A Case-control Study Comparing and Correlating iNOS Expression among Various Clinicopathological Variants of Oral Leukoplakia and Oral Squamous Cell Carcinoma: A Immunohistochemistry Study

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Background and Objective: The role of inducible nitric oxide synthase (iNOS) has been implicated in various pathological processes including oral carcinoma. Oral premalignancy being its precursor lesion is also expected to show similar pattern. This study attempts to appraise the iNOS expression in various clinicopathological stages and grades of oral leukoplakia (OL) and oral squamous cell carcinoma (OSCC). Materials and Methods: A case-control study design was adopted for this study with a total sample of 90 subjects, distributed equally into the three study groups, namely controls, OL, and OSCC. Clinical staging and histopathological grading for both the case groups were performed. Representative tissue samples from all groups were obtained and studied for iNOS expression using immunohistochemistry (IHC). Data were presented in mean and percentages accordingly. Inferential analysis was performed using Kruskal–Wallis test, Mann–Whitney test, and Spearman rank correlation test. Results: Significant (P < 0.001) difference was observed among the groups, where 83.3% of OSCC and 73.3% of OL epithelial cells showed iNOS expression. The normal cells did not show up any expression. The expression was found to rise with the progressing clinical stages of OL (P < 0.05) and OSCC (P < 0.01). Similar pattern was observed with respect to advancing dysplasia in OL (P < 0.01) and cell differentiation in OSCC (P < 0.01). Significant positive correlation was found in clinicopathological categories of OL and OSCC. Considering the risk assessment, iNOS staining was found to be significantly raised in advanced cases of OSCC (P < 0.01) and high-risk cases of OL (P < 0.01). Conclusion: Increased expression of iNOS can be an early diagnostic marker in OL and as prognostic marker in OSCC.

Keywords: Immunohistochemistry staining, inducible nitric oxide synthase, nitric oxide synthase, oral cancer, oral premalignant

INTRODUCTION

Alarming reports from the Global Cancer Observatory (GCO) show an increase in the burden of cancer arising in the lip and oral cavity worldwide. It is ranked as the 16th most common cancer with 177,384 counts of deaths in 2018, for both genders of all ages.[1] On further exploration of the cancer statistics, Asia accounts for 64.2% of the...
The end products of lipid peroxidation have shown to increase the free radical production further. Eventually, these entities cause mutations in the essential regulatory genes, thus contributing to oral carcinogenesis.\[18]\n
Another aspect of iNOS-induced NO in the development of OC is its influence on the process of angiogenesis in a hypoxic environment.\[19]\n
It is considered as an imperative for tumor survival and progression as it builds numerous new channels for the dissemination of neoplastic cells.\[20]\n
As NO has a possible behavior of a signaling molecule in the tumor microenvironment, its possibility as a carcinogenic marker needs to be explored.

This facet of NO has been explored in previous studies, but with conflicting results regarding its expression.\[21,22]\n
Also, very few studies are available, which have evaluated the simultaneous expression of iNOS in premalignancy and oral malignancy.\[23]\n
To the best of our search, no study has attempted to correlate the clinical staging of PMD with iNOS expression.

In the view of the lacunae, as aforementioned, this study aimed to evaluate and compare iNOS expression among the different clinicopathological categories of patients with OL and OC. The null hypothesis for this study states that an equal amount of iNOS expression was observed in the healthy controls, OL, and OC patients.

**Materials and Methods**

**Study and sample characteristics**

This research has been approved from the institutional ethics board. This study has an analytical study of the case-control design. Subjects from the two case groups were recruited from the outpatient clinic of oral diagnosis and medicine clinic. Each group comprised 30 patients of OL and oral squamous cell carcinoma (OSCC), respectively. Inclusion of the patients in case groups was carried out after the due histopathological conformation. Consequently, histopathological grading was performed for the patients of each case group. To prevent bias for the confounding factor, 30 age- and gender-matched subjects with no history of tobacco consumption were included in the control group. Patients with a previous history of treatment for OL and OSCC and those with chronic systemic diseases such as diabetes mellitus, hypertension, liver dysfunction, or other carcinoma and pregnancy were excluded from the study.

