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IMMUNOHISTOCHEMICAL EVALUATION OF LAMININ-5 AND MYOFIBROBLASTS IN EPITHELIAL DYSPLASIA AND ORAL SQUAMOUS CELL CARCINOMA

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Abstract

Background: Oral squamous cell carcinoma is one of the most prevalent cancers worldwide. The transformation of the normal epithelial cell into a tumor cell bestows upon them certain features at the cellular and molecular level which aids in its survival and proliferation. Invasion of the altered tumor cells through the basement membrane into the connective tissue stroma and their subsequent spread and metastasis is an important prognostic indicator. Laminin-5 is a protein associated with a migratory phenotype in epithelial neoplastic cells. Along with laminin, the stromal myofibroblasts play a significant role in tumor invasion, due to its ability to modify the extracellular matrix.

Aim: To evaluate the role of laminin 5 and stromal myofibroblasts in oral epithelial dysplasia and oral squamous cell carcinoma.

Methods: Paraffin-embedded archival samples of 25 normal, 30 oral epithelial dysplasia and 30 oral squamous cell carcinoma (OSCC) were evaluated for laminin-5 and α- smooth muscle actin (SMA) using standard immunohistochemistry. Semi-quantitative assessment of the expression of laminin and alpha SMA was done in all the study samples. The area of staining and the staining intensity was evaluated in order to determine the staining index which were then statistically analyzed between the three groups.

Results: All the cases of laminin showed cytoplasmic staining in the basal cell layer and basement membrane. Expression of laminin was observed in the basal cell layer of normal and epithelial dysplasia study group and mainly around the tumor islands in OSCC group. α- SMA was seen with increasing intensity with increasing grade of the disease. Comparison of laminin expression between the three groups showed a statistically significant decrease in the staining index from normal to epithelial dysplasia to OSCC ($p < 0.01$). Statistical comparison of α-SMA in between the three groups using Kruskal- Wallis test showed a significant increase in the expression of α-SMA from normal to epithelial dysplasia to OSCC ($p < 0.01$)

Conclusion: Decreased laminin expression in the basement membrane and increased expression of α-SMA favors tumor invasion, establishment of an invasive phenotype of neoplastic cells and a permissive environment for tumor invasion.

Key words: Epithelial dysplasia, Oral cancer, Laminin, Alpha- Smooth muscle actin, Immunohistochemistry, Myofibroblasts

Introduction

Oral cancer is a significant disease affecting humans and the most common form is the oral squamous cell carcinoma (OSCC) which accounts for approximately 3% of all malignancies and more than 90% of cancers of the oral cavity and oropharynx [1].OSCC is a neoplasm of epithelial origin with high prevalence rate in developing countries of the world[2]. The disease is highly reported to occur in India and possess a multifactorial etiology with tobacco and alcohol being the primary risk factor. The use of tobacco in smokeless and smoking form is one of the important determinants of OSCC with the common intra-oral site being the alveolo-gingivo-buccal complex. In general, OSCC can arise *de novo* or from pre-existing oral lesions collectively referred to as oral potentially malignant disorders[3]. Basal membrane components are not only an important structural barrier but also act as barrier against neoplastic invasion in SCC, thus avoiding tumor cell dissemination[4].

Laminin-5 (Ln-5) is the major component of the basement membrane in most adult tissues and is a heterotrimer composed of three different laminin chains (α3, β3, and γ2 chains)[5]. Formerly called as kalinin, nicein, epiligrin or ladsin, laminin-5 is now designated as laminin 332 using a recently introduced simplified nomenclature[6]. The major functions of Ln-5 include binding of epithelial cells to the basement membrane through the formation of

hemidesmosomes and the migration of epithelial cells during wound repair. In addition, Ln-5 has been implicated in tumor progression[7]. Several literature data have suggested the potential role of laminin- 5 in OSCC[8, 9], breast cancer[10] and cervical adenocarcinoma[11] among others.

