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Expression of Inducible Nitric Oxide Synthase in Oral Squamous Cell Carcinoma and Relationship to Regional Lymph Node Metastasis

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I. Introduction:

Oral squamous cell carcinoma (OSCC) is the most prevalent malignant neoplasm all over the world.[1]These malignancies are often still not detected until a late stage.[2]Inducible NOS is an enzyme that has been implicated in the tumorigenesis of various neoplasms. The role of NO generated by the iNOS is very complex. However, the pathways regulating iNOS expression seem to vary in different cells or different species.[3]There are few reports in the literature regarding the expression and relationship of iNOS with cervical lymph node metastasis in OSCC. **Methods:**The present study included forty formalin-fixed, paraffin-embedded tissue blocks of OSCC; with and without regional lymph node metastasis.The sections were examined and graded histopathologically according to WHO criteria (2010) and were subjected to the Streptavidin- Biotin Complex (ABC) immunohistochemical staining procedure as described by Wood and Wrank (1981).iNOS immunostained sections were examined under light microscope at x10 followed by x40 magnification as expression was evaluated.**Results:** Within tumor cells, iNOS expression revealed to be upregulated with increased malignancy grade. In addition, the rate of expression of iNOS in cases having lymph node metastasis is significantly higher than those having no lymph node metastasis ($P<0.05$). **Conclusions:** The expression of iNOS was absent in normal mucosal keratinocytes as well as iNOS expression was inversely proportional to tumor differentiation, but that wasn't statistically significant.iNOS is a reliable marker for lymph node metastasis in OSCC irrespective of the histologic grade and finally, iNOS expression correlates with nodal metastasis in oral squamous cell carcinoma.

Oral squamous cell carcinoma constitutes at least 90% of all oral malignancies. According to World Health Organization (WHO) 2017, it is the sixth commonest cancer in males, and the tenth commonest site of cancer in females. Evaluation of cervical lymph node metastasis is an integral point to determine the surgical approach and the possible use of chemotherapy in patients with oral cancer.[4]

Nitric oxide (NO), a free-radical gas, is a short-living molecule which is involved in a multitude of biological processes including inflammation and cancer.[5]NO can react with other radicals to form cytotoxic compounds, such as peroxynitrite, which can cause DNA damage and protein modifications.[6]NO can also react directly with a variety of enzymes and other proteins to either activate or inhibit their functions.[7]NO is generated by NO synthases (NOS) that convert L-arginine to L-citrulline in tumor cells, macrophages and fibroblasts.[8]

It has been reported that an enhanced expression of iNOS was seen in epidermal squamous cell carcinoma[9]and breast cancer[10]and in gynecological malignancies.[11] From these reports; iNOS may seem to play an unknown role in tumor growth, metastasis and angiogenesis. On the other hand, it has been reported that over expression of the iNOS gene can suppress tumor growth and metastasis of melanoma and renal carcinoma cell.[12]Moreover, it has also been reported that NO can induce apoptosis in these tumor cells by suppressing nuclear factor 1 beta (NF-1β) activity[13]or caspase.[14]It seems that iNOS may be related to the antitumor effect as well as to carcinogenesis.

This study was undertaken to assess the expression of iNOS in OSCC and its relationship to cervical lymph node metastasis.

II. Material And Methods

Materials:

A) Tissue Specimens:

Fortyformalin-fixed, paraffin-embedded tissue blocks; twenty blocks of OSCC with regional lymph node metastasis & twenty blocks of OSCC without regional lymph node metastasis obtained from the archives of Oral Pathology Department, Faculty of Dentistry, Tanta University.Approval for this research was obtained from Research Ethics Committee Faculty of Dentistry, Tanta University.

B) Immunohistochemical (IHC) Reagents:

- Primary mouse monoclonal IgG_1 antibodies against human iNOS*.
- Strept-Avidin Biotin Complex Universal Kit*.

* *Manufacturer: R & H systems a biotechne brand, USA Public domain,*

Methods:

A) Histopathological Examination:

Serial paraffin sections were cut using a microtome with a thickness of 4µm and mounted on positively charged (Opti-plus) slides for the staining procedure. A section from each specimen was stained by routine haematoxylin and eosin to confirm the diagnosis, and the most representative sections for immunostaining were selected.

B) IHC Technique:

The slides were subjected to the following Streptavidin- Biotin Complex (ABC) immunohistochemical staining procedure as described by Wood and Wrank (1981).

Evaluation of Immunostaining:

1) Semi-quantitative analysis:

iNOS expression was evaluated according to the scoring scale as modified from Martin et al. (2007).

