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Original Article

Immunohistochemical analysis of syndecan-1 in leukoplakia and oral submucous fibrosis

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ABSTRACT

Background: Syndecan-1 is abundant in normal tissues and reduced in squamous cell carcinomas. Leukoplakia and oral submucous fibrosis (OSF) are oral pre-cancerous disorders that have potential for malignant transformation. The aim of this study was to evaluate the expression of syndecan-1 in leukoplakia and OSF and to identify its role as a reliable marker for predicting malignant changes.

Materials and Methods: Expression of syndecan-1 was examined immunohistochemically in 42 cases of oral leukoplakia with or without epithelial dysplasia, 28 cases of OSF and 10 cases of normal oral epithelia as control. Mann-Whiteny '*U*' test was used for statistical analysis and the level of significance was fixed at $P < 0.05$.

Results: Intense syndecan-1 expression was observed in nine cases with normal epithelium. Immunopositivity was lost gradually as the extent of epithelial dysplasia increased. The significant reduction in syndecan-1 expression was observed in leukoplakia as epithelial dysplasia progressed from moderate or severe. Similarly, significant reduction was observed in staining intensities in OSF with dysplasia.

Conclusion: The results reveal that down-regulation of syndecan-1 expression is associated with dysplastic changes in leukoplakia and OSF. Thus syndecan-1 can be considered as marker for predicting malignant changes.

Key Words: Immunohistochemistry, leukoplakia, oral submucous fibrosis, syndecan-1

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INTRODUCTION

Leukoplakia, candidiasis, lichen planus, and oral submucous fibrosis (OSF) are the potential malignant disorders (PMDs) of the oral cavity. Among these, leukoplakia is a pre-malignant lesion with a high-risk of malignant transformation[1] and OSF, a pre-malignant condition with a moderate to high-risk of malignant development.[2,3] The term dysplasia is very commonly associated with pre-malignant conditions and can be expressed as abnormal growth or development of cells leading to abnormal size, shape or abnormal organization of adult cells, finally, leading to malignant conditions. The dysplasia is histologically classified as mild, moderate, and severe based upon the changes in the epithelium.[4]

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Etiology of the oral leukoplakia in most of the cases is idiopathic but in some cases initiation occurs due to tobacco use, cadidiasis, viruses, vitamin deficiencies, etc.[5] It can be described as a greyish white patch developed in the oral cavity, which cannot be scrapped off. OSF is a chronic fibroelastic change resulting in the formation of collagen fibers in the lamina propria. Although, the pathogenesis of the disease is considered as multifactorial with areca nut chewing, ingestion of chillies, genetic and immunological processes, nutritional deficiencies;

areca nut is found to be the main etiological factor for developing OSF.[6] Long-term population based studies confirmed that the rate of transformation of pre-malignant nature of OSF to malignancy is high as 7.6% and that of leukoplakia is 3.6-17.5%.[7-10] Survival rates of patients with oral malignancies are among the worst of all cancer sites. Prevention and early detection of PMDs have the potential of not only decreasing the incidence but also in improving the survival of those who develop oral cancer.[11] Therefore, recent studies in this field have focused on the development of biomarkers for early detection, disease monitoring, and determining the prognosis of patients with oral malignancies.[12] A more aggressive tumor behavior and worse prognosis may be signified by changes in a range of biomarkers, which include reduced E-cadherin expression, laminin-γ2 chain expression, and decreased tumor cell transmembrane proteoglycan syndecan-1 expression.[13]

Syndecans are a family of heparan sulfate proteoglycan receptors that participate both in cell-to-cell and cell-to-matrix adhesion.[14] The syndecan family is composed of four closely related proteins (syndecans-1 to 4). Syndecan-1, also known as CD138, is a prototype member of the family, which has been studied most extensively. Syndecan-1, is suggested to function as matrix receptor transducting information between the extracellular matrix and inside of the cell.[15,16] In stratified squamous epithelia, it is proposed to function as a cell–cell adhesion molecule.[17-19] Syndecan-1 is abundant in normal epithelial cells and tissues, localizing to both basal and suprabasal cell layers. Expression of syndecan-1 is induced during keratinocyte differentiation, and reduced in squamous cell carcinomas (SCCs). Its strong expression was observed on the keratinocytes of normal oral epithelium and epithelial hyperplasia.[20]