Epithelial mesenchymal transition (EMT) is the process by which epithelial cells adopt a mesenchymal phenotype or fibroblast-like properties. The transition of the neoplastic epithelial cells to acquire fibroblasts like properties is a significant event in the metastatic potential of the tumor[12]. Myofibroblasts are differentiated fibroblasts that express alpha smooth muscle actin (α- SMA) and have intermediate characteristics among classic fibroblasts and smooth muscle cells. They have the ability to modify the extracellular matrix and thus actively participate in tumor invasion and metastasis[13]. Several studies have determined the role of myofibroblasts in various malignancies including OSCC[14- 16].

Invasion of the altered tumor cells through the basement membrane into the connective tissue stroma and their subsequent spread and metastasis is an important prognostic indicator. Various proteins found in the basement membrane and the components of the stromal tissue undergoes modification and alteration and these alterations may aid in better understanding of the carcinogenesis model. Thus, the need of the present study is to evaluate the role of basement membrane proteins and stromal myofibroblasts in epithelial dysplasia and OSCC have a role in tumor progression to determine the presence of any therapeutic and prognostic implications.

MATERIALS AND METHODS:

An in-vitro tissue based immunohistochemical study was carried out in Department of Oral Pathology and Microbiology of our institution. The study samples consisted of paraffin embedded tissue specimens of the desired lesions obtained from the department archives; the selected specimen was examined for adequacy of the tissue material followed by confirmation of the diagnosis using routine H & E stain. The samples were divided into three groups namely: Group I (n=25) comprising of normal, uninflamed oral mucosal tissue obtained from healthy patients undergoing routine dental treatment (third molar impaction and gingivectomy); Group II included histopathologically confirmed cases of oral epithelial dysplasia of varying grades and group III (n=30) comprised of histopathologically confirmed cases of oral squamous cell carcinoma as per Broder's classification. The inclusion criteria were (i) histopathologically confirmed cases of oral epithelial dysplasia and oral squamous cell carcinoma and (ii) presence of adequate amount of tissue showing epithelium and sufficient connective tissue for IHC staining. Insufficient tissue lacking adequate depth, tissue specimen with inconclusive diagnosis, recurrent cases, cases with other concurrent oral lesions and previous history of therapy (surgery, chemotherapy and radiotherapy) were excluded from the study.

The selected samples were then immunohistochemically analyzed for the expression of laminin-5 and α- SMA using standard techniques. Assessment of immunohistochemical stained sections was carried out using binocular compound microscope attached with a camera (Motic microscope attached to a computer with Motic advanced images 3.2 software). The IHC stained slides were evaluated by observing the staining pattern in the basal cells and basement membrane of epithelial dysplasia and for OSCC under high power (x400 magnification). For SMA, the positive expression of stromal myofibroblasts were evaluated for both the groups. The SMA staining was observed mainly in sub-epithelial zone of group II and around the basement membrane and tumor islands in Group III cases.

Semi-quantitative assessment of the expression of laminin and alpha SMA was done in all the study samples. The area of staining and the staining intensity was evaluated in order to determine the staining index. The area of staining was graded based on the percentage of positive expression in each slide as follows: +1= 0-25%; + 2= 25-50%; + 3= 51-75%; + 4= 76-100%. The staining intensity was recorded as: 0 = no staining; 1 = mild staining; 2 = moderate staining; 3 = intense staining. Following this the staining index was calculated as the sum of area of staining and staining intensity[17].

All data was entered in Microsoft Excel (MS office version 2010) and tabulated. Data analysis was done using Windows PC based software "MedCalc Statistical Software" version 18.2.1 (MedCalc Software bvba, Ostend, Belgium; http://www.medcalc.org; 2014). All testing was done at alpha 0.05 (95% confidence levels). The three groups (normal, epithelial dysplasia and OSCC) were analyzed for differences by using the Kruskal-Wallis test. Post-hoc Scheffe's test was used for pair-wise comparisons between the three groups. Correlation between alpha-SMA and laminin was done using non-parametric Spearman's correlation.

