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Expression of Erythropoietin and Erythropoietin Receptor in Oral Squamous Cell Carcinoma

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Abstract: ***Background: Despite advancement in treatment modalities for oral squamous cell carcinoma, this tumor still represents a challenge for researchers because of great tendency of tumor to metastasize and recur. Many parameters have been previously studied in order to predict the prognosis of such devastating tumor and to achieve the best treatment outcomes. Erythropoietin is multifunctional growth hormones primarily function to stimulate erythropoiesis through binding to its specific erythropoietin receptor. Questions about existence of both proteins have been raised in malignant tumors. This study was planned to investigate the expression of erythropoietin and erythropoietin receptor in oral squamous cell carcinoma and to correlate the expression with various parameters. Materials and Methods: erythropoietin and erythropoietin receptor expression were inspected by immunohistochemistry in 50 surgical cases of oral squamous cell carcinoma. The resulting expression labelling indicies then correlated with various clinicopathological parameters. Results: oral squamous cell carcinoma specimens showed positive expression of erythropoietin and erythropoietin receptor. Erythropoietin receptor expression but not erythropoietin was correlated significantly with gender and tumor site. Non-significant statistical relations were reordered regarding the expression of both proteins with other clinicopathological parameters. Conclusions: current study confirmed the expression of erythropoietin and its receptor in oral squamous cell carcinoma.***

Keywords: oral squamous cell carcinoma, erythropoietin, erythropoietin receptor, immunohistochemistry

1. Introduction

Squamous cell carcinoma (SCC) is the most predominant oral epithelial tumor constituting more than 90% of entire oral malignancies (1).The origin of this devastating tumor is from the surface epithelium of the oral cavity with a characteristic squamous differentiation (2). Multiple genetic mutations that modify the functions of proto-oncogenes, tumor suppressor genes, genes of apoptosis or DNA repair genes are required for the development of oral squamous cell carcinoma (OSCC) (3)**.** These genetic alterations give the tumor cells growth and survival benefits by increasing the construction of growth factors, number of cell surface receptors, increasing levels of transcription or intracellular messenger factor, and increased ability to evade the apoptosis (4). In spite of considerable progresses in diagnosis and treatment of OSCC in past decades, the prognosis still poor (5). Erythropoietin is a low molecular weight (34 kDa) glycoprotein hormone whose fundamental function is the regulation of RBC production through stimulation of erythroid progenitor cells proliferation, survival, and differentiation in the bone marrow (6). The liver is the main site of EPO production in fetus. At birth, there is shifting in the site of production from the liver to the kidney and, in adults, the major production sites are peritubular fibroblasts of the renal cortex ***(7).*** Serum level of EPO can be elevated several hundred folds due to hypoxia which represents the primary stimulator of EPO production ***(8).***EPO perform its effect through binding to its specific receptor erythropoietin receptor (EPOR) ***(9).***The major signaling pathways activated by erythropoietin include JAK/STAT, RAS/MAP kinase, protein kinase, P13K pathways ***(10).*** Erythropoietin are found to be expressed in several non-hematopoietic cells and tissues like vascular endothelial cells, uterus, heart, GIT, kidney, central nervous system and the solid tumors where it performs protective function in anti-apoptotic and/or mitogenic manner ***(11,12,13).*** In tumors, many functions suggested for EPO. It was documented that EPO has "anti-apoptotic effect, can stimulates angiogenesis, promotes drug resistance and increases cell proliferation" ***(14).*** Increased level of EPO expression was detected in many tumors such as; "squamous cell carcinomas of head and neck region, renal carcinomas, breast cancer, Wilms tumors, melanoma and cerebellar hemangioblastomas" ***(15,16).***Erythropoietin receptor is a member of a large family of receptors termed type I cytokine receptor which comprises in addition, receptors for other hematopoietic growth factors such as "growth hormone, prolactin, G-CSF, GM-CSF, thrombopoietin, and many interleukins". These cytokine receptors share several features, "including an extracellular ligand- binding domain with two pairs of conserved cysteine residues and a conserved motif, WSXWS located close to the transmembrane domain; a single transmembrane domain; and an intracellular domain lacking catalytic activity" ***(11).*** The vast majority of EPOR are present on the surface of erythroid precursor cells, erythroid burst forming units and erythroid colony forming units in bone marrow. Nevertheless, detected expression of EPOR was documented in a variety of tissues like "heart, brain, liver, and others" ***(10).*** In addition to the normal tissues, EPORs have been noticed in several tumor tissues such as prostate ***(6),*** breast cancer ***(17),*** renal cancer ***(14),*** hepatic cellular carcinoma ***(18),*** and melanoma ***(19)***. Efforts have been made by many researchers in order to discover dependable parameters that can be utilized as prognosticators of metastatic spread to the lymph nodes, recurrence, and reduced disease survival in patients with OSCC. Of these which were studied broadly and their relations to poor prognosis were documented are TNM stage, tumor grade, depth of invasion, pattern of tumor invasion, tumor budding, lymphovascular invasion and perineural invasion.

