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Immunohistochemical Expression of Macrophages and EGFR in Relation to HPV-16 Infection in a Group of Iraqi Patients with OSCC

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Abstract: ***Background: Oralsquamous cell carcinoma (OSCC) is the commonest malignant tumor of the oral cavity. The human papilloma virus (HPV) associated OSCC underlines the importance of the immune system for the pathogenesis of oral cancer. Macrophages are involved in various aspects of host defense mechanisms and pathophysiological conditions. Moreover, the role of growth factors-driven signaling in the pathogenesis of human cancer has been long established. Based on these facts it could be hypothesized that there may be an association between EGFR and macrophages with the HPV infection in OSCC patients. Objectives: The aim of this study was to investigate the impact of EGFR and macrophages expressions on HPV 16 expression in Iraqi OSCC patients. Materials and Methods: Sixteen Formalin-fixed, paraffin embedded (FFBE) tissue blocks were enrolled in this study. Serial sections were obtained from tissue blocks of OSCC biopsies. The expression of HPV16, EGFR and macrophages was investigated immunohistochemically. Results: The results revealed that 7cases (43.75%) showed positive brown cytoplasmic staining for macrophage, while the positivity rate of the EGFR immune staining showed 13 cases (81.75%) with positive brown membranous and/or cytoplasmic expression. Only two cases were HPV16 positive, There was a highly significant correlation between Macrophages and EGFR IHC expressions (p=0.006). Conclusions: The results of this study suggest that the high expression of EGFR and macrophages could increase the malignancy of oral squamous cell carcinomas (OSCC). In addition the inverse correlation between HPV-16 expression with respect to the EGFR and Macrophage expressions candidates themas promoting therapeutic targets to eliminate HPV infection. Further studies are recommended on larger population to support these findings.***

Keywords: Human; Human Papilloma virus 16,18; Squamous Cell Carcinoma; Epidermal growth factor receptor , Macorphages

1. Introduction

Head and neck squamous cell carcinoma (HNSCC) is the sixth most common cancer, with annual incidence of 600,000 cases worldwide [1]. Anatomically, head and neck cancer include the oral cavity, the pharynx , the larynx, the paranasal sinuses, the nasal cavity and the salivary glands.Beyond distinction by anatomic sites, HNSCC is divided into two broad classes: human papillomavirus (HPV)-associated (HPV+) and HPV-negative (HPV-) disease. (Tim and Erica, 2016)

Oral squamous cell carcinoma(OSCC) is the commonest malignant tumor of the oral cavity, accounting for more than 90% of these malignancies. In Iraq oral cancer remains a highly lethal and disfiguring disease. Patients at their fifth decade of life were the most commonly affected with a male to female ratio of 2:1 (Al-Talabani and Al-Rawi, 2002).

Papillomaviruses are diverse group of viruses that have been found in more than 20 different mammalian species, as well as in birds and reptiles. Because of their medical importance, the human papillomaviruses (HPV) have been most extensively studied, and more than 100 different types have now been identified (Bernard, 2005)

The role of Human Papilloma Virus (HPV) in the etiology of cervical cancer is firmly established. HPV infection has also been postulated as a potential risk factor for OSCC. An in situ hybridization study confirmed the presence of HPV DNA in oral premalignancies and oral carcinomas, thereby suggesting a causal association of HPV and carcinogenesis in oral lesions as well (Saad et al, 2011). Several studies have detected HPV DNA in a considerable proportion of oral cancers, with wide variations from 0% to 100% prevalence in oral tissues, perhaps reflecting the inherent variations in the different populations, as well as the detection methods used (Hussain and Faris,2010).