RESULTS:

The present study was carried out to evaluate the expression of laminin 5 and stromal myofibroblasts in normal, epithelial dysplasia and OSCC study groups. Semi-quantitative assessment of laminin and α- SMA was done in all 85 cases. All the cases of laminin showed cytoplasmic staining for the basal cell layer and basement membrane. Expression of laminin was observed in the basal cell layer of normal and epithelial dysplasia study group and mainly around the tumor islands in OSCC group. α- SMA was seen

with increasing intensity along with the increasing grade of the disease. Staining index for both was calculated by the area of staining and intensity of staining as described in methodology earlier. The staining for laminin was intense in the basal cell layers and basement membrane of normal mucosal tissue (Fig. 1). Staining was less in epithelial dysplasia (Fig.2) whereas laminin staining was discontinuous in OSCC around the peri-tumoral islands. (Fig 3)

The average staining index for laminin in normal group was 4.4 ± 0.67; in epithelial dysplasia it was 2.6 ± 0.71 and in OSCC group it was 2.5 ± 0.62 (Table 1). The results of the comparison using Kruskal Wallis test showed a statistically significant decrease in the staining index from normal to epithelial dysplasia to OSCC ($p < 0.01$) (Table 1). Intergroup comparison was performed using Post-hoc Scheffe test and the results indicated that the staining index was significantly reduced in epithelial dysplasia and OSCC when compared with normal controls ($p < 0.01$) while the decreased expression in OSCC than epithelial dysplasia was not statistically significant (Table 2).

Evaluation of myofibroblasts through the immunohistochemical expression of α-SMA revealed a mild staining in normal (Fig 4), moderate staining in subepithelial zone of oral epithelial dysplasia (Fig 5) and intense staining in OSCC especially around the tumor islands (Fig 6). The mean and standard deviation of expression of α-SMA in normal group was 2.7 ± 0.73, in epithelial dysplasia it was 5.3 ± 0.96 and in OSCC group it was 6.2 ± 0.72 (Table 3). Statistical comparison of α-SMA in between the three groups using Kruskal- Wallis test showed a significant increase in the expression of α-SMA from normal to epithelial dysplasia to OSCC ($p < 0.01$) (Table 3). Post-hoc Scheffe test to evaluate the inter-group difference showed a statistically significant increase in α-SMA expression in epithelial dysplasia and OSCC when compared to normal. Also, the increased expression in OSCC than epithelial dysplasia was statistically significant ($p < 0.01$) (Table 4).

To evaluate the relation of laminin and α-SMA expression in the three groups, Spearman's co-efficient correlation analysis was employed. Correlation analysis of all the tissue samples together showed an inverse relationship of laminin and α-SMA ($r = -0.730$) which was statistically significant ($p < 0.001$) (Table 5). Additionally, regression analysis was done to evaluate the strength of association between α-SMA and laminin in the three groups. The analysis revealed statistical significance in normal and epithelial dysplasia ($p < 0.01$) while in OSCC the association was not significant ($p = 0.06$).

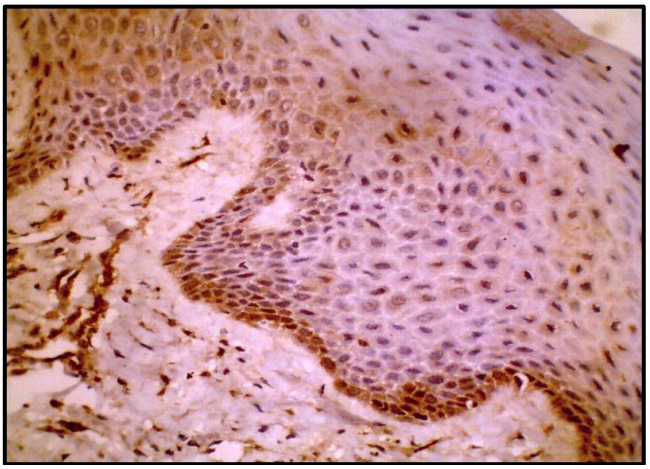

Figure 1: Photomicrograph (40X) showing laminin expression in basal cells and basement membrane of normal oral mucosa.

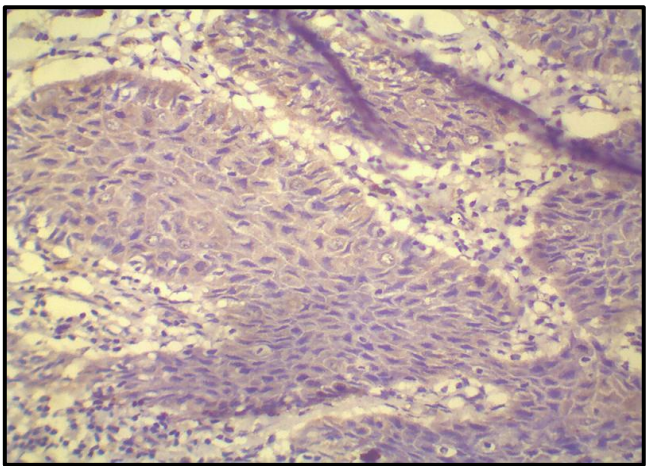

Figure 2: Photomicrograph (40X) showing laminin expression in oral epithelial dysplasia.