2. Materials and Methods

Patients

The sample of this study comprises biopsy specimens taken from fifty patients with primary oral squamous cell carcinomas retrieved from the archives of oral pathology laboratory of the oral diagnosis department at the college of dentistry/ Baghdad University and Ghazi Al-Hariri hospital laboratory. For each specimen, theclinical and histopathological data were documented.

Immunohistochemical staining

The primary antibodies used in the immunohistochemical procedure were Rabbit polyclonal anti-erythropoietin (ab217371, dilution 1/100) and mouse monoclonal anti-erythropoietin receptor (ab56310, dilution 1/10) antibodies from Abcam (Cambridge, UK). Mouse and Rabbit specific HRP/DAB detection IHC kit ab80436 (Abcam®, UK) was used for detection of primary antibodies. 4um sectionwas taken from each tissue block and then placed on glass slide. The sections were deparaffinized, rehydrated in graded alcohols, exposed to endogenous peroxidase block in 3% hydrogen peroxide and boiled in citrate buffer at 90Cº for 7 minutes. The slides were incubated with primary antibody overnight at 4Cº, and then washed and incubated with secondary antibody at 37Cº for 25 minutes. Finally, the sections were developed with DAB chromogen and counterstained with hematoxylin. Cytoplasmic or membranous brown staining was considered positive. Erythropoietin and erythropoietin receptor labeling indices were counted as a ratio of immunostaining positive cells to thetotal number of cells counted.

Statistical analyses

ANOVA test or student's T test were used as appropriate to compare the differences in mean expression labelling indicies among different clinicopathological parameters in oral squamous cell carcinoma. A p value less than 0.05 was considered statistically significant.

3. Results

The demographical, clinical, and histopathological characteristics for the 50 patients included in this study are shown in table 1. Male gender was the predominant comprising 54% of all cases and the male to female ratio was 1.17/1. The minimum, maximum, and mean ages were 17, 85, and 54.9 years respectively. The predominant tumor site was the tongue accounts for 46% of the cases followed by in descending order of frequency: buccal mucosa, alveolar ridge, lower lip, and floor of the mouth. Regarding tumor stage, the chief stage recorded was stage IV which accounts for 32% of all cases. Out of 50 cases, 29 cases were well differentiated, 15 cases were moderately differentiated, and 6 cases were poorly differentiated. The pattern of tumor invasion was classified into four categories pattern III was the most prevalent comprising 38% (19 cases) followed by pattern IV, II, and I. In this study, the tumor depth ranged from 1.25mm to 8.75mm. The tumor sample was divided into two groups: ≥ 4mm and < 4mm based on researches that considered 4 mm as a cutoff point for increased likelihood of loco-regional recurrence and distant metastasis. LVI and PNI were present in 30 (60%) and 24 (48%) cases respectively. The intensity of tumor budding was divided into two categories depending on previous reports that correlate number of greater than 5 tumor buds with worse prognosis and poor survival. Lymphocytic infiltration was estimated at the invasive tumor front and divided into 3 grades. By which, Grade 1 was the dominant with a frequency of 37 cases (74%). In tumor cells, EPO expression was brown granular cytoplasmic and occasionally nuclear. The intensity of EPO staining was frequently higher in the tumor cells at the invasive front than in other areas. In addition, smooth muscle cells, endothelial cells, fibroblasts, and some inflammatory cells showed positive expression for EPO. Dissimilarly, normal epithelial cells lack such staining. The mean EPO LI score was (41.72±12.93). Males have higher expression index than females. Concerning EPOR, brown cytoplasmic and/ or membranous expression was regarded as positive immunostaining. Negative or extremely weak expression was noticed in presumably normal epithelium compared to the intense expression in dysplastic and cancerous areas. High intensity of EPOR expression was detected in more differentiated parts of tumor islands. In addition, positive expression was detected in the vascular and stromal components of the tumor. Among histopathological parameters, EPO expression showed non-significant statistical relations. For EPOR, Noticed difference in mean labelling index was observed between well and poorly differentiated tumors and the predominance tend to favor the well-differentiated tumor. Yet, significant statistical relation not reached. EPOR expression however correlate significantly with gender, and tumor site as illustrated in table 1.

