Comparative Evaluation of Immunohistochemical Expression of Endothelin A Receptor between Oral Squamous Cell Carcinoma and Normal Oral Mucosa

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Abstract

Background: Recent research has provided evidences indicating the importance of endothelin axis in carcinogenesis. According to our knowledge, there are little information about endothelin A receptor (ETA) expression in oral squamous cell carcinoma (OSCC). So, the aim of the present study was to evaluate the immunohistochemical expression of ETA in OSCC and normal oral mucosa (NOM).

Methods: In this cross-sectional study, studied group composed of paraffin-embedded tissue blocks of 21 OSCCs and 20 NOMs. Four micron sections were prepared from tissue blocks and stained with ETA antibody using immunohistochemistry (IHC). Percentage of stained cells and staining intensity were compared between OSCC and NOM groups and also between different grades of OSCC using Mann-Whitney, Chi-Square and Kruskal-Wallis statistical tests.

Results: In OSCC group, all cases showed positive staining for ETA while in NOM group, 17 cases showed no staining. Comparison of the percentage of stained cells and staining intensity for ETA revealed a significant difference between OSCC and NOM groups ($P<0.001$). There was also a significant difference between different grades of OSCC with respect to the percentage of stained cells ($P=0.01$) so that with increase in grade, ETA expression was also increased.

Conclusion: The results of this study support the role of ETA receptor in carcinogenesis process and progression of OSCC.

Introduction

Oral squamous cell carcinoma (OSCC) is the most common malignant neoplasm of the oral cavity that comprises 90% of oral cancers (1,2). OSCC represents a public health problem. This cancer is extremely aggressive and immediately invades its underlying tissues and causes significant morbidity and mortality. In recent years, several studies have studied potential biomarkers of OSCC progression and prognosis. Targeted therapy against these biomarkers may help to overcome this cancer and lessen the morbidity and mortality caused by this cancer (1).

Endothelin (ET) axis includes three isoforms (ET-1, ET-2, ET-3) and ET receptors, which are Endothelin A (ETA) and Endothelin B (ETB) receptors, play an important physiologic role as regulators of vascular tone, tissue development and differentiation, cell proliferation and hormone production (3,4).

Recent research about endothelin axis has provided evidences of the importance of this axis in cancers (4). Forexample, in the study by Alaizari et al (2013), overexpression of endothelin-1 was observed in high-grade OSCCs compared to low-grade OSCCs (2) or in the study by Ishimoto et al (2012), overexpression of both endothelin receptors (ETA and ETB) was observed in tumor cells of tongue cancer specimens (3). Therefore, endothelin axis involves not only in vascular biology but also in carcinogenesis.

Components of endothelin system can help to tumors growth and progression via direct and indirect mechanisms (2). It seems that direct mechanism of neoplastic cells predominantly affects tumor cell proliferation, migration, invasion and resistance to apoptosis while indirect mechanism regulates different kinases involved in cell proliferation, survival, angiogenesis, epithelial-mesenchymal transition and cell invasion and mobility (2,5). Present studies show overexpression of ETA and ETB in lung, colon and skin cancers (3).

At present, treatments of OSCC include surgery with/without radiotherapy, and to a lesser extent, chemotherapy (1). According to the important role of endothelin axis in cancer biology (2) and the presence of ETA receptor antagonists, these antagonists can be used as a new therapeutic opportunity in targeted therapy for oral cancers (4).

The important role of endothelin axis in tumor biology have attracted much attention but there is little information about ETA receptor expression in OSCC. Therefore, the aim of the present study was to evaluate immunohistochemical expression of ETA receptor in oral squamous cell carcinoma (OSCC) and normal oral mucosa cases (NOMs) in order to provide the basis for future targeted therapies.

Materials and Methods

In this cross-sectional retrospective descriptive-analytical study, the samples included paraffin-embedded tissue blocks of 21 OSCCs (8 well-differentiated OSCCs, 8 moderately-differentiated OSCCs and 5 poorly-differentiated OSCC) that were retrieved from the archive of Oral and Maxillofacial Pathology Department. Tissue blocks of 20 NOMs (gingival tissue without clinically and histologically inflammation or with minimal inflammation caused by crown lengthening surgery) was used as control group.

In OSCC group, tissue sections of 4μ thickness were prepared from each tissue blocks and stained with hematoxylin and eosin (H&E) to ensure the accuracy of the diagnosis and to define histopathologic grade of OSCCs. The criteria used for diagnosis of OSCC and determination of its grade were based on the Neville et al (6). Based on these criteria, OSCCs were divided into three histopathologic grades: well-differentiated, moderately-differentiated, and poorly-differentiated. Patients with OSCCs who had received preoperative radiotherapy or chemotherapy and those with recurrent OSCC were excluded from the study. Tissue blocks without enough cancerous tissue or with improper quality and fixation were also excluded from the study. Patients who underwent excisional biopsies were included in the study (2).