**Study protocol**

The objectives and purpose of the study were explained to the subjects prior to the initiation of the study.
Subsequently verbal as well as written informed consent was obtained. The study followed the principles of the declaration of Helsinki. Subjects were given the option to leave the study at any given point. The detailed case history was recorded with an emphasis over the personal history related to tobacco consumption. The comprehensive examination was performed with the intended outcome of providing oral leukoplakia staging (OLEP staging)\cite{24} and tumor, node, and metastasis (TNM)\cite{25} staging for OL and OSCC, respectively. Tissue samples were received from incision biopsy for immunohistochemistry (IHC) staining for evaluating iNOS expression as described by Varghese et al.\cite{26}

**Interpretation of immunohistochemistry slides**

The assessment of iNOS staining was twofold, including the quantitative and qualitative, which was performed by two independent observers. The inter- and intra-examiner variability was assessed with Cronbach α measuring 0.85 and 0.81, respectively, showing a high level of agreement. Initially, the percentage of tissue stained was assessed, where the positive expression was considered only if more than 25% of cells were involved. According to the amount of staining, they were graded. In the second part of the evaluation, the intensity was examined, and the grading was performed accordingly. A comprehensive score was generated by combining the two grades.\cite{27}

**Statistical analysis**

For the sample size calculation, a post hoc analysis was performed using G Power 3.1.9.2 software (Heinrich-Heine-Universität Düsseldorf, Germany). It was kept at a confidence interval (α) 0.05, the effect size of 0.5, and 2 Df (degree of freedom). The sample size achieved a statistical power of 0.92. Collected data were entered into Excel spreadsheets (Microsoft, Redmond, WA, USA) for editing and coding. The qualitative data were expressed in percentages, whereas the quantitative data were expressed in mean with standard deviation. For comparison between the groups and different clinicopathological variants, Kruskal–Wallis test was used. Spearman rank correlation test was performed for the significant variables. Association was considered statistically significant when $P < 0.05$. Data were analyzed using the Statistical Package for the Social Sciences (SPSS) software program, version 21.0 (IBM, Chicago, Illinois).

**RESULTS**

This study aspires to evaluate and correlate the expression of iNOS in patients with various clinicopathological categories of OL and OSCC.

**Analysis of biographic and habit-related data**

In this study, the mean age of patients with OL was 43.80 ± 11.12, whereas the average age of OSCC patients was 62.07 ± 10.18. In both the study groups, there was male predominance when gender was compared. This study reestablishes the age and gender distribution available in the literature, which puts oral malignancy highly prevalent in old age and male predominance. The habit of quid placement with the development of OL lesion can be well correlated in this study where majorities (60%) of the lesion were found on buccal mucosa. Lesions of OSCC were mostly recorded in the vestibule (40%), with tongue reported with the least number (13.3%) of cases. However, the presence of habit in our entire sample supports the fact of them

| S. no. | Variable                          | Study group       |
|-------|----------------------------------|-------------------|
|       |                                  | Group A | Group B | Group C |
| 1.    | Sample size                      | 30      | 30      | 30      |
| 2.    | Age (expressed as mean ± SD)     | 38.97 ± 9.694 | 43.80 ± 11.124 | 62.07 ± 10.184 |
| 3.    | Gender                           | Female | 7 (23.3) | 8 (26.7) | 12 (40) |
|       |                                  | Male    | 23 (76.7) | 22 (73.3) | 18 (60) |
| 4.    | Duration of habit                | Not applicable | 19.50 ± 10.009 | 8.07 ± 3.483 |
| 5.    | Frequency of habit               | Not applicable | 8.07 ± 3.483 | 8.40 ± 3.519 |
| 6.    | Site of lesion                   | Buccal mucosa | 18 (60) | 6 (20) |
|       |                                  | Alveolus | 4 (13.3) | 8 (26.6) |
|       |                                  | Vestibule | 5 (16.6) | 12 (40) |
|       |                                  | Tongue   | 3 (10) | 4 (13.3) |

SD = standard deviation
being a major risk factor in the development of oral premalignant and malignant lesions [Table 1].

**Inferential and causal analysis of inducible nitric oxide synthase expression among the study groups**

A significantly ($P < 0.001$) higher percentage (83.3%) of patients with OSCC showed iNOS expression in comparison to patients with OL (73.3%). On the contrary, none of the samples of the control group showed expression [Table 2].

Later comparative evaluation was performed among the clinicopathological variants of OL. Firstly, the evaluation was performed within the histopathological grades. A significant ($P < 0.01$) increase in the number of cases with expression was seen along with the deteriorating grades of dysplasia. Similarly, a significant ($P < 0.05$) increasing percentage of cells with positive expression was found with the advancing clinical OLEP stages. These results of the study laid the path to conduct the correlation analysis. A significant positive correlation was seen between the expression level with histopathological grades ($P < 0.01; r = 0.580$) and clinical stages ($P < 0.05; r = 0.592$) [Table 3].