Earlier studies noted that syndecan-1 levels correlate with malignancy in various tissues. It has been shown that mRNA and protein of syndecan-1, decreased in a wide variety of cancers. An evident correlation has been found between stage and grade of cancer and the extent of syndecan loss in SCC (skin, head and neck, esophageal, or lung), lip carcinogenesis, oral cancer in dysplasia and SCC of oral cavity, as well as adenocarcinomas (gastric and colorectal).[4,12,21-28] In epithelial dysplasia, immunopositivity is lost gradually along with the increase in the degree of hyperplasia.[4] The down-regulation of syndecan-1 in dysplasias suggests malignant transformation of the epithelium. However, syndecan-1 is up-regulated in breast and liver cancers.[29] Therefore, the relationship between cancer and syndecan-1 expression is still unclear. It may depend on the location of cancer and influence of other cells or proteins, or some other factors. The altered expression of syndecan-1 in SCC and pre-malignant lesions may have prognostic value in determination of the clinical outcome of the lesions.[26]

Immunohistochemical (IHC) staining is widely used in the diagnosis of abnormal cells such as those found in cancerous tumors. The aim of the IHC technique is to evaluate variation in the expression of antigenic constituent associated with normal, pre-malignant and malignant conditions. The expression of syndecan-1 in normal or malignant cells is monitored using IHC staining technique. The intensity of staining is found to be directly proportional to the density of expression of syndecan-1. This is a reliable and sensitive technique, which uses monoclonal antibodies to detect and visualize antigens in cells and tissues.

In India, there is predominance of oral leukoplakia and OSF, which have significant risk of malignant transformation.[11] In literature, very less information is available on comparative account of expression of syndecaqn-1 in case of leukoplakia and OSF. Therefore, this study was undertaken to evaluate the expression of syndecan-1 in oral leukoplakia and OSF with or without dysplasia as compared to normal oral mucosa.

MATERIALS AND METHODS

This study was conducted in the Department of Oral Pathology and Microbiology. Histologically diagnosed 42 cases of oral leukoplakia and 28 cases of OSF were recruited for the study. The leukoplakia cases were categorized as: Hyperplasia without dysplasia (n = 10), mild dysplasia (n = 11), moderate dysplasia (n = 10), and severe dysplasia (n = 11). The OSF cases were categorized as: Without dysplasia (n = 15) and with dysplasia (n = 13). Ten cases of normal oral mucosa served as a positive control for expression of syndecan-1. Histologically pre-malignancy was classified as mild, moderate, and severe dysplasia.[10] The study design was approved by the Institutional Ethics Committee and informed consent was obtained from the study population.

Tissue specimens and staining techniques

Two to three serial sections of tissue (5 μm thickness) were embedded in paraffin blocks were mounted on

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silanized slides. The slides were de-paraffinized by heating on the slide warmer at 60°C for 15-20 min. The sections were subsequently rehydrated by giving them two changes of xylene for 5 min each. The sections were transferred to absolute and 95% alcohol respectively for 3 min each before being immersed in distilled water for 30 s. For the antigen heat retrieval, the sections were placed in a metal rack and were immersed into a 3l capacity pressure cooker containing about 2l of citrate buffer solution. The pressure cooker was then sealed and brought to full pressure, which maintained for 2 min. The slides were left in hot buffer solution for cooling.

IHC staining

The staining was carried out using Dako EnVision™ + system, HPR (DAKO Corporation, USA) technique. All the reagents were equilibrated to room temperature (24-28°C) prior to immunostaining. Likewise, all incubations were performed at room temperature; with a humidifying chamber being used to incubate sections for prolonged time with reagents. The tissue sections were not permitted to dry during the staining procedure. After tapping off the excess buffer from the slide, the sections were covered with peroxidase block for 5 min, gently washed with distilled water and then placed in a Tris-buffered saline (HiMedia Labs) bath for 5 min. Excess buffer was tapped off and optimally diluted primary antibody (anti-CD138, DAKO, Denmark) was used to cover the sections. The sections were incubated overnight in a humidifying chamber kept in a refrigerator. Next day, the sections were rinsed with distilled water and placed in fresh buffer bath for 5 min. Excess buffer was tapped off and the tissue sections were incubated with streptavidin peroxidase for 15 min. The slides were washed gently with distilled water and then placed in buffer bath for 5 min. Excess buffer was tapped off and the tissue sections were covered with freshly prepared 3,3-diaminobenzidine + chromogen solution. Slides were then rinsed with water for 5 min. The slides were immersed in Harri's hematoxylin bath for 15 min, washed gently under running water, and dedifferentiated by dipping in 1% acid alcohol. The slides were again washed in running tap water and allowed to dry. Slides were dehydrated and dipped in xylene, and mounted in Dibutyl Phthalate in Xylene, a non-aqueous permanent mounting medium.

