Original Article

Alteration in the Expression of Basement Membrane Components in Oral Epithelial Dysplasia and Oral Squamous Cell Carcinoma

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Abstract

Background: The basement membrane is a dynamic structure that undergoes quantitative and qualitative changes during the progression from oral epithelial dysplasia to squamous cell carcinoma (SCC), which is essentially important during invasion. **Materials and Methods:** Basement membrane expression was determined using periodic acid–Schiff (PAS), a histochemical stain, and type IV collagen immunohistochemical marker, in 70 diagnosed cases of oral epithelial dysplasia and oral SCC. **Results:** Continuous basement membrane expression was found in various grades of oral epithelial dysplasia using PAS staining technique. In type IV collagen staining, continuous basement membrane expression was found in mild and moderate dysplasia, but in severe dysplasia, discontinuous basement membrane expression was found in few cases. In oral SCC, discontinuity and absence of basement membrane expression increased with the increasing grades of oral SCC and no case in oral SCC showed continuous basement membrane expression. Statistically significant difference was seen between control and oral SCC ($P < 0.05$) and between oral epithelial dysplasia and oral SCC ($P < 0.001$) but statistically non-significant differences were found between control and oral epithelial dysplasia ($P > 0.05$). **Conclusion:** Alteration in the expression of type IV collagen in the basement membrane in oral epithelial dysplasia and oral SCC indicates that the loss of continuity of the subepithelial basement membrane parallels the progression of the neoplastic transformation process in oral epithelium.

Keywords: Basement membrane, Periodic acid–Schiff, Type IV collagen.

Introduction

Basement membrane is composed mainly of two networks of macromolecules; the glycoprotein components, that is, laminin and fibronectin; and the collagenous components which include type IV collagen and to some extent collagen type VII. Basement membrane acts as a structural barrier for the dysplastic epithelial cells to prevent the progression of epithelial dysplasia to carcinomas.[1] In epithelial malignancies, breaks and defects in the basement membrane have been thought to be associated with the loss of basement membrane components.[2] Various histochemical staining techniques have been developed to visualize the basement membrane.[3] Several lines of evidence indicate that the reactivity of Schiff reagent with the high proportion of carbohydrate macromolecules (glycoprotein and proteoglycans) within the epithelial basement membrane makes the periodic acid–Schiff (PAS) technique a valuable mean of assessing basement membrane.[4] Ozzello and Speer[5] for the 1st time assessed the basement membrane in carcinoma of breast using PAS stain.

Recently, immunohistochemical methods with antibodies directed against specific components of basement membrane provided a specific tool for the diagnostic investigations of the basement membrane in normal and pathological conditions.[6] Type IV collagen is a non-fibrillar collagen that makes up

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about 50% of all basement membrane components.[7] It was first recognized to be a basement membrane component by Kefalides in 1971.[8] It forms a structural backbone of the basement membrane and considered to be the protective component against the invasion and metastasis.[9] Anti-collagen IV antibody has been commercially recognized as a specific antibody against basement membrane component type IV collagen.

The aim of this study was to assess and compare the alteration in the expression of basement membrane components using both PAS and type IV collagen antibody staining in oral epithelial dysplasia and oral squamous cell carcinoma (SCC).

Materials and Methods

Seventy formalin-fixed, paraffin-embedded blocks formed the study sample. These were divided into three main groups (Groups I–III). Group I or control group (normal gingival tissue) comprised 10 cases; Group II (oral epithelial dysplasia) included 30 cases, out of which 10 cases of mild dysplasia formed Group IIa, 10 cases of moderate dysplasia formed Group IIb, and 10 cases of severe dysplasia formed Group IIc; Group III (oral SCC) included 30 cases, out of which 15 cases of well-differentiated SCC formed Group IIIa and 15 cases of moderately differentiated SCC formed Group IIIb. Three sections were cut from all paraffin-embedded tissue blocks for hematoxylin and eosin (H&E) staining, PAS staining, and type IV collagen immunohistochemical staining. Using H&E stained sections, all the histological slides were reviewed.