Subsequently, a similar analysis was performed with respect to OSCC clinicopathological stages and grades. A significant increase in iNOS expression was found in advancing histopathological grades ($P < 0.01$) and clinical TNM stages ($P < 0.01$). On performing causal analysis, a significant positive correlation ($P < 0.001$) was observed among the iNOS expression and progressing clinical stages ($r = 0.638$) and a deteriorating level of cell differentiation ($r = 0.609$) [Table 4].

### Table 2: Comparative evaluation of inducible nitric oxide synthase levels among the study groups

| S. no. | Study group | Number of cases | No. (%) of cases showing positive for iNOS | $P$ Value |
|--------|-------------|----------------|------------------------------------------|-----------|
| 1      | Group A     | 30             | 0                                        | 0.000*    |
| 2      | Group B     | 30             | 22 (73.33)                               |           |
| 3      | Group C     | 30             | 25 (83.3)                                |           |

iNOS = inducible nitric oxide synthase

* $P < 0.001$

### Table 3: Frequency distribution table showing comparative and correlation analysis of inducible nitric oxide synthase expression among different clinicopathological types of oral leukoplakia

| S. no. | Variable                                   | Category          | Number of cases | No. (%) of cases showing positive for iNOS | $P$ Value | $P$ Value (correlation coefficient) |
|--------|--------------------------------------------|-------------------|-----------------|-------------------------------------------|-----------|-----------------------------------|
| 1      | Clinical stages (OLEP staging)             | Stage I           | 6 (20.0)        | 3 (50)                                    | 0.015*    |                                   |
|        |                                            | Stage II          | 8 (26.7)        | 6 (75)                                    |           | 0.001† (0.592)                    |
|        |                                            | Stage III         | 4 (13.3)        | 3 (75)                                    |           |                                   |
|        |                                            | Stage IV          | 12 (40.0)       | 10 (83.33)                                |           |                                   |
| 2      | Histopathological grades (dysplasia)       | Mild              | 9 (30.0)        | 6 (66.6)                                  | 0.008†    | 0.001† (0.580)                    |
|        |                                            | Moderate          | 10 (33.3)       | 7 (70)                                    |           |                                   |
|        |                                            | Severe            | 11 (36.7)       | 9 (81.81)                                 |           |                                   |

OLEP = oral leukoplakia staging, iNOS = inducible nitric oxide synthase

* $P < 0.05$ and † $P < 0.01$

### Table 4: Frequency distribution table showing comparative and correlation analysis of inducible nitric oxide synthase expression among different clinicopathological types of oral squamous cell carcinoma

| S. no. | Variable                                   | Category        | Number of cases | No. (%) of cases showing positive for iNOS | $P$ Value | $P$ Value (correlation coefficient) |
|--------|--------------------------------------------|-----------------|-----------------|-------------------------------------------|-----------|-----------------------------------|
| 1      | Clinical stages (TNM staging)              | Stage I         | 3 (10.0)        | 1 (33.3)                                  | 0.0061†   | 0.000† (0.638)                    |
|        |                                            | Stage II        | 6 (20.0)        | 5 (83.3)                                  |           |                                   |
|        |                                            | Stage III       | 8 (26.7)        | 7 (87.5)                                  | 0.0000‡   |                                   |
|        |                                            | Stage IV        | 13 (43.3)       | 12 (92.30)                                |           |                                   |
| 2      | Histopathological grades (level of differentiation) | Well            | 4 (13.3)        | 3 (75)                                    | 0.0000‡   |                                   |
|        |                                            | Moderate        | 12 (40.0)       | 9 (75)                                    |           |                                   |
|        |                                            | Poorly          | 14 (46.7)       | 13 (92.85)                                |           |                                   |

TNM = tumor, node, and metastasis staging, iNOS = inducible nitric oxide synthase

† $P < 0.01$ and ‡ $P < 0.001$
Evaluation of iNOS expression was studied among the merged groups of clinical stages of both study groups. Significantly ($P < 0.01$) increased expression was observed in the high-risk group of OL cases in comparison to low-risk group patients. Likewise, advanced OSCC cases outscored ($P < 0.01$) the early-stage OSCC subjects. A significant positive correlation ($P < 0.01$) was observed in both the study groups among iNOS expression and clinical risk assessment.