The human papilloma virus (HPV) associated OSCC underlines the importance of the immune system for the pathogenesis of oral cancer. It is assumed that the favorable prognosis in HPV positive cases might be associated with an immune response against viral antigens. This observation outlines the potential ability of the immune system to engage oral cancer.(Falk et al;2014)

Macrophages are involved in various aspects of host defense mechanisms and pathophysiological conditions, such as chronic inflammatory disease and cancer The functional competence of macrophages is acquired after the exposure of these cells to stimuli in the tissue microenvironment. Bacterial cellular components, such as lipopolysaccharide (LPS), and the type 1 helper T cell (Th1)-derived cytokine interferon-gamma (IFN-γ) polarize classically activated macrophages, which are referred to as M1 macrophages. These macrophages produce large amounts of proinflammatory cytokines, such as IL-12 and tumor necrosis factor-alpha (TNF-α), reactive oxygen intermediates and reactive nitrogen intermediates, which

contribute to the antimicrobial and antitumor activities of macrophages. In contrast, Th2-derived IL-4 and IL-13 induce macrophage polarization to the alternatively activated (M2) phenotype that participates in anti-inflammatory processes, tissue remodeling, scavenging effects, and angiogenesis. These macrophages generally show low amounts of IL-12 production, impaired nitric oxide induction, enhanced expression of angiogenic cytokines, such as vascular endothelial cell growth factor (VEGF), and proteolytic enzymes. (Kazumasa et al; 2011)

The role of growth factors-driven signaling in the pathogenesis of human cancer has been long established. Sporn and Roberts, 1985 elaborated the theory of autocrine secretion: cancer cells generally exhibit a reduced requirement for exogenously supplied growth factors to maintain a high rate of proliferation.(Nicola et al;2006) Different families of growth factors and growth factor receptors have been shown to be involved in the autonomous growth of cancer cells. Among these, the epidermal growth factor receptor (EGFR) and the EGF-family of peptide growth factor have a central role in the pathogenesis and progression of different carcinoma types (Salomon et al., 1995; Normanno et al., 2001) .(Nicola et al;2006)

The hypothesis on which the present study was based is the existence of a link between EGFR and macrophages in the life cycle of HPV16.In addition on the concept of that EGFR could be a useful biomarker for macrophages classification.

2. Materials & Methods

This retrospective study enrolled 16formalin-fixed, paraffin embedded(FFBE) tissue blocks which have been diagnosed as OSCC were obtained from the archives of the department of Oral & Maxillofacial Pathology/ College of Dentistry/ University of Baghdad and Al-Shaheed Ghazi Hospital/ Medical City ,during the period from (1975-2013),which were previously studied for the IHC expression of HPV16,18 by Muhsin2015.

Immunohistochemistry Staining procedure:
An immunohistochemcial staining was performed using anti-EGFR and Anti-macrophage biomarkers .Negative and positive control slides were included in each IHC run (as recommended by the manufacturer).Immunodetection was performed according to manufacture instructions of Cambridge Science Company using EXPOSE Mouse and Rabbit Specific HRP/DAB Detection IHC kit (ab80436). All tests were carried out on 5 µm formalin- fixed ,paraffin-embedded sections. Sections were dewaxed and rehydrated then subjected to antigen retrieval. Endogenous peroxidase activity and non-specific binding were blocked by incubation with 3% hydrogen peroxide and protein block, respectively. Slides were then incubated sequentially with primary antibodies using a dilution of (1/100) for both biomarkerfor 1hour at 37C and secondary antibody was applied for 10 minutes at room temperature followed by incubation with Streptavidine-HRP for 10 minutes at 37C.Diaminobenzidinehydrochloride (DAB) was used as the chromogen to visualize peroxidase activity. Sections were counterstained with Mayer's hematoxylin for 30 seconds, dehydrated and mounted..(Areej et al;2013)

Assessment of IHC results
In each tissue section five representative fields were selected, with an average of 1000 tumor cell per case and 200 tumor cells per field, for evaluating the EGFR and Macrophage immunoexpression .

The extent of EGFR immunostaining was graded and scored as follows: 0 points for negative staining of the considered cells, (1) <10%, (2)10-50%, (3)51-80% and (4)≥ 80% positive staining of the considered cells.(Laimer *et al;*2007)
To evaluate the positively stained macrophages, three high-power magnification fields (400×) with the most abundant distribution were selected from each specimen. The positively stained and unstained cells were counted. The data were expressed as the mean percentage of the ratio of the number of positive cells relative to the total number of cells for one microscopic field and the scoring was calculated as the percentage of positive cells.3

Statistical Analysis:
Statistical analysis was done using SPSS (Statistical package for social sciences) Version 10 and Excel application.
Inferential statistics:Pearson correlation (Chi square test) was performed to find out the relation between each marker and the clinicalopathological parameters, as well as the relation between bothmarkers. P value (<0.05) was considered statistically significant, and (< 0.000) as highly significant.