2) Image analysis using ImageJ software:

Assessment of the staining intensity using ImageJ software Modified from Varghese et al. (2007).

Statistical analysis:

The data obtained were then statistically analyzed using (IBM SPSS17.0 version software for windows). Correlations among groups were done using one way analysis of variance (one way ANOVA) test. Subsequent comparisons between groups were assessed using Mann-Whitney U test. Significance of changes in iNOS expression versus histopathological grade was tested by person correlation test as well as non-parametric Kendall's tau-b and spearman's rho test. The level of statistical significance was at $p < 0.05$.

III. Results

Histo-pathological findings :

A) Conventional H & E staining of OSCC

By light microscope examination of 20 cases of OSCC without regional lymph node metastasis, 7 cases out of the 20 cases (35%) were diagnosed as well-differentiated, 6 cases (30%) were diagnosed as moderately differentiated and the remaining 7 cases (35%) were diagnosed as poorly differentiated.

By light microscope examination of 20 cases of OSCC with regional lymph node metastasis, 8 cases out of the 20 cases (40%) were diagnosed as well-differentiated, 7 cases(35%) were diagnosed as moderately differentiated and the remaining 5 cases (25%) were diagnosed as poorly differentiated.

B) Immunohistochemical staining of iNOS:

The iNOS expression was observed in 70% of specimens without lymph node metastasis while iNOS expression was observed in all specimens with lymph node metastasis (100%).In addition, most of the inflammatory cells including macrophages, giant cells and sometimes endothelial cells were found to be iNOS positive.

Analysis of iNOSimmnuohistochemical expression:

A) Semi Quantitative analysis:

- **Well differentiated OSCC**

i. Cases without lymph node metastasis

Seven cases showed variety of intensity in iNOS expression ranged from 0 to 1.Out of the seven cases, four cases with SCC exhibited no staining with a percentage of 57.1% while three cases

exhibited weak positive staining with a percentage of 42.9% and none of them showed strong or moderate iNOS expression.

ii. Cases with lymph node metastasis

Eight specimens showed intensity of iNOS expression ranged from 1 to 3. Five cases showed weak positive intense reaction with a percentage of 62,5% while two cases expressed moderate positive staining with a percentage of 25% and only one case exhibited strong iNOS expression 12.5%

- **Moderately differentiated OSCC**

i. Cases without lymph node metastasis

Six cases showed variety of the intensity of iNOS expression ranged from 0 to 2.Out of the six cases, two cases has intensity of 0 with negative iNOS expression 33.3% while 3 cases exhibited weak positive staining with a percentage of 50% and only one specimens has intensity of 2 with moderate iNOS expression 16.7%

ii. Cases with lymph node metastasis

Seven specimens with lymph node metastasis showed intense reaction of iNOS expression ranged from 1 to 3. Only one case has intensity of 1 with weak positiveiNOS expression 14.3% while four cases expressed moderate positive staining with a percentage of 57.1% and two cases has intensity of 3 with strong iNOS expression 28.6%