**DISCUSSION**

Chronic inflammation-induced carcinogenesis is a well-established fact wherein mediators such as iNOS, vascular endothelial growth factor (VEGF), p53, cytokines, NO, and reactive oxygen species (ROS) play a major role. iNOS is known for its production of NO in large amounts under chronic inflammatory conditions.$^{[6,28]}$ Their raised expressions have already been well established in various malignancies including head and neck squamous cell cancer (HNSCC).$^{[9]}$ However, inconsistencies with respect to iNOS expression in PMD are noted.$^{[7,21,22]}$ In addition, correlation among clinical stages and histological grades are not well explored. Thus, this study aimed at evaluating the iNOS expression in OL and OSCC and also attempts to correlate the levels along the clinical stages and the histopathological grades.

**Intergroup comparison of inducible nitric oxide synthase expression**

The percentage of cells showing iNOS expression was found to be significantly ($P < 0.001$) different between the three study groups. None of the normal cells showed the positive expression. On the contrary, 73.3% and 83.3% of cases showed expression in OL and OSCC, respectively [Table 2]. In the OSCC specimens, the staining was intense in the center of tumor islands and in the invasive tumor front [Figure 1A–C]. In the dysplastic epithelium specimens of OL, epithelial staining was also seen in the cytoplasm of premalignant keratinocytes but appeared less intense [Figure 2A and B]. It has been reported that an enhanced expression of iNOS is seen in the various cancers including OSCC and plays an important role in tumor growth, angiogenesis, and metastasis.$^{[9]}$ This has been shown to occur in premalignant conditions as breast and cervical dysplasia.$^{[29]}$ In a similar study, the overexpression of iNOS was found to be correlated with cervical lymph node metastasis and angiogenesis.$^{[10]}$

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**Figure 1:** (A) Well-differentiated OSCC showing inducible nitric oxide synthase (iNOS) staining ($×40$ magnification). (B) Moderately well-differentiated OSCC showing iNOS staining ($×40$ magnification). (C) Poorly differentiated OSCC showing iNOS staining ($×40$ magnification)

**Figure 2:** (A) Moderate dysplasia showing inducible nitric oxide synthase (iNOS) staining ($×10$ magnification). (B) Severe dysplasia showing iNOS staining ($×10$ magnification)
Comparative analysis of inducible nitric oxide synthase expression in the oral leukoplakia

In this study, all grades of dysplasia showed expression of iNOS with significantly \( (P < 0.01) \) varying percentages of involved number of cells. Cells with severe dysplasia showed the maximum expression (83.3%) and the minimum expression (66.6%) was reported in the case of mild dysplasia. A significant \( (P < 0.01) \) positive correlation has been established with the increasing severity of dysplasia.

Consistent results were reported in studies, which dealt with oral epithelial dysplasia.\cite{27,31,32} Also, Chen et al.\cite{21} found similar results when studied iNOS protein expression among specific PMD such as oral submucous fibrosis (OSMF), verrucous hyperplasia (VH), and neoplastic lesion such as verrucous carcinoma (VC). Aforementioned studies also reported absence of expression in the normal buccal mucosa. In another study, serum NO samples were investigated in contrast to the tissue samples in this study.\cite{31} They found significantly higher expression in OSCC and PMD when compared with control subjects. However, the difference between OSCC and OPMD was not significant. This shows that angiogenesis is crucial in the initial stages well before the frank tumor invasion, as depicted by severe dysplasia and in situ lesions.

The probable reason for the observations of this study could be explained by the fact that the activation of iNOS may occur because of interplay between the abnormal epithelial cells and macrophages in the dysplastic epithelium.\cite{33} However, the exact pathway for the mechanism of activation is yet to be unfolded. It has been suggested that high NO levels are required in the initial phase of tumor growth to trigger angiogenesis and step-up permeability.\cite{10} So, it can be assumed that the higher percentage of iNOS expression seen in this study indicates toward its role in the transformation of locally aggressive lesion into invasive tumor.

This study makes an edge over the available literature by reporting the significant \( (P < 0.01) \) positive correlation found between the iNOS expression and advancing OLEP clinical stages of OL. This observation adds to the available evidence that iNOS expression which in turn is linked with the NO-induced angiogenesis becomes more pronounced in the advanced stage where the need for angiogenesis is extreme.