Table 1: Erythropoietin and erythropoietin receptor expression labeling indicies and their correlations with various clinicopathological parameters

Age	*minimum* *maximum*	17 years 85 years	42.51±11.88 41.13±13.82	0.713	36.71±13.41 35.82±15.53	0.834
Gender	*Male* *Female*	27 (54%) 23 (46%)	44.25±13.01 38.73±12.46	0.134	41.37±13.54 30.13±13.52	***0.005****
Tumor site	*Tongue* *Buccal mucosa* *Alveolar ridge* *Lower lip* *Floor of the mouth*	23 (46%) 12 (24%) 8 (16%) 4 (8%) 3 (6%)	38.43±11.22 43.83±13.50 42.87±9.07 49.00±16.97 45.66±26.57	0.506	35.00±11.67 31.16±16.24 35.12±8.96 58.75±17.57 38.33±17.03	***0.017****
Tumor stage	*I* *II* *III* *IV*	12 (24%) 13 (26%) 9 (18%) 16 (32%)	43.75±13.41 41.69±7.9 41.55±20.86 40.31±11.22	0.927	38.08±15.01 36.15±9.80 36.33±22.43 34.75±13.23	0.952

Tumor grade	*Well* *Moderate* *poor*	29 (58%) 15 (30%) 6 (12%)	42.06±13.78 43.33±11.89 36.00±11.50	0.499	39.17±15.88 35.26±10.54 24.16±10.99	0.065
LVI	*Present* *Absent*	30 (60%) 20 (40%)	43.40±13.18 39.20±12.45	0.265	34.93±15.17 38.10±13.70	0.456
PNI	*Present* *Absent*	24 (48%) 26 (52%)	40.91±13.27 42.46±12.83	0.678	36.04±17.99 36.34±10.79	0.942
DOI	*≥4 mm* *<4 mm*	22 (44%) 28 (56%)	42.00±14.28 41.50±14.23	0.894	38.90±11.63 34.07±16.37	0.247
POI	*Mode 1* *Mode 2* *Mode 3* *Mode 4*	8 (16%) 10 (20%) 19 (38%) 13 (26%)	39.62±18.59 39.80±14.45 44.84±9.48 39.92±12.85	0.631	37.25±16.69 37.70±10.38 39.73±13.19 29.23±16.85	0.238
TB	*<5 buds* *≥5 buds*	7 (14%) 43 (86%)	45.71±11.81 41.06±13.12	0.384	39.71±11.72 35.62±14.99	0.496
LI	*Grade 1* *Grade 2* *Grade 3*	37 (74%) 6 (12%) 7 (14%)	40.13±13.44 47.33±10.48 45.28±11.32	0.337	37.40±13.15 37.33±18.15 28.85±18.48	0.362

Abbreviations: LVI: lymphovascular invasion; PNI: perineural invasion; DOI: depth of invasion; POI: pattern of invasion; TB: tumor budding; LI: lymphocytic infiltration.