Another 4μ sections were prepared from tissue blocks and stained with ETA antibody (Novocastra™ Liquid Mouse Monoclonal Antibody Endothelin-1 Receptor (ETA); Leica Biosystems, Newcastle, United Kingdom, Product Code:

NCL-L-ETA, Clone: RJT24, Ig Class: IgG2b) using immunohistochemistry. Sections of ductal breast carcinoma were used for positive control and primary antibody was removed for negative control. In immunohistochemical staining, tissue sections were immersed in citrate buffer 10 mM (pH 6) for antigen retrieval and were heated in the microwave oven at 95°C for 5 minutes. 3% hydrogen peroxide solution was poured on the glass slides for blocking the endogenous activity. Bonding of primary antibody was detected by streptavidin-biotin immunoperoxidase method, and diaminobenzidine was used as a chromogen (2). Stained slides were examined using Olympus CX21 light microscope (Olympus Corporation, Tokyo, Japan) at ×100 and ×400 magnifications by two independent pathologists. In this evaluation, percentage of stained cells (7) and staining intensity (8) for ETA immunomarker were taken into account. Cytoplasmic staining for the immunomarker was considered positive (8). The average of the reported scores was considered as the percentage of stained cells in each case. In the case of disagreement on the score of staining intensity, another pathologist (the third one) evaluated the specimen and final report was based on the opinions of two of the three pathologists.

In OSCC group, five fields were selected as hot spots (fields in which tumor cells had the highest staining intensity) at ×100 magnification under light microscope; in these fields percentage of stained cells was counted at ×400 magnification. The average of these five selected fields was recorded as the final percentage of stained cells for each case. In NOM group, selection of hot spots and calculation of the percentage of stained cells were performed at epithelium. Percentages of stained cells were semi-quantitatively categorized into four groups as follows:

Negative ($\leq 25\%$), weak positive (26-50%), positive (51-75%) and strongly positive ($> 75\%$) (7).

Staining intensity of tumor cells and normal mucosa was also semi-quantitatively categorized into four groups as follows:

Negative (score 0): absence of staining; weak positive (score 1+): weak/hardly appreciable cytoplasmic staining in the most of cells or light brown staining; moderately positive (score 2+): moderate cytoplasmic staining in the most of cells or oak brown staining; and strongly positive (score 3+): strong cytoplasmic staining in the most of cells or dark brown staining (8).

Finally, data were analyzed using Kruskal-Wallis, Mann-Whitney and Chi-square statistical tests by SPSS version 20. Statistically significant level was considered at $P< 0.05$.

Ethical Approvals

The study has been independently reviewed and approved by the Ethics Committee of Babol University of Medical Sciences (Code: MUBABOL.REC.1395.155).

Results

The average age of patients with OSCC was 65 years. Of those, 7 patients (33.3%) were females and 14 (66.7%) were males.