Interpretation of staining

Presence of brown colored end product at the site of target antigen was indicative of positive reactivity. The negative tissue control demonstrated absence of specific staining. Normal oral mucosa was taken as a positive control with each batch of staining as syndecan-1 is strongly positive in stratified squamous epithelium. Intensity of staining was recorded as negative, weak, moderate, and intense and they were assigned numerical values of 0, 1, 2, and 3, respectively, for the purpose of statistical analysis.

Statistical analysis

The statistical analysis was carried out using Mann-Whiteny '*U*' test between normal and different histological groups of leukoplakia as well as OSF; and also between the individual groups of leukoplakia and in between individual groups of OSF. The level of significance was fixed at 5%.

RESULTS

Observations were made on the basis of intensity of staining in the three different layers of epithelium (stratum basale, stratum spinosum, and superficial layer). The expression of syndecan-1 in basal layer was same in the normal epithelium and the other groups; while no expression of syndecan-1 was seen in the superficial layer in any of the groups. It was only in the spinous cell layer where different intensities were seen. Figure 1 shows intense staining in the spinous layer of the normal epithelium as compared to the weak staining observed in case of moderate dysplatic oral epithelium of leukoplakia [Figure 2]. As the severity of dysplasia in leukoplakia increased the intensity of syndecan-1 expression was weak [Figure 3]. Similarly, moderate staining intensity was observed in non-dysplatic oral epithelium of OSF [Figure 4] and weak staining intensity in dysplatic oral epithelium of OSF [Figure 5].

Table 1 shows IHC staining intensities and comparison between different groups. Intense staining was seen in majority cases of normal epithelium, hyperplasic epithelium without dysplasia, and mild dysplasia. When syndecan-1 expression was compared between normal and other groups, there was statistically significant ($P < 0.01$) difference in moderate and severe dysplasia subgroups. Furthermore, mild dysplasia subgroup showed significant difference when compared to moderate and severe dysplasia ($P < 0.01$). However, no association between leukoplakia with moderate and severe dysplasia subgroups was found.

Table 2 shows staining intensities for OSF. In OSF without dysplasia, 12 showed intense staining out

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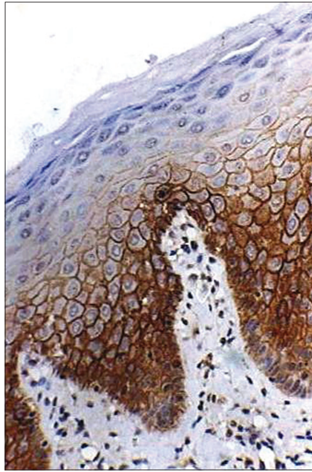

Figure 1: Intense staining in spinous cell layer of normal oral epithelium (×250)

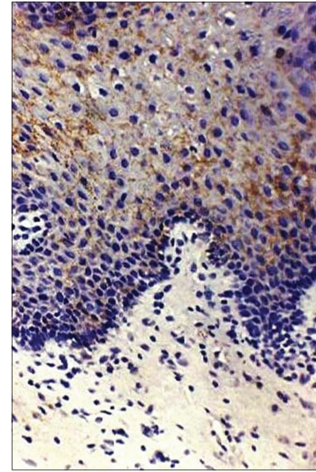

Figure 2: Weak staining intensity in moderately dysplastic oral epithelium of leukoplakia (×250)