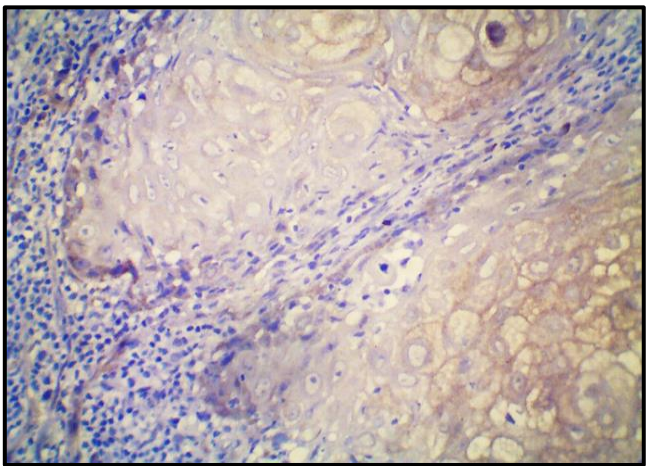

Figure 3: Photomicrograph (10X) showing laminin discontinuity around the tumor islands of OSCC.

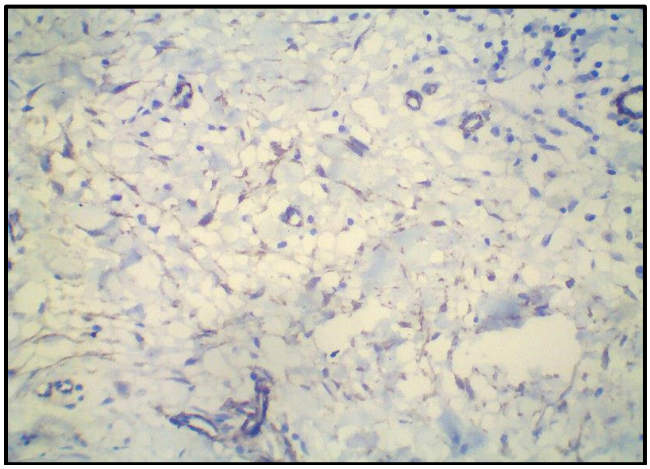

Figure 4: Photomicrograph (40X) showing α-SMA expression in connective tissue of normal oral mucosa.

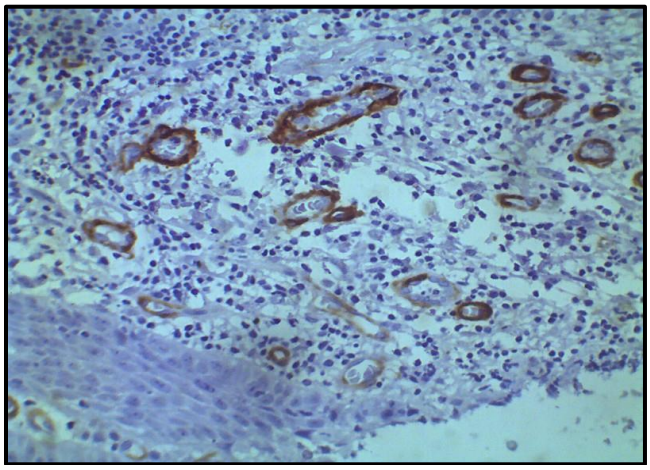

Figure 5: Photomicrograph (40X) showing α-SMA staining for stromal myofibroblasts in sub-epithelial region of oral epithelial dysplasia