3. Results

The study sample was previously employed to identify the expression of HPV16 by Muhsin2015 ,in which only 2 cases showed positive expression of HPV16 and all cases showed negative expression of HPV18.

Immunostaining results demonstrated that 7 cases(43.75%) showed positive brown cytoplasmic staining for macrophage (Figures 1&2) and the remaining9 cases(56.25%) were negative .while the positivity rate of the EGFR immunostaining revealed that13 cases(81.75%) showed positive brown membranous and/or cytoplasmic EGFR immunostaining(Figures 3-6)and only 3 cases (18.75%) were negative.

Regarding the correlation of Macrophages and EGFR IHC expression with the clinicopathological parameters, there was a highly significant correlation with the age , gender, site and a significant correlation with the grade for both markers, as clarified in table(1) and table (2).

Table 1: Correlation of Antimacrophage expression and the clinicopathological parameters

Parameters	+ve N(%)	-ve N(%)	Total	Chi-square (P-value)
Age				12.192
35-50	4(57.14%)	1(11.11%)	5	P<0.001
51-80	3(42.86%)	8(88.89%)	11	H.S
Gender				12.289
Male	3(42.86%)	3(33.33%)	6	P<0.001
Female	4(57.14%)	6(66.67%)	10	H.S
Site				
Tongue	3(42.86%)	6(66.67%)	9	17.87
Gingiva	2(28.57%)	0(0%)	2	P<0.001
Buccal mucosa	2(28.57%)	2(22.22%)	4	H.S
Maxilla	0(0%)	1(11.11%)	1	
Grade				3.438
Well diff.	2(28.57%)	5(55.56%)	7	P=0.046
Moderately diff.	2(28.57%)	4(44.44%)	6	S
Poorly diff.	3(42.86%)	0(0%)	3	
Total	7	9	16	

Table 2: Correlation of EGFR expression and the clinicopathological parameters

Parameters	+ve N(%)	-ve N(%)	Total	Chi-square (P-value)
Age				10.109
35-50	3(23.08%)	2(66.67%)	5	P<0.001
51-80	10(76.92%)	1(33.33%)	11	H.S
Gender				10.199
Male	4(30.77%)	2(66.67%)	6	P<0.001
Female	9(69.23%)	1(33.33%)	10	H.S
Site				
Tongue	8(61.54%)	1(33.33%)	9	13.25
Gingiva	2(15.38%)	0(0%)	2	P<0.001
Buccal mucosa	2(15.38)	2(66.67%)	4	H.S
Maxilla	1(7.7)	0(0%)	1	
Grade				2.027
Poorly diff.	7(53.85%)	0(0%)	7	P=0.049
Moderate diff.	4(30.77%)	2(66.67%)	6	S
Well diff.	2(15.38%)	1(33.33%)	3	
Total	13	3	16	

In the present study, the possible impact of Macrophagesand EGFR expression on the life cycle of HPV16 was considered. Interestingly, in spite of the low HPV16 positivity (two cases, 12.5%), the results revealed a significant correlation with the Macrophages and a highly significant correlation with the EGFR expression, as demonstrated in tables (3&4) respectively.

Table 3: Correlation between Antimacrophage and HPV16 IHC expression

	+Ve	%	-Ve	%	Total	Chi-square (P-value)
Anti-macrophage	7	43.75	9	56.25	16	4.144 p=0.042
HPV16	2	12.5	14	87.5	16	S

Table 4: Correlation between EGFR and HPV16 IHC expression

	+Ve	%	-Ve	%	Total	Chi-square (P-value)
EGFR	13	81.25	3	18.75	16	7.594 p=0.006
HPV16	2	12.5	14	87.5	16	HS

Correlating the expression rate of either markers, a significant correlation was observed between them, P-value 0.035, as shown in (Table 5).