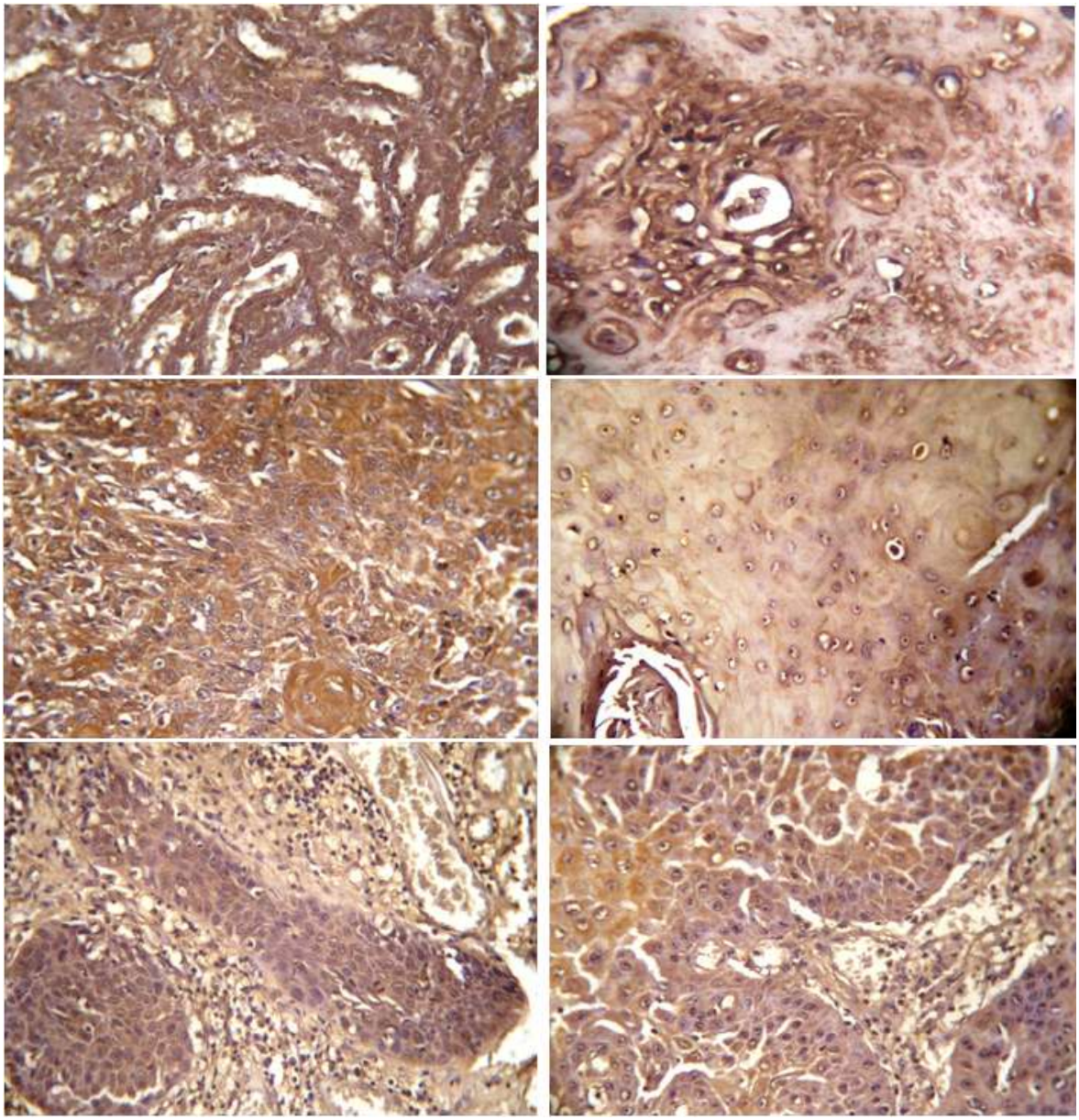

Figure 1: A. positive control of EPO (rat kidney tissue), B. and C. EPO expression in OSCC D. positive control of EPOR (placenta) E and F. EPOR expression in OSCC. (X20)

