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