Table 5: Correlation between Anti- Macrophage andEGFR IHC expression

Biomarkers	+ve N(%)	-ve N(%)	Total	Chi-square (P-value)
Anti-Macrophage	7(43.75%)	9(56.25%)	16 (100%)	3.654
EGFR	13(81.75%)	3(18.75%)	16 (100%)	P=0.035
				S

4. Discussion

Macrophages are involved in various aspects of host defense mechanisms and pathophysiological conditions, such as chronic inflammatory disease and cancer (Kazumasa et al;2011)

The macrophages were classified as M1 (classically activated) and M2 (alternatively activated) macrophages based on the expression of macrophage gene products, including receptors, cytokines, and effector molecules, induced by classical macrophage-activating stimuli such as Th1- derived IFN or the Th2-derived anti-inflammatory cytokines IL-4 and IL-13 [12, 19, 42]. M1 macrophages produce large amounts of pro-inflammatory cytokines, reactive oxygen intermediates and reactive nitrogen intermediates, such as nitric oxide (NO), which contribute to the anti-tumor activity of macrophages [12]. In contrast, M2 macrophages have been suggested to contribute to angiogenesis, tissue remodeling. (Kazumasa et al;2015).

As seen in table (1), it is obvious that all the three poorly differentiated OSCC cases showed positive antimacrophage expression, which may indicate the shifting of macrophage polarization in the lymph nodes from the anti-tumor M1 type to the tumor-promoting M2 type in correlation with the increasing severity of the tumor; this is in agreement with Falk et al; 2014 This supports the suggestion that the infiltrated TAMs in OSCC have an M2 phenotype and hence they may participate in the development of OSCC. (Kazumasa et al;2011).

Moreover; it is logical to find high expression of EGFR in tongue since it provides high surface area to be exposed to carcinogens also its non-keratinized surface Table (2) which is relatively the same for the expression of Macrophage in table (1) (Khashman,2008).

In the current study, the recorded high positivity rate of EGFR expression coincides with Ang who found that the Epidermal growth factor receptor (EGFR/ErbB1/HER1) over expression is present in the majority of oral squamous cell carcinoma (OSCC) tumors,(IYO,2016).also(seta et al,2010).

As demonstrated in table (2) EGFR expression in the studied cases was adversely associated with tumor progression as it was found in descending pattern from well to moderately then to poorly differentiated tumor (53.84%) , (30.74%)&(15.38%) respectively, this is in accordance with IYO KIMURA,2015 who suggested that the loss of EGFR expression is associated with the acquirement of an invasive phenotype in OSCC..(IYO,2016).Furthermore ; our results agree with other researchers who found that the activation

of (EGFR) in tumor cells accelerates tumorigenesis .(Gang,2015)

The role of inflammation in human papillomavirus (HPV) infection and disease is complex since it involves responses capable of preventing initial infections, clearing those ongoing as well as promoting persistence and progression of associated lesions.(Enrique **et al;,2010)**.

In this study, The significant correlation of macrophages with the expression of HPV shown in table (3) supports the outcome of John*et al*, 2005 who found that Macrophages kill E6-expressing cells by both TNFand Nitric Oxide-Dependent Mechanisms.(John et al;2005)

As revealed in table (4),the correlation between HPV infection and the EGFR expression was highly significant which supports the hypothesis of the inverse relation of HPV life cycle with the EGFR expression and this is in accordance with the finding of Ma Lingli (Ma L et al; 2014).

A significant correlation was found between the expression of both EGFR and Macrophage as shown in table. This correlation supports the theory Takashia et al,2016 who found that Increased expression of EGFR is caused by M2 macrophages, we thought that the elevated EGFR expression might be due to switching of macrophages from the M1 to the M2 phenotype so that we can use the expression of EGFR to detect the M phenotype (Takahisa et al;2016).

In conclusion, the results of our study suggests that the high expression of EGFR and macrophages Increase malignancy of oral squamous cell carcinomas (oscc). Also we found inverse correlation between HPV-16 expression and EGFr , Macrophage expression which may be a promoting therapeutic targets to elimeinate HPV infection .We recommend further studies on a population to support these findings.

5. Acknowledgments

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Figure1&2 Immunohistochemical expression of antimacrophage in oral squamous cell carcinoma (Original magnification 400x.)