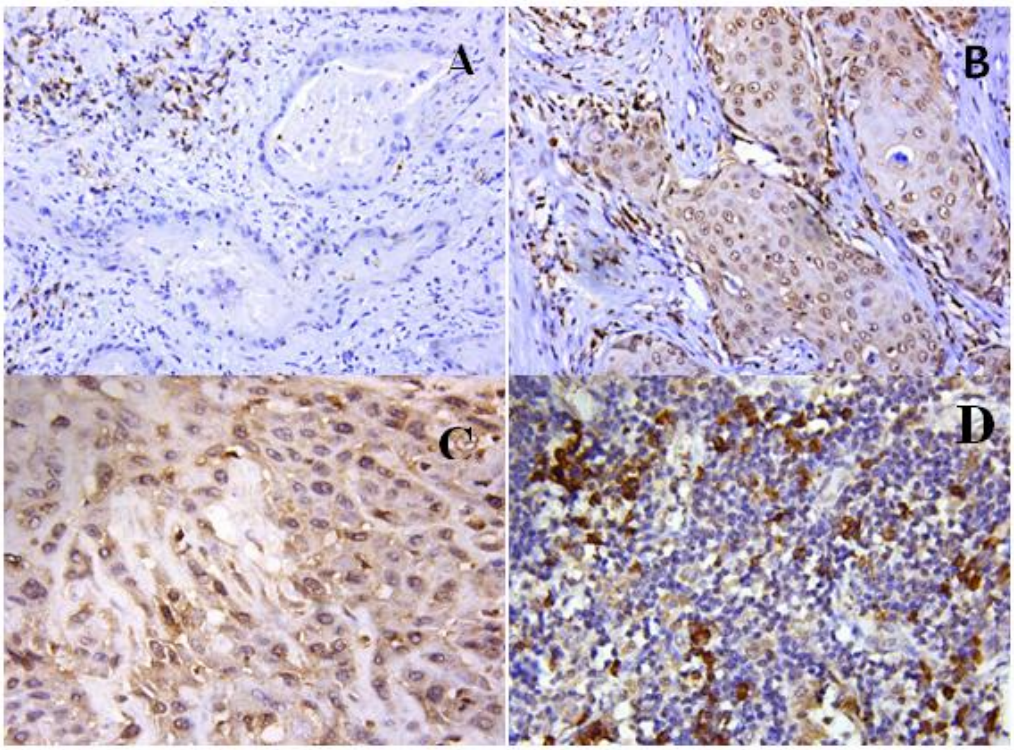
- **Poorly differentiated OSCC**

i. Cases without lymph node metastasis

The intensity of iNOS expression ranged from 1 to 2 with mean value of (23.400±16.62). Four cases showed weak staining with a percentage of 57.1% while three cases exhibited moderate positive staining with a percentage of 42.9%

ii. Cases with lymph node metastasis

The intensity of iNOS expression ranged from 2 to 3.Out of the five cases, two cases exhibited moderate staining with a percentage of 40% while three cases expressed strong staining with a percentage of 60% and none of them showed weak iNOS expression.



(A) photomicrograph shows well-differentiated OSCC with negative cytoplasmic immunostaining of keratin pearls. (B) reveals moderately-differentiated OSCC with positive (+2) cytoplasmic immunostaining of malignant epithelial cell nests.(C) photomicrograph shows poorly-differentiated OSCC with high positive (+3) cytoplasmic immunostaining of malignant scattered epithelial cells. (D) unveils the same lesion of (C) in metastatic lymh node.

IV. Discussion

It has been proved that there is an increase in the expression of iNOS in a variety of neoplasms and this pathway is involved in various stages of tumorigenesis such as cellular proliferation, evasion from p53 dependent apoptosis and tumor induced angiogenesis.[15] NO production may promote cancer progression by providing a selective growth advantage to tumor cells with mutant p53, Induction of angiogenesis by modulating angiogenic factors, thus promoting tumorigenic activity or tumoricidal action by activating p53.[16]

In the present study, there is an increased expression of iNOS on OSCCs but the expression was absent in normal mucosal keratinocytes and these results are in agreement with findings of Connelly et al.[17], Karthik et al.[18] and Chen [19] These findings indicate the role of NO in neoplastic transformation and carcinogenesis. In addition, absence of iNOS from normal epithelium may be due to that epithelial cells require factors, conditions, and/or exposures to maintain continuous high-level expression of iNOS.[18]

In the present study, a positive correlation was seen between lymph node metastasis and iNOS expression and the association of lymph node metastasis with positive iNOS expression was statistically significant and these results in agreement with findings of Augustine et al. [20] , Karthik et al. [18] , Sappayatosok et al. [21] and Brennan et al. [22] The proposition can be made that there are enhanced growth and metastasis via NO‑mediated pathogenesis as NO causes induction of angiogenesis and enhanced tumor growth and expression has been shown to be associated with extra‑capsular spread[22] and also Continuous generation of NO by iNOS in the stroma of tumors contribute to the activation of matrix metalloproteinases (MMPs), which assist in the breakdown of basement membrane, the loss of integrity and degradation of collagenous and non-collagenous proteins of stromal tissue and bone, which in turn preconditions the stroma and facilitate the process of invasion.[23]

In this study, OSCCs showed all stain intensities (no staining, weak, moderate and strong) and iNOS expression was inversely proportional with tumor differentiation, but that wasn't statistically significant. These findings are in agreement with observations of Augustine et al.[20] and the findings of Sappayatosok et al.[21] that reported correlation of iNOS expression with tumor staging but not tumor grading.

In contrast, Karadayi et al.[24] revealed iNOS expression in gastric adenocarcinoma and there was a significant difference between iNOS and histological grade. Moreover, Tschugguelet al.[25] demonstrated that expression of iNOS in human breast cancer depends on tumor grade, and that was statistically significant. These findings may be due to the different concentrations of iNOS in different tissues of the body as the effects of NO in tumor seem to depend on the activity, localization of NOS isoforms concentration, duration of NO exposure and cellular sensitivity to NO.[26]

Accordingly, all the findings of the current study suggested a potential role of iNOS in metastasis of OSCC in regional cervical lymph node.

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