4. Discussion

Erythropoietin (EPO) was first suggested as a late acting growth factor that stimulates red blood cell production through binding and activating an erythropoietin receptor (EPOR) on the surface of committed erythroid progenitor cells resulting in their survival, proliferation and differentiation ***(20).***However, EPO is now considered to be a pleiotropic hormone that exhibits an anti-apoptotic action on numerous cells and tissues, including malignant ones ***(10).*** In this study, we reported the expression of both erythropoietin and erythropoietin receptor in tissues from oral squamous cell carcinoma. The expression pattern for EPO was granular mainly cytoplasmic and occasionally nuclear which was more intense in cells at the invasive tumor front and near necrotic foci. Similar pattern of expression was mentioned by other earlier reports in squamous cell carcinoma or other tumors ***(21,6,9)***. We detected cytoplasmic/or membranous expression for erythropoietin receptor. Beside tumor cells, EPO and EPOR expression was noticed in endothelial cells of tumor vasculatures. This expression suggesting the possible role of both proteins in induction of angiogenic process in oral squamous cell carcinoma. It was reported that EPO encourage vascular growth during tumor evolution ***(22)*** and its expression was correlated with microvessel density in tongue SCC ***(23)*** or tumor angiogenesis in both neuroblastoma, and melanoma ***(18,24).*** Further study revealed that EPO analogue stimulates neovascularization in colorectal liver metastases of hepatectomized and non-hepatectomized mice ***(25).*** Similarly, angiogenesis found to be enhanced by EPO administration via JAK2/STAT3/VEGF in pituitary adenoma ***(26).*** On the other hand, Hardee et al, found that active EPOR expression significantly encourage angiogenesis and growth of tumor cells ***(27).*** However, Doubts about the existence of expression or functionality of EPOR in tumor tissue was declared by some researchers. They stated that most results that proved EPOR expression by immunohistochemistry are based on utilization of non-specific antibodies that offer false-positive outcomes and discovery of an antibody with total specificity is rare (***28, 29).*** Some authors debated the specificity of C-20 antibody which is raised against intracellular COOH terminus of EPOR since it can identifies several proteins not related to EPOR such as heat shock protein 70 family ***(30).*** Another study reported that there was unnoticeable or low EPOR level in normal or tumor tissue from skin, lung, breast, colon, and ovary ***(20).*** By using RT-PCR and microarray analysis techniques, Sinclair and his co-workers found no signs for EPOR gene amplification in tumor tissues ***(31).*** In contrast to the outcomes obtained by many studies (Chiu etal, Wang etal, Li etal, and Seibold etal) this study revealed no association between EPO expression with patients age or higher tumor stage. Our study however, showed lower level of EPO expression in OSCC compared to that obtained by Chiu etal. ***(9).*** These differences in outcomes may be related to the patient's selection, sample size, disparities in clonality and concentration of primary antibodies or to the subjectivity in interpretation of data obtained because consensus scoring by researchers is usually unfeasible in routine IHC. Regarding EPOR expression we found positive correlations with gender and tumor site. Lower lip recorded the higher level of EPOR expression. However, Despite high levels of EPOR expression, no association with tumor depth, pattern of invasion, lymphovascular invasion, perineural invasion, or tumor buds have been recorded. This questioned the role of EPOR in tumor invasion but factors such as absence of cofactors necessary for EPOR transferring to the cell membrane (membranous expression considered as a prerequisite of the EPOR functionality) or downstream signaling pathways may be absent or repressed should be considered. Our results revealed significant correlation between EPO and EPOR in oral squamous cell carcinoma. The co-expression of both proteins in oral squamous cell carcinoma suggests the involvement of an autocrine EPO-EPOR signaling loop.

5. Conclusions

Positive expression of EPO and EPOR has been detected in OSCC. This expression was not associated with pathological parameters related to invasive and metastatic ability of oral squamous cell carcinoma cells.

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