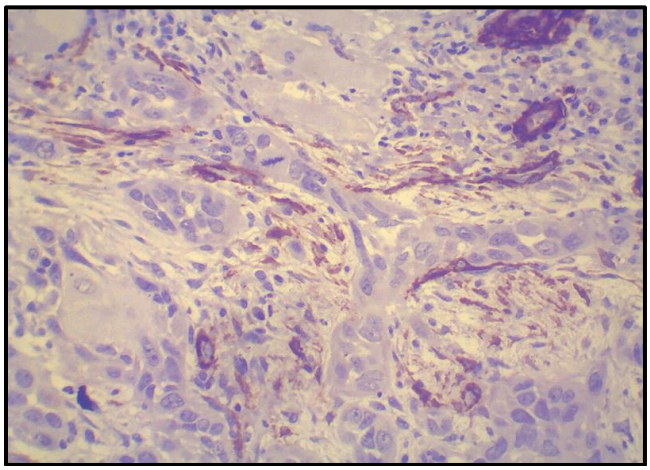

Figure 6: Photomicrograph (40X) showing α-SMA positivity for stromal myofibroblasts around the tumor islands in IHC stained section of OSCC.

Table 1: Mean, standard deviation and Kruskal Wallis test to determine significance of laminin expression in normal, epithelial dysplasia and OSCC study groups

	N	Mean	Standard deviation	Significance level
Group 1	25	4.4	0.67	
Group 2	30	2.6	0.71	**P <0.001**
Group 3	30	2.5	0.62	

Table 2: Post-hoc Scheffe's test to determine the intergroup difference of laminin expression

Group	n	Average rank	Different (p<0.05) from other groups
(1) 1	25	73.18	(2) (3)
(2) 2	30	32.93	(1)
(3) 3	30	30.38	(1)

Table 3: Mean, standard deviation and Kruskal Wallis test to determine significance of α-SMA expression in normal, epithelial dysplasia and OSCC study groups and Krusal

	N	Mean	Standard deviation	Significance level
Group 1	25	2.7	0.73	
Group 2	30	5.3	0.96	**p <0.001**
Group 3	30	6.2	0.72	

Table 4: Post-hoc Scheffe's test to determine the intergroup difference of α-SMA expression

Group	n	Average rank	Different (p<0.05) from other groups
(1) 1	25	16.72	(2) (3)
(2) 2	30	51.55	(1) (3)
(3) 3	30	68.23	(1) (2)

Table 5: Spearman's correlation analysis of laminin and α-SMA between normal, epithelial dysplasia and OSCC study groups

Sample size	85
Spearman's coefficient if rank correlation (rho)	-0.730
Significance level	**p<0.0001**
95% Confidence Interval for rho	-0.814 to -0.616

Discussion:

In the present study, the staining index of laminin expression was significantly decreased in oral epithelial dysplasia and OSCC when compared to normal. However, no statistically significant difference was observed on comparing between oral epithelial dysplasia and OSCC. In normal oral mucosal tissue, the staining of the laminin protein was continuous and intense confirming the basement membrane continuity. In epithelial dysplasia (group II), intracytoplasmic staining was observed in basal cells as well as in the basement membrane which was less intense as compared to normal control group and in OSCC (group III) laminin expression was discontinuous at the basement membrane zone and decreased intensity was observed at the tumor host interface. The study results were in accordance with several other results obtained from the literature[18, 19].

Progressive decrease in laminin expression in the oral epithelial dysplasia and OSCC could be attributed to the E-cadherin expression loss. The altered distribution of laminin and collagen IV in premalignant and malignant lesions of oral epithelium are associated with the progression of OSCC[20]. The decrease in laminin expression in epithelial dysplasia could probably be due to the cytological alterations observed in the overlying dysplastic epithelium. In OSCC, decreased and disrupted laminin expression in the basement membrane zone and around the tumor islands suggests the loss in the basement membrane continuity, promotion of cell migration and invasion. This may occur after the gamma 2 chain has been cleaved by MMPs secreted by cancer cells or neighboring stromal cells. This discontinuity may also be because of unequal enzymatic degradation of the basement membrane by the tumor cells.