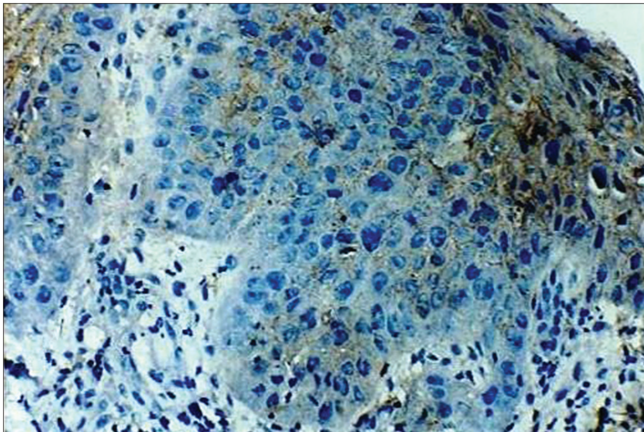

Figure 3: Weak staining intensity in severely dysplastic oral epithelium of leukoplakia (×250)

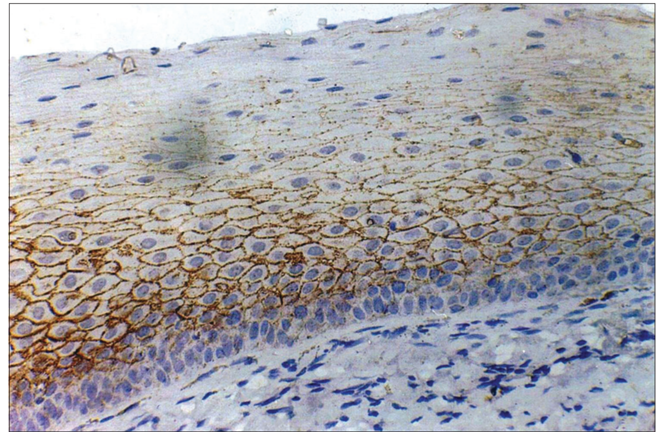

Figure 4: Moderate staining intensity in non-dysplastic oral epithelium of oral submucous fibrosis (×250)

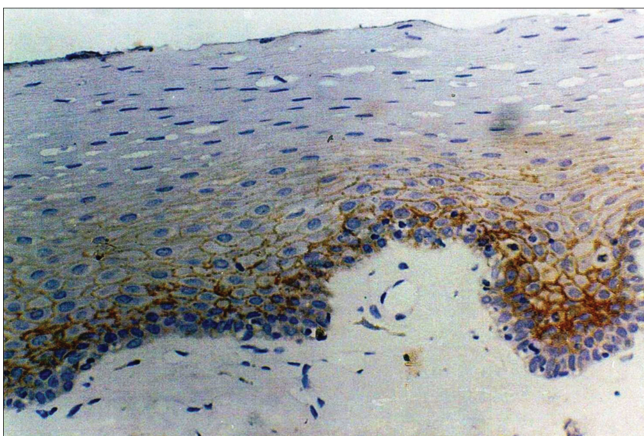

Figure 5: Staining intensity in dysplastic oral epithelium of oral submucous fibrosis (×250)

of 15 cases and three showed moderate staining. However, in OSF with dysplasia group out of 13 cases 10 were with moderate staining, two with weak staining and only one with intense staining.

The results of comparison for syndecan-1 expression between normal epithelium and OSF with and without dysplasia suggested a statistical correlation of OSF with dysplasia group ($P < 0.01$). Furthermore, comparison of OSF with and without dysplasia subgroups reported a highly significant difference.

DISCUSSION

Leukoplakia is an oral pre-cancerous lesion that may develop into SCC. The cellular changes in the epithelium known as dysplasia are often observed in oral pre-malignancies. The epithelial dysplasia points toward the possible subsequent development of malignancy. OSF is a chronic disorder characterized by fibrosis of the lining mucosa of the upper digestive tract involving the oral cavity. It may cause atrophy in the epithelium, increasing carcinogen penetration. As mentioned earlier, syndecan-1 is highly expressed in

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Table 1: Staining intensities and comparison of syndecan-1 expression between normal and different subgroups of leukoplakia as well as individual subgroups of leukoplakia

Type	Intensity of staining				Intergroup comparison		
	(0)	(1)	(2)	(3)	Normal with subgroups of leukoplakia	Mild dysplasia with other groups	Moderate dysplasia with severe dysplasia
Normal oral epithelium	-	-	1	9	-		
Hyperplasic epithelium without dysplasia	-	-	3	7	0.4727		
Mild dysplasia	-	-	3	8	0.5262		
Moderate dysplasia	-	4	6	-	0.0004*	0.0014*	
Severe dysplasia	-	7	4	-	0.0002*	0.0004*	0.378