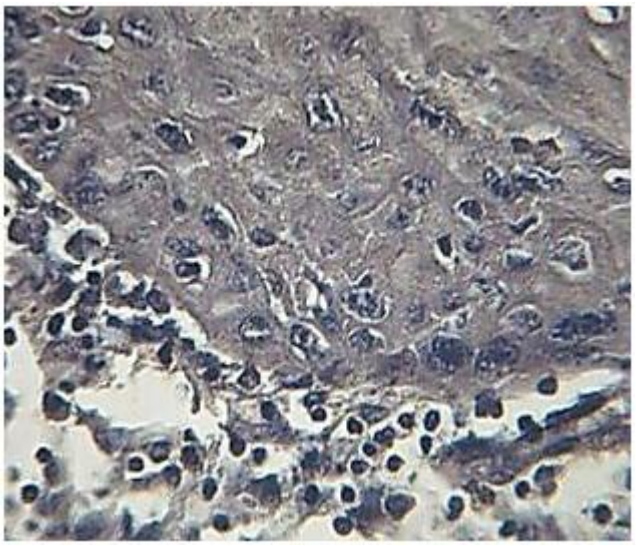

Figure 1

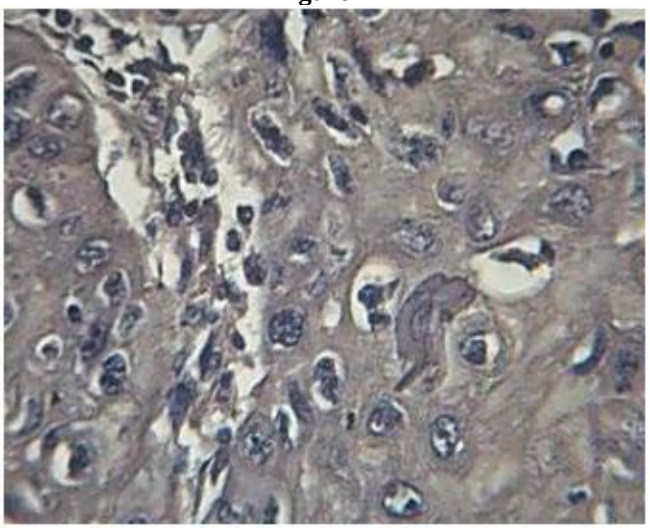

Figure 2

Figure3, 4, 5, 6

Immunohistochemical expression of epidermal growth factor receptor (EGFR) in oral squamous cell carcinoma(Original magnification 400x.)