17 cases of NOMs didn't stain for ETA immunomarker while all cases of OSCC showed positive staining for this marker (Figures 1-3). Means ± standard deviations (SD) of the percentage of stained cells for ETA in OSCC and NOM groups were 0.74 ± 0.20 and 0.08 ± 0.14, respectively. Means ± SD of the percentage of stained cells in well-differentiated, moderately differentiated and poorly-differentiated OSCCs were 0.55 ± 0.15, 0.82 ± 0.12 and 0.96 ± 0.04, respectively.
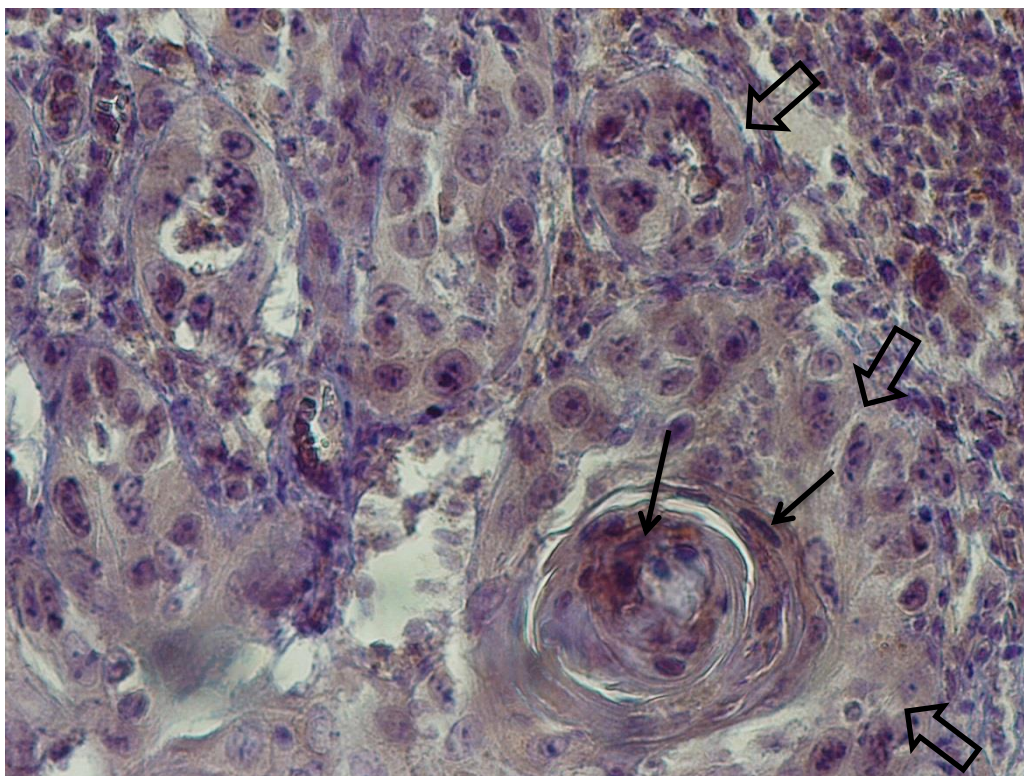
Figure 1. Well-differentiated OSCC with weak staining intensity for ETA (×400 magnification).
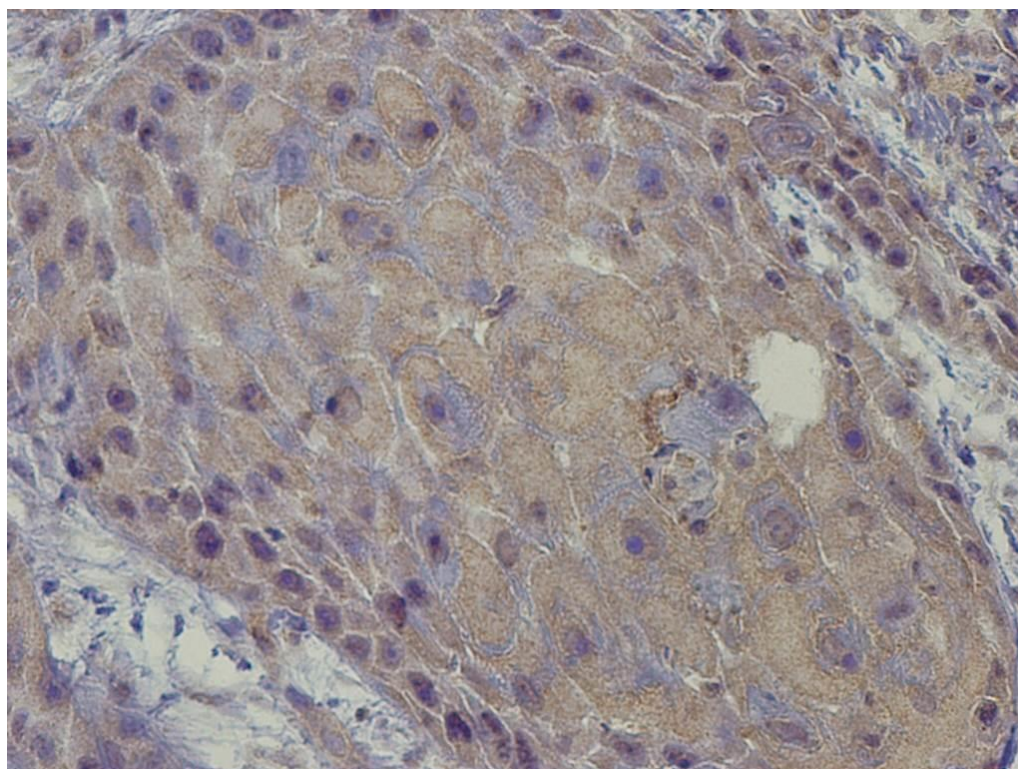