Comparative analysis of inducible nitric oxide synthase expression in the oral squamous cell carcinoma

A significantly \( (P < 0.01) \) increasing pattern of iNOS expression was observed among the advancing clinical stages and histopathological grades of OSCC [Table 4]. In vivo studies have been able to reveal a possible role for NO formed as a result of activation of iNOS favoring the tumor’s local growth and distant migration. It has shown the ability to promote the development of new blood vessel by upregulating the VEGF.\cite{8,22} This phenomenon is seemed to be propelled by the ischemia present in the tumor proper and thus hypoxia is looked on as the initial trigging factor for iNOS.\cite{34} Hence, the iNOS expression is generally seen within the tumor or in the close proximity of the growing malignant tissue. Also, evidence from the various studies has emerged in support of tumor lymphangiogenesis as an important mechanism by which tumor cells gain entry to the lymphatic system and spread to lymph nodes in head and neck cancer.

The results of this study depict a significant \( (P < 0.001) \) positive correlation with the escalating clinical stages and depreciating differentiation of cells [Table 4]. Other authors have reported similar to our results and suggested there use as a prognostic marker.\cite{9} In contrast to this study, few researchers found no correlation among the histopathological grades,\cite{27} whereas others reported correlation only with the lymph node status.\cite{33}

Comparative analysis of inducible nitric oxide synthase expression among the merged clinical stages of oral leukoplakia and oral squamous cell carcinoma

The clinical stages of OLEP and TNM were merged on the basis of associated risk and prognosis, respectively. This evaluation among the merged stages will help in viewing the expression on the scale of involved risk. Stages I and II of OLEP were merged to form “low risk,” whereas Stages III and IV were merged and called “high-risk” category. Similarly, first two stages of TNM were called as “early OSCC,” whereas later two stages were combined to form “advanced OSCC.” Significantly \( (P < 0.01) \) increased expressions were observed in the high-risk and advanced OSCC categories when compared with their respective counterparts [Table 5]. Likewise results were observed when studies were conducted with respect to breast cancer and HNSCC.\cite{8,29}

A steep drop in the prognosis of patients with HNSCC has been recorded when cervical lymph nodes are found to be involved as a consequence of metastasis. The situation gets even worse when the tumor cells encroach the extracapsular areas.\cite{8,9} This can be well correlated with the progressing TNM stages. The further spread of neoplastic cells from the lymph nodes again depends on the neoangiogenesis.\cite{8,9} In addition to the factors required for angiogenesis, they
also need to produce various lytic enzymes that will be required in damaging the cellular and intercellular matrix. This will pave the path for cells to migrate to distant locations in the body.[8] Studies conducted with experimental animals have been successful in showing the reduction in the blood vessel density when samples were treated with iNOS inhibitors in comparison with untreated control.[36] The additional evidence that supports the aforementioned facts comes from the studies showing significantly concomitant raised expression of VEGF mRNA and iNOS in tumors with lymph node metastases. On the contrary, some authors found no correlation with iNOS expression and lymph node metastasis.[9]

Considering the results of this study, the null hypothesis is rejected as significant differential pattern of iNOS staining was seen among the groups. NO has been known to induce carcinogenesis through various interlinked mechanism; hence, future studies are warranted with intention to compare markers of different pathways. This will improve the understanding of NO’s role in carcinogenesis.

**CONCLUSION**

From this study, it can be concluded that in OL group a proportional increase was observed in iNOS expression as the clinical stages and grades are progressing. Similarly, in OSCC 83.3% of the cases have shown significantly high levels of iNOS protein staining as compared to dysplasia and normal epithelium. The iNOS expression was higher in high-risk OL and advanced OSCC cases. These findings depict that iNOS can be considered as a candidate for early diagnostic marker in OL and prognostic marker in OSCC.

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Nil.

**Conflicts of interest**
There are no conflicts of interest.

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**Table 5: Comparative and correlation analysis of inducible nitric oxide synthase expression among the merged clinical stages of oral leukoplakia and oral squamous cell carcinoma**

| S. no. | Clinical stages        | Number of cases (%) | $P$ Value | $P$ Value (correlation coefficient) |
|--------|------------------------|---------------------|-----------|----------------------------------|
| 1.     | Low risk (Stages I + II) | 14 (46.6)           |           | 0.005$^\dagger$ (0.500)         |
| 2.     | High risk (Stages III + IV) | 16 (53.3)         | 0.007$^\dagger$ |                               |
|        | Early OSCC (Stages I + II) | 9 (30)             |           | 0.001$^\dagger$ (0.592)         |
|        | Advanced OSCC (Stages III + IV) | 21 (70)          |           |                                 |

OSCC = oral squamous cell carcinoma

$^\dagger P < 0.01$

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