In present study the α-SMA expression for myofibroblasts were found to be increased in epithelial dysplasia (group II) when compared to normal (group I) which was statistically significant. When compared between epithelial dysplasia (group II) and OSCC (group III) α-SMA expression for myofibroblasts were found to be increased in OSCC and was statistically significant. Increased expression of α-SMA for myofibroblasts was noted in OSCC (group III) when compared between all the groups. This was in accordance with various studies[21, 22]. A possible reason for this increase in myofibroblasts in epithelial dysplasia is that the genetically altered epithelium may have an inductive effect of the adjacent stroma to produce myofibroblasts. In OSCC, an increase in myofibroblasts population causes proteolytic degradation of the stromal components thereby aiding in the spread of tumor cells. Additionally, myofibroblasts may contribute to cytokine production and tumor angiogenesis.

The stromal reaction to the altered overlying epithelium varies from increased inflammatory response, altered collagen fiber remodeling and vasculature and to some extent the presence of myofibroblasts. The possible reason for an increased myofibroblasts is that that the genetically altered epithelium may have an inductive effect on the adjacent stroma to produce myofibroblasts[14]. Additionally, the changes in the composition and organization of the stromal micro-environment associated with the cytokine release may aid in the formation of myofibroblasts[23]. The exact role of these cells however is unknown.

Etemad-Moghadam et al (2009) showed that the presence of myofibroblasts was significantly higher in oral squamous cell carcinomas compared to both dysplasia and normal mucosa. These findings show the presence of myofibroblasts in the stroma of oral squamous cell carcinoma but not dysplasia and normal mucosa, suggesting further investigation to clarify the role of myofibroblasts in the carcinogenesis process[14]. Chaudhary et al (2012) found an increase expression as the disease progresses from oral premalignancy to verrucous carcinoma and to invasive OSCC thereby suggesting that the proliferation of myofibroblasts may be used as a stromal marker of premalignancy and malignancy[22]. Analysis of α-SMA expression for myofibroblasts proliferation can be used as a stromal marker for predicting behavior in oral pre-cancer and cancer.

The transdifferentiation of oral fibroblasts to myofibroblasts could occur secondary to the release of transforming growth factor-β1, that are released by OSCC cells. Analysis of myofibroblasts expression in varying grades of OSCC may indicate that the loss of cellular differentiation affects the number of myofibroblasts in the tumor stroma[15, 21]. Stromal destruction in OSCC occurs either through cancer cell-prompted destruction or due to cooperation between cancer cell and surrounding stroma. Myofibroblast appearance in invasive cancer and tumor desmoplasia are important reflection of the tumor–host interaction, especially in aggressive cancers. The transdifferentiation of myofibroblasts is generally induced during the invasive stage of SCC and further loss of tumor differentiation would not affect the number of these cells[23].

Statistical correlation of laminin-5 and alpha-SMA in oral epithelial dysplasia and OSCC showed an inverse relationship between the two wherein the decreased expression of laminin was associated with an increase in myofibroblasts population. Franz et al (2010) demonstrated that laminin α2 chain significantly decreased while α3, α4, α5 and γ2 chains and also α-smooth muscle actin (ASMA) significantly increase with rising grade of oral squamous cell carcinoma[24]. It was hypothesized that mediated by myofibroblasts, OSCC development is associated with a stromal upregulation of laminin isoforms possibly contributing to a migration promoting microenvironment. It is possible that mesenchymal cells contribute to the promotion of tumor cell migration as well as vessel formation in OSCC by providing and organizing pro-migratory laminin-5 fragment[25]. Epithelial-mesenchymal interaction plays a key role in oral carcinogenesis. An inverse relationship between laminin 5 and α-SMA expression probably suggest that the epithelial and mesenchymal components have a synergistic effect for tumor invasion.

Conclusion:

It can be concluded that the decrease laminin expression in the basement membrane of oral squamous cell carcinomas and the structural change may affect basement membrane dynamism and favor tumor invasion. Thus, the expression of laminin in basement membrane may be a useful

parameter to evaluate tumor histologic differentiation and aggressiveness. Also, laminin can be adopted as a useful marker in evaluating the histological differentiation and aggressiveness of oral carcinoma. The increase in the expression of SMA as the disease progresses from oral premalignancy to OSCC suggests that the proliferation of myofibroblasts may be used as a prognostic marker of premalignancy and malignancy. Additionally, myofibroblasts could be useful as a potential target for chemotherapeutic regimen in oral squamous cell carcinoma.

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