(0) No staining, (1) Weak staining, (2) Moderate staining, (3) Intense staining. *Statistically significant

Table 2: Staining intensities and comparison of syndecan-1 expression between normal epithelium and oral submucous fibrosis with or without dysplasia as well as individual subgroups of OSF

Type	Intensity of staining				Inter group comparison	
	(0)	(1)	(2)	(3)	Normal with other OSF groups	Dysplasia group with OSF without dysplasia
Normal oral epithelium	-	-	1	9	-	
OSF with dysplasia	-	2	10	1	0.0008*	
OSF without dysplasia	-	-	3	12	0.698	0.0008*

(0) No staining, (1) Weak staining, (2) Moderate staining, (3) Intense staining. *Statistically significant. OSF: Oral submucous fibrosis

fibroblastic and epithelial cells. It is especially high in keratinocytes whereas low in endothelial and neural cells.[26] Being highly expressive in epithelial cells syndecan-1 has been reported to be a prognostic factor for tumor progression and survival in various types of malignant tumors. This suggests the suitability of syndecan-1 as cancer biomarker. A number of studies have demonstrated prognostic significance of syndecan-1 in oral cancers.[11,20,21,24,30-33]

This study was undertaken to examine the intensity of expression of syndecan-1, in normal stratified squamous epithelium as compared to oral leukoplakia and OSF. The observations were made on the basis of intensity of staining. The results showed no significant difference in staining intensity of syndecan-1 in normal epithelium and epithelial hyperplasia without dysplasia as well as leukoplakia with mild dysplasia suggesting occurrence of very little cellular disturbance in these two conditions as compared to normal epithelium.

In epithelium of leukoplakia with moderate dysplasia syndecan-1 expression was moderate in about 60% cases, whereas remaining 40% showed weak staining and there was no case observed with intense staining. Similar results about weak staining were noted in another study on syndecan-1 expression in dysplastic oral epithelium.[20] Results of moderate and severe dysplastic epithelium in leukoplakia suggested a remarkable relation in the expressibility of syndecan-1. These were suggestive of progressively decreased adhesion with increased grade of dysplasia.

The results of syndecan-1 expression in the OSF with dysplasia group were different as moderate expression was observed in 10 cases and weak expression in 2 cases. The pattern of syndecan-1 expression in OSF without dysplasia was comparable to the normal epithelium. Syndecan-1 immunopositivity was lost gradually as the extent of epithelial dysplasia increased. This was more evident in cases of leukoplakia with severe dyspalsia as well as OSF with dysplasia. This suggests that syndecan-1 expression may be one of the most important prognostic factors in oral cancers. However, its reliability has been questioned in some studies.[23]

The development of malignant epithelial tumors is associated with reduced intercellular adhesion, disturbed differentiation and cellular changes in the epithelial layer suggesting that the expression and function of cell adhesion molecules could also change during malignant transformation. Thus, low expression levels of syndecan-1 with increased dysplasia can be attributed to its role in cell–cell and cell–matrix interactions.[20] The malignant changes in cell morphology change cell–cell adhesion properties. Loss of syndecan-1 from malignantly transformed cells could be one mechanism by which tumor cells loosen their attachment to each other and to the extracellular matrix and become non-responsive to the signals coming from their microenvironment.[26] Due to changed morphology and reduced cell adhesion

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the expression of syndecan-1 may be altered. With alterations in expression of syndecan-1 in leukoplakia and OSF disorders of the oral cavity the changes in the cell morphology can be predicted. The changes in cell morphology are related to dysplastic condition at the cellular level. This suggests that it is possible to determine the degree of dysplasia by evaluating intensity of syndecan-1 expression. Therefore, altered expression of syndecan-1 in pre-malignant disorders of stratified epithelia suggests that syndecan-1 has prognostic value in the determination of the clinical outcome of these lesions.

CONCLUSION

This study demonstrated that reduction in expression of syndecan-1 is a useful biomarker for assessing dysplastic changes, which may lead to malignancy.

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