Figure 2. Moderately-differentiated OSCC with moderate staining intensity for ETA (×400 magnification).
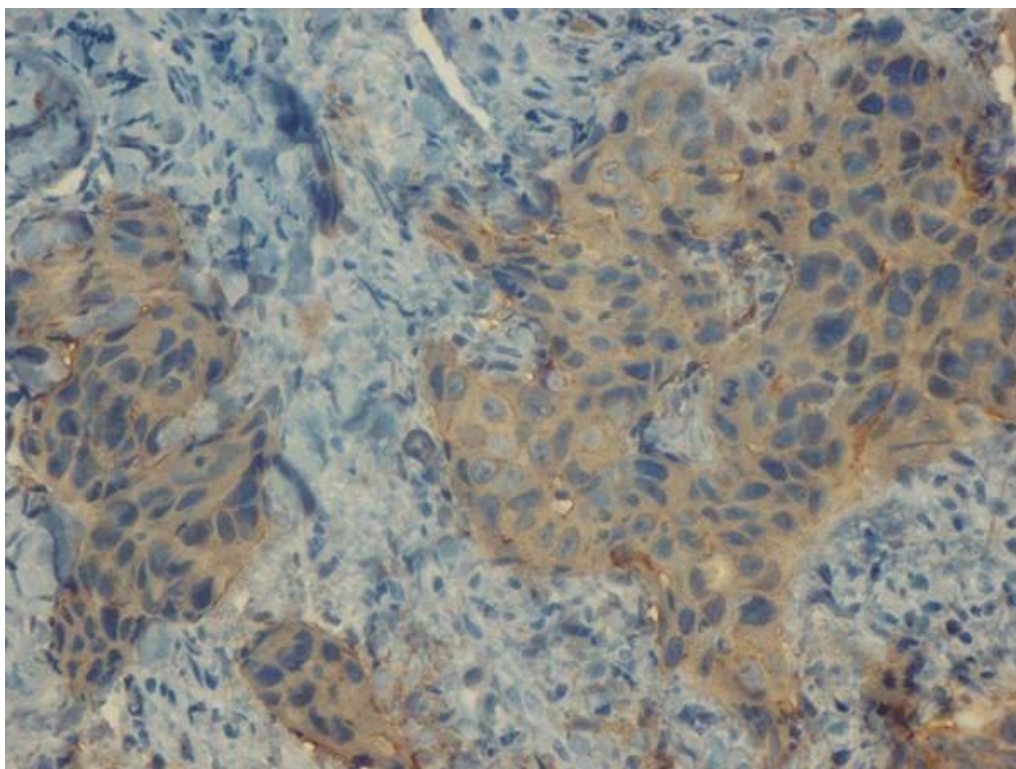
Figure 3. Poorly-differentiated OSCC with moderate staining intensity for ETA (×100 magnification).

Table 1 shows percentages of stained cells for ETA in NOM and OSCC groups. Statistical analysis shows that there was a significant difference the percentage of stained cells between NOM and OSCC groups (P<0.001); in other words, the percentages of stained cells in OSCC group were significantly higher than NOM group. OSCCs in strongly positive category of percentage of stained cells were greater than positive, weak positive and negative categories .Table 1 also shows percentages of stained cells for ETA in different grades of OSCC groups. Statistical analysis shows that there was a significant difference in the percentage of stained cells between different grades of OSCC (P<0.05); in other words,

by increase of OSCC grade, the percentages of stained cells were significantly increased . All of poorly-differentiated OSCCs were placed in the strongly positive category.

Table 2 shows staining intensities for ETA in the groups. Statistical analysis shows that weak positive and moderately positive categories of staining intensity were observed significantly more than strongly positive category (P<0.05).

Table 2 also shows staining intensities for ETA in different grades of OSCC. Statistical analysis shows that there was no significant difference in staining intensity between different grades of OSCC (P>0.05)

Table 1. Categories of percentage of stained cells for ETA in normal oral mucosa (NOM) group and oral squamous cell carcinoma (OSCC) group.

Group	Category	Percentage of Stained Cells				P-value	
		Negative	Weak Positive	Positive	Strongly positive		
NOM		17	3	0	0		
		85%	15%	0%	0%		
OSCC	Well-differentiated	0	4	3	1		
		0%	50%	37.5%	12.5%		
	Moderately-differentiated	0	0	3	5	0.01	<0.001
		0%	0%	37.5%	62.5%		
	Poorly-differentiated	0	0	0	5		
		0%	0%	0%	100%		
	Total	0	4	7	10		
		0%	19%	33.4%	47.6%		

Table 2. Categories of staining intensity for ETA in normal oral mucosa (NOM) group and oral squamous cell carcinoma (OSCC) group.