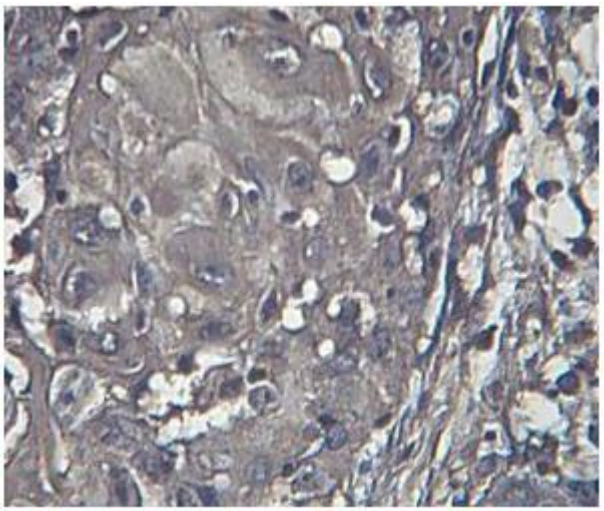

Figure 3

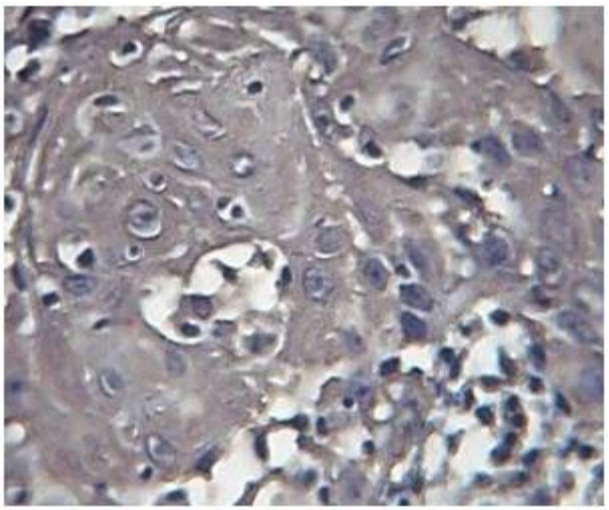

Figure 4

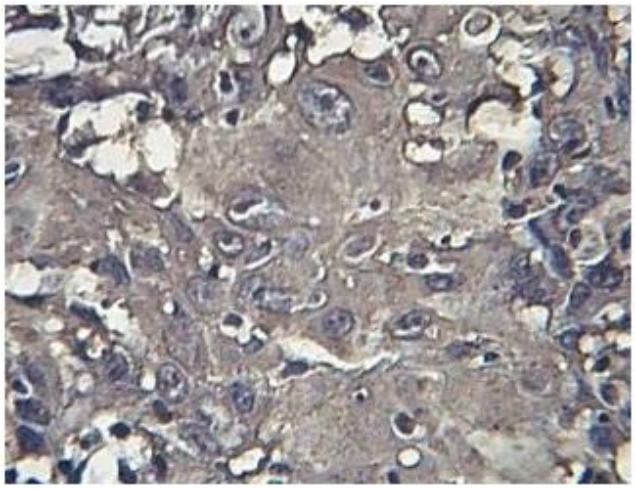

Figure 5

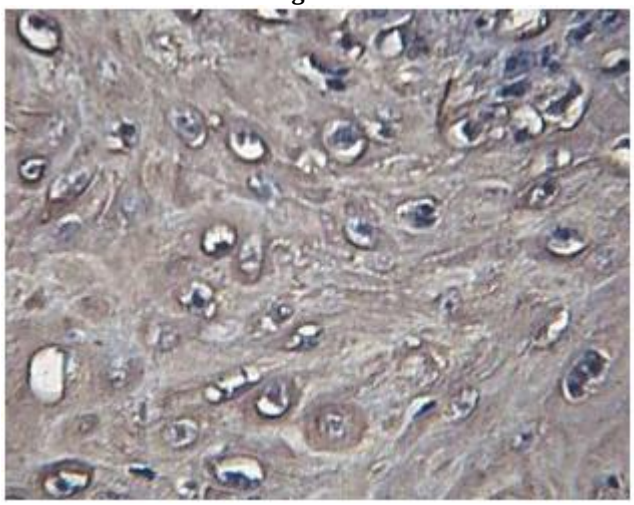

Figure 6

References

[1] Tim N. Beck, and Erica A. Golemis . Genomic insights into head and neck cancer. Beck and Golemis Cancers of the Head & Neck 2016; 1(1): DOI 10.1186/s41199-016-0003-z.

[2] Al-Talabani NG and Al-Rawi NH. Squamous cell carcinoma of oral cavity, a clinicopathological analysis of 1425 cases from Iraq. J Alrafidain Dent. 2002,1,1-6.

[3] Bernard HU. The clinical importance of the nomenclature, evolution andtaxonomy of human papillomaviruses. J Clin Virol 2005;32S:S1–S6.

[4] Saad Hasan Mohammed Ali,Athraa Yahya Al-Hijazi,Basim M.Khashman. P53-tumor suppressor gene overexpression in human papilloma virus-infected patients with oral squamous cell carcinoma. Journal of Baghdad College Dentistry 2011; 23(special issue):

[5] Hussain Gadelkarim Ahmed, and Faris Merghany Eltoom . Detection of Human Papilloma Virus Types 16 and 18 among Sudanese Patients with Oral Squamous Cell Carcinoma. The Open Cancer Journal 2010; 3 : .

[6] Falk Wehrhan, Maike Büttner-Herold, Peter Hyckel, Patrick Moebius, Raimund Preidl, Luitpold Distel, Jutta Ries, Kerstin Amann, Christian Schmitt, Friedrich W Neukam and Manuel Weber. Increased malignancy of oral squamous cell carcinomas (oscc) is associated with macrophage polarization in regional lymph nodes – an immunohistochemical study. BMC Cancer 2014; 14(522): .

[7] Kazumasa Mori , Miki Hiroi , Jun Shimada and Yoshihiro Ohmori. Infiltration of M2 Tumor-Associated Macrophages in Oral Squamous Cell Carcinoma Correlates with Tumor Malignancy. cancers 2011; 3(): doi:10.3390/cancers3043726.

