الفتره الزمنيه من تموز 2013 لغاية ايلول 2015. باستخدام تقنية التهجين بالموقع شخص فايرس الايبشتاين بار في 33.7% (27 مريض من اصل 80) والمرشح اللمفاوي تم تشخيصه في 61.2% (49 مريض من اصل 80). وفقا لما جاء ، فان اعمار المرضى كانت تتراوح مابين 27-76 سنه مع متوسط عمر 59 عاما. وكانت نسبة الرجال الى النساء 3:1 (60 رجل و20 امراة).شمل التمايز الورمي على 63 عينه جيدة التمايز و 6 عينات متوسطة التمايز و 11 عينه ضعيفة التمايز الورمي.من بينهم 60 مدخنا للتبغ و20 غير مدخن .تمت دراسة 80 عينة منها 38 عينة لسرطانات اللسان و29 عينة من سرطانات اللثه و13 عينة لسقف الفم .في حين 59 عينة (73.7%) وقعت ضمن الحالة الورمية I,II وماتبقى 21 عينة كانت ضمن الحاله III,IV للورم.اظهرت نتائج هذه الدراسة ارتباط عالي المعنوية لكل من فايروس الابشتاين بار وكذلك المرشح اللمفاوي مع كل من العوامل المذكورة من حيث العمر و الجنس والحاله المرضيه ودرجة التمايز الورمي وموقع الخلايا الحرشفيه المصابه وتعاطي التدخين عند $p < 0.01$