Group	Category	Categories of Staining Intensity				P-value	
		Negative	Weak positive	Moderately positive	Strongly positive		
NOM		17	2	1	0		
		85%	10%	5%	0%		
OSCC	Well-differentiated	0	7	1	0		
		0%	87.5%	12.5%	0%		
	Moderately differentiated	0	3	4	1	0.19	<0.001
		0%	37.5%	50%	12.5%		
	Poorly-differentiated	0	2	3	0		
		0%	40%	60%	0%		
	Total	0	12	8	1		
		0%	57.1%	38.1%	4.8%		

Discussion

Oral squamous cell carcinoma (OSCC) is the most common malignant neoplasm of the oral cavity. Recent research about the role of endothelin axis has provided evidences indicating the importance of endothelin A (ETA) receptor in cancers. So, the aim of the present study was to evaluate immunohistochemical expression of ETA in patients with OSCC and those with normal oral mucosa (NOM).

In this study, percentage of stained cells in OSCC group was significantly higher than NOM group that is suggestive of the probable role of ETA in carcinogenesis process of OSCC.

It was also revealed that with increase of OSCC grade, ETA expression was also significantly increased. This finding suggests a relationship between ETA expressions and OSCC grade. Therefore, it is suggested that increase in ETA expression probably help to progression of OSCC and increase of its invasive properties. In the study by Ishibashi et al (2003), high expression of endothelin protein reduced the

recurrence-free survival in patients with esophageal SCC. They concluded that measurement of endothelin expression by a simple immunohistochemistry analysis may help to predict prognosis of patients with esophageal SCC (9). Increase in ETA expression due to the increase of OSCC grade in the present study is considered as a negative prognostic factor, which is consistent with the study by Ishibashi et al (٩).

In Awano et al (2006) study on endothelin axis components (ET-1, ETA, ETB and endothelin-converting enzyme 1 [ECE-1] isoforms) in human OSCC cells, expressions of all components were observed in OSCC cells but only ET-1, ETB and ECE-1 increased in comparison with normal epidermal keratinocytes. They concluded that endothelin system regulation in OSCC cells may provide a new therapeutic protocol for oral cancer (10). The results of the present study are to some extent inconsistent with Awano et al (2006) study although in their study other endothelin axis components increased in OSCC in comparison with normal keratinocytes. The reason for this inconsistency can be due to using paraffin-embedded tissue blocks in this study while they used OSCC cell lines, in other words, it can be due to different methods used for detecting ETA expression because they used RT-PCR, immunoblot, ELISA and immunofluorescence to detect endothelin axis components while in this study immunohistochemistry was used to detect ETA protein expression.

In Pickering et al (2007) study, overexpression of endothelin-1 protein and mRNA was observed in OSCCs in comparison to normal control group (11). The results of the present study are to some extent consistent with Pickering et al (2007) study because both ET-1 and ETA are components of endothelin axis and ET-1 exert at least some of its effects by bonding to ETA receptor.

In Hinsley et al (2012) study, ET-1 increased migration of head and neck SCC cells through releasing EFGR ligands from fibroblasts. They concluded that endothelin axis activation in head and neck SCC may help to SCC progression by stimulating cancer cell motility via increasing epithelial-stromal interactions (12), which is consistent with the present study.

In Ishimoto et al (2012) study, both ETA and ETB endothelin receptors were overexpressed in tumor cells of tongue cancer specimens. They concluded that endothelin signaling pathway may play an important role in cell growth in SCC (3), which is consistent with the present study because in both studies, ETA was overexpressed in tumor cells of OSCCs.

Alaizari et al (2013), observed endothelin-1 immunoreactivity in all of the studied samples and reported significantly more immunoexpression in higher grade of OSCCs compared to that in the lower grade of OSCCs. They concluded that overexpression of endothelin-1 can increase invasive properties of poorly-differentiated OSCCs (2), which is consistent with the results of this study.

Salem et al (2015), reported that endothelin-1 and ETA were expressed significantly more in all of cutaneous squamous cell carcinoma (CSCC) and psoriasis compared to the control and basal cell carcinoma groups. They concluded that overexpression of ET-1 and ETA implies to their involvements in keratinocyte proliferation in CSCC and psoriasis (8). The results of their study are consistent with the result of this study in the field of overexpression of ETA in SCCs.

Cong et al (2016), also reported the overexpression of ETA in hepatocellular carcinoma tissues and cells (13). Results of their study support the role of ETA in cancer progression, which is consistent with results of this study indicating the role of ETA in OSCC progression.

Conclusion

Regarding the significant difference in ETA expression between NOM and OSCC groups, it seems that this receptor involves in carcinogenesis process in OSCC. On the other hand, with respect to the significant increase of ETA

expression in higher grades of OSCC, this receptor probably helps to progression of OSCC.

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