[8] Kazumasa Mori, Shigeki Haraguchi, Miki Hiori, Jun Shimada and Yoshihiro Ohmori. Tumor-associated macrophages in oral premalignant lesions coexpress CD163 and STAT1 in a Th1-dominated microenvironment. BMC Cancer 2015; 15:573. DOI 10.1186/s12885-015-1587-0.

[9] Nicola Normanno , Antonella De Luca , Caterina Bianco , Luigi Strizzi , Mario Mancino , Monica R. Maiello , Adele Carotenuto , Gianfranco De Feo , Francesco Caponigro , David S. Salomon. Epidermal growth factor receptor (EGFR) signaling in cancer. Gene (366)2006.

[10] Areej Atiyah Hussein,Basim Mohammed Khashman and Saad Muhmood Hussain. Role of Tgf-β1and Gremlin-1in the Pathogenesis of Chronic HCV Infection and Hepatocellular Carcinoma. INDIAN JOURNAL OF APPLIED RESEARCH 2013; 3(9): .

[11] Laimer K, Spizzo G, Gastl G, Obrist P, BrunhuberTh, Fong D, Barbieri V, Jank S, Doppler W, Rasse M, Norer B: High EGFR expression predicts poor prognosis in patients with squamous cell carcinoma of the oral cavity and oropharynx: A TMA-based immunohistochemical analysis. Oral Oncology 2007, 43:193-198 .

[12] Khashman Basim M. Molecular and Virological study of Human papilloma virus in Iraqi patients with oral squamous cell carcinoma. A master thesis in Molecular virology, Department of Medical Microbiology, College of Medicine, University of Baghdad, 2008.

[13] IYO KIMURA, HIROKO KITAHARA, KAZUHIRO OOI, KOROKU KATO, NATUYO NOGUCHI, KUNIO YOSHIZAWA, HIROYUKI NAKAMURA and SHUICHI KAWASHIRI. Loss of epidermal growth factor receptor expression in oral squamous cell carcinoma is associated with invasiveness and

epithelial-mesenchymal transition. ONCOLOGY LETTERS 11 : 201-207,2016.

[14] Seta A Sarkis1, Bashar H Abdullah1, Ban A Abdul Majeed2 and Nazar G Talabani ,Head & Neck Oncology 2010, 2:13.

[15] Gang Zhao, Liping Liu, D. Brent Polk, Richard M. Peek, Xishan Hao, Hui Li, Fang Yan. Activation of EGF receptor in macrophages inhibits M2 polarization and suppresses proliferation and epithelial-to-mesenchymal transition in gastric epithelial cells. [abstract]. In: Proceedings of the 106th Annual Meeting of the American Association for Cancer Research; 2015 Apr 18-22; Philadelphia, PA. Philadelphia (PA): AACR; Cancer Res 2015;75(15 Suppl):Abstract nr 981. doi:10.1158/1538-7445.AM2015-981.

[16] Enrique Boccardo, Ana P.Lepique and Luisa L.Villa. The role of inflammation in HPV carcinogenesis. Carcinogenesis 2010; 21(11): .

[17] John M. Routes, Kristin Morris, Misoo C. Ellison, and Sharon Ryan. Macrophages Kill Human Papillomavirus Type 16 E6-Expressing Tumor Cells by Tumor Necrosis Factor Alpha- and Nitric Oxide-Dependent Mechanisms. JOURNAL OF VIROLOGY 2005; 79(1): 116–123.

[18] Ma L, Wang D, Wufuer A, Wu R, Zhang S, Wang R. Relationship between human papilloma virus infection and expression of p16 and EGFR in head and neck squamous cell carcinoma and their prognostic significance. China Journal of oncology 2014; 36(1): .

[19] Takahisa Yamaguchi, Sachio Fushida,Yasuhiko Yamamoto,Tomoya Tsukada, Jun Kinoshita, Katsunobu Oyama, Tomoharu Miyashita, Hidehiro Tajima, Itasu Ninomiya, Seiichi Munesue, Ai Harashima, Shinichi Harada, Hiroshi Yamamoto,Tetsuo Ohta. Tumor-associated macrophages of the M2 phenotype contribute to progression in gastric cancer with peritoneal dissemination. Gastric Cancer 2016; 19: 1052–1065.