Immunohistochemical staining for type IV collagen was performed on poly-L-lysine coated slides using mouse type IV collagen antibody (Biogenix, India) overnight at 4°C followed by secondary antibody for 30 min. 2.5 mg/ml pepsin digested enzyme (Biogenex chemical, Milmont Drive, Fremont, USA, EK000-5KE) was used for optimal antigen retrieval for 7 min at 37°C. Imaging analysis system (Nikon Research Microscope, ECLIPSE 80i) was used to assess the staining patterns. Negative controls with an omission of the antiserum from the primary incubation were also included. Stained blood vessels in the connective tissue were taken as positive control.

Evaluation of pas staining and type IV collagen immunohistochemical staining

Freshly stained sections were used for immediate observation. All slides were assessed under light microscope in whole of the tissue section. Intense magenta color staining of the basement membrane indicated as positive PAS staining. Intense brown color staining in the basement membrane indicated as positive type IV collagen staining. The evaluation of positive reactions was performed using the criteria modified from Zuk *et al*.,[3] Fenyvesi,[10] and Arduino *et al*.[11]

Basement membrane staining was evaluated semi-quantitatively and graded as absent staining (−: Score 0); minimal staining, when present in 1–20% of basement membrane (+/−: Score 1); large interruption, when 21–50% of basement membrane staining was evident (++: Score 2); focal interruption, when greater than 50% of basement membrane staining was evident (+++: Score 3); completeness of staining, discrete staining (++++: Score 4); and completeness of staining, intense staining (+++++: Score 5). The slides were evaluated by two observers, both and the interobserver variability came out to be nil in the present study.

Data were analyzed and subjected to statistical analysis. Means and standard deviations for PAS and type IV collagen score were calculated for all the groups. Data were further examined for statistical significance (*P* value) using Mann–Whitney test and Spearman's rho correlation.

Results

The mean value score of basement membrane using PAS stain and type IV collagen stain is tabulated in Table 1.

Using Mann–Whitney U-test in PAS stain, significant differences were seen between control and oral SCC ($P < 0.003$) and between oral epithelial dysplasia and oral SCC ($P < 0.001$), but statistically non-significant differences were found between control and oral epithelial dysplasia ($P > 0.877$). Between various subgroups, statistically significant differences were seen between control and well-differentiated SCC ($P < 0.008$), control and moderate differentiated SCC ($P < 0.010$), mild dysplasia and well-differentiated SCC ($P < 0.023$), mild dysplasia and moderately differentiated SCC ($P < 0.025$), moderate dysplasia and well-differentiated SCC ($P < 0.001$), moderate dysplasia and moderately differentiated SCC ($P < 0.001$), severe dysplasia and well-differentiated SCC ($P < 0.008$), and between severe dysplasia and moderately differentiated SCC ($P < 0.008$). However, statistically non-significant difference was seen between control and mild dysplasia ($P > 0.790$), control and moderate dysplasia ($P > 0.465$), control and severe dysplasia ($P > 0.926$), mild dysplasia and moderate dysplasia ($P > 0.283$), mild dysplasia and severe dysplasia ($P > 0.740$) and moderate dysplasia and severe dysplasia ($P > 0.631$), and between well-differentiated SCC and moderate differentiated SCC ($P > 0.967$).

Using Mann–Whitney U-test in type IV collagen stain, highly significant differences were seen between control and oral SCC ($P < 0.001$) and oral epithelial dysplasia and oral SCC ($P < 0.001$), but statistically non-significant differences were found between control and oral epithelial dysplasia ($P > 0.572$). Between various subgroups, significant differences were seen between control and severe dysplasia ($P < 0.030$), mild dysplasia and severe dysplasia ($P < 0.037$), moderate dysplasia and severe dysplasia ($P < 0.007$), control and well-differentiated SCC ($P < 0.002$), control and moderately differentiated SCC ($P < 0.001$), mild dysplasia and well-differentiated SCC ($P < 0.005$), mild dysplasia and moderately differentiated SCC ($P < 0.002$), moderate dysplasia and well-differentiated SCC ($P < 0.001$), and between moderate dysplasia and moderately differentiated SCC ($P < 0.001$), but statistically non-significant difference was seen between

Table 1: Mean values of PAS and type IV collagen staining score in all the groups

Study groups	Subgroups	PAS			Type IV collagen		
		Min.	Max.	Mean±SD	Min.	Max.	Mean±SD
Group I (control)		0	4	3.20±1.687	1	5	3.70±1.494
Group II (oral epithelial dysplasia)	Mild dysplasia IIa	0	5	3.20±1.549	0	5	3.70±1.829
	Moderate dysplasia IIb	3	4	3.90±0.316	2	5	4.00±1.155
	Severe dysplasia IIc	0	5	3.40±1.430	0	5	1.80±1.619
	Total dysplasia II	0	5	3.50±1.225	0	5	3.17±1.802
Group III (ORAL SCC)	Well-differentiated SCC IIIa	0	3	2.27±0.961	0	3	1.73±1.387
	Moderately differentiated SCC IIIb	1	3	2.33±0.724	0	3	1.07±1.163
	Total SCC III	0	3	2.30±0.837	0	3	1.40±1.303

PAS: Periodic acid–Schiff, SCC: Squamous cell carcinoma

control and mild dysplasia ($P > 0.719$), control and moderate dysplasia ($P > 0.626$), mild dysplasia and moderate dysplasia ($P > 1.000$), well-differentiated SCC and moderately differentiated SCC ($P > 0.214$), severe dysplasia and well-differentiated SCC ($P > 0.887$), and between severe dysplasia and moderately differentiated SCC ($P > 0.227$).

On comparing the overall PAS and type IV collagen staining score, statistically significant correlation was found in control ($P < 0.011$) and in oral SCC ($P < 0.022$), but no significant correlation was found in oral epithelial dysplasia ($P > 0.082$).

Discussion

Tumor invasion involves the process of derangement in the proper sorting of cell populations and causing a violation of normal tissue boundaries.[12] In tumor biology, the basement membrane plays an important role in controlling migration, invasion, and metastasis of tumor cells.[13] In the histological assessment of oral epithelial dysplasia and oral SCC, it is difficult to evaluate the early stage of basement membrane destruction stained with H&E. Hence, to overcome this problem, histochemical PAS stain and recently immunohistochemical stain type IV collagen have been used for the basement membrane identification.[14]

In the present study, the expression of basement membrane in PAS stain as well as in type IV collagen immunohistochemical stain of control group was intense and continuous (Figure 1a and b). No case in control showed absence of staining, while the PAS staining technique showed absence of staining in two cases, which is not in accordance with histopathology of normal tissue (Table 2). Hence, type IV collagen staining is more accurate than PAS staining technique.

Oral epithelial dysplasia stained with PAS

Out of total 30 cases of oral epithelial dysplasia stained with PAS, 23 cases showed continuous basement membrane expression, which includes 7 (70%) of the 10 cases each of mild dysplasia and severe dysplasia and 9 (90%) moderate dysplasia. Discontinuous staining expression was found in total of 5 (16.6%) cases which include 2 (20%) cases of mild and severe dysplasia and only 1 (10%) case of moderate dysplasia. The absence of staining was evident in total of 2 (0.6%) cases

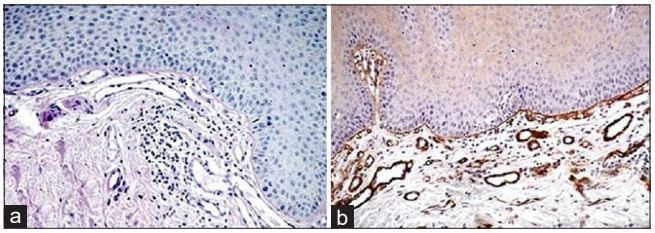

Figure 1: Photomicrograph showing basement membrane (a) Periodic acid–Schiff and (b) type IV collagen staining in normal gingival tissue

which include 1 (10%) case of mild dysplasia and 1 (1%) case of severe dysplasia (Table 2). An insignificant increase in the mean PAS stain score was observed in oral epithelial dysplasia (Figure 2a and b), when compared to the control and also as the severity of oral epithelial dysplasia increases. Similar findings was reported by Fuentes *et al*.[15] This increase in mean score may be due to staining of carbohydrate macromolecules, that is, proteoglycan and the carbohydrate side chain of type IV collagen other than glycoprotein, when oxidized in periodic acid for prolonged period.[16]

Oral epithelial dysplasia cases stained with type IV collagen stain

Out of total 30 cases, 17 cases showed continuous basement membrane expression, which included 7 (70%) cases of mild dysplasia, 8 (80%) cases of moderate dysplasia, and 2 (20%) of severe dysplasia. Discontinuous staining expression was found in total of 10 (33.3%) cases, which included 2 (20%) cases of mild dysplasia, 2 (20%) cases of moderate dysplasia, and 6 (60%) cases of severe dysplasia. The absence of staining was evident in total of 3 (10%) cases which include 1 (10%) case of mild dysplasia and 2 (20%) cases of severe dysplasia. An insignificant decrease in the mean type IV collagen staining shows the insignificant decrease in the mean score in oral epithelial dysplasia, when compared to the control. This finding was in accordance with the study conducted by Visser *et al*.[14] on laryngeal dysplasia. When compared with control group, an insignificant increase in mean score was observed in mild and moderate dysplasia whereas significant decrease in severe dysplasia was observed, similar results were reported by Mori *et al*.[17] in esophageal lesions. This suggests that excessive deposition of type IV collagen serves as a selective template

Table 2: Pattern of PAS and type IV collagen staining in all the groups

Study groups	Subgroups	PAS			Type IV collagen		
		Continuous	Discontinuous	Absent	Continuous	Discontinuous	Absent
Group I (control)		8	0	2	8	2	0
Group II (oral epithelial dysplasia)	Mild dysplasia IIa	7	2	1	7	2	1
	Moderate dysplasia IIb	9	1	0	8	2	0
	Severe dysplasia IIc	7	2	1	2	6	2
	Total dysplasia II	23	5	2	17	10	3
Group III (oral SCC)	Well-differentiated SCC IIIa	0	14	1	0	10	5
	Moderately differentiated SCC IIIb	0	15	0	0	9	6
	Total SCC III	0	29	1	0	19	11

PAS: Periodic acid–Schiff, SCC: Squamous cell carcinoma

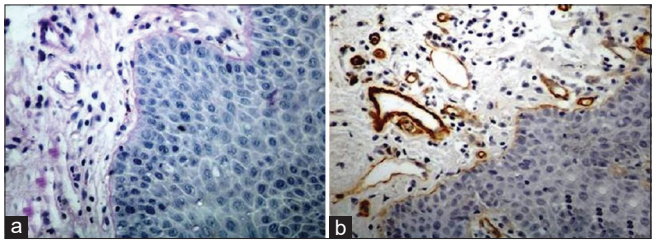

Figure 2: Photomicrograph showing basement membrane (a) Periodic acid–Schiff staining and (b) type IV collagen staining in severe dysplasia

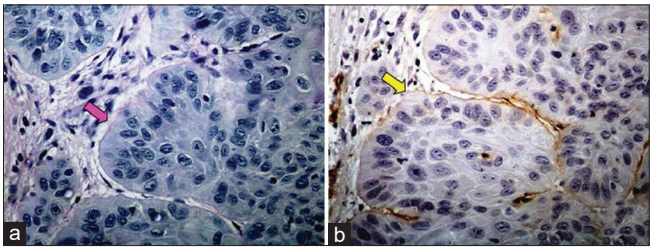

Figure 3: Photomicrograph showing basement membrane with arrow (a) Periodic acid–Schiff staining and (b) type IV collagen staining in well-differentiated squamous cell carcinoma

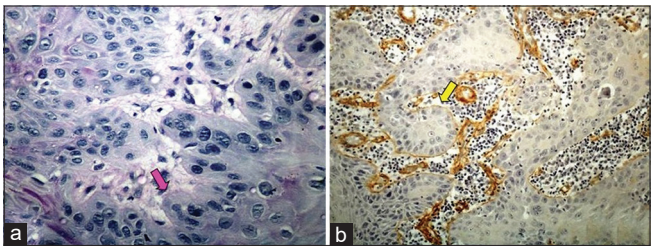

Figure 4: Photomicrograph showing basement membrane with arrow (a) Periodic acid–Schiff staining and (b) type IV collagen staining in moderately differentiated squamous cell carcinoma

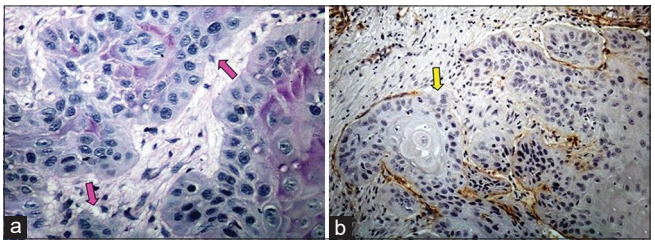

Figure 5: Photomicrograph showing discontinuous basement membrane with arrow in oral squamous cell carcinoma (a) Periodic acid–Schiff staining and (b) type IV collagen staining

for adhesion of altered cells to epithelial connective tissue interface for cancer progression.[18]

Oral SCC stained with PAS

A significant decrease in mean PAS stain score was observed in the transition from control to oral SCC and also in transition from oral epithelial dysplasia to oral SCC, which is in accordance with other studies reported by Marcos *et al.*,[13] Tosios *et al.*,[19] and Arcadi.[16] Out of total 30 cases of oral SCC, 29 (96.6%) cases showed discontinuous staining expression, which include 14 (93.3%) of the total 15 cases of well-differentiated SCC and 15 (100%) of total 15 cases of moderately differentiated SCC. The absence of staining was evident in 1 (0.03%) case, that is, in well-differentiated SCC (Table 2, Figures 3a and 4a), suggesting that it may be due to hydrolysis of glycoprotein component by the plasmin induced by tumor cell plasminogen activator.[20] Furthermore, when the comparison was done between various grades of oral epithelial dysplasia and oral SCC, the decrease in the mean value of PAS score came out to statistically significant in all comparisons, suggesting the alteration in the basement membrane components due to increase in enzymatic activity of tumor cells due to increased proliferation.[16]

Oral SCC stained with type IV collagen stain

A significant decrease in mean type IV collagen staining score was observed in the transition from control to oral SCC and also in transition from oral epithelial dysplasia to oral SCC (Figure 5). This decrease in mean score in oral SCC may be due DNA hypermethylation in the promoter region of type IV collagen gene which leads to diminished expression of type IV collagen in cancer tissue.[21] Similar findings were observed by Lee *et al.*[22] in pancreatic adenocarcinoma cases.

Out of total 30 cases, 19 (63.3%) cases showed discontinuous staining expression, which included 10 (66.6%) of the total 15 cases of well-differentiated SCC and 9 (60%) of total 15 cases of moderately differentiated SCC (Figure 3b and 4b).

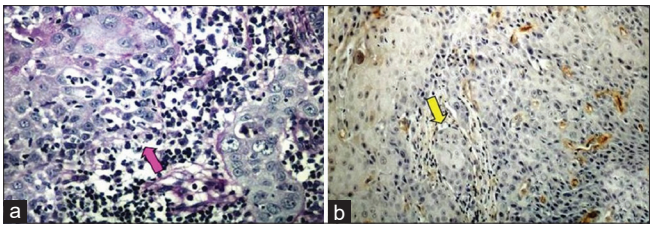

Figure 6: Photomicrograph showing absent basement membrane with arrow in oral squamous cell carcinoma (a) Periodic acid–Schiff staining and (b) type IV collagen staining

The absence of staining was evident in total of 11 (36.6%) cases, which include 5 (33.3%) cases of well-differentiated SCC and 6 (40%) cases of moderately differentiated SCC. No cases in oral SCC showed continuous pattern in both the techniques (Table 2 and Figure 6).

Conclusion

Thus, from this study, we concluded as the lesions change their behavior from normal to various grades of oral epithelial dysplasia to various grades of oral SCC, expression of basement membrane components undergoes alterations, demonstrated by loss of continuity of basement membrane. On progression from control to oral epithelial dysplasia, an inverse correlation was derived between PAS and type IV collagen staining score and a direct correlation was derived between PAS staining score and type IV collagen staining score on progression from oral epithelial dysplasia to oral SCC. This study suggested that discontinuity of basement membrane is best demonstrated by type IV collagen marker as compared to routine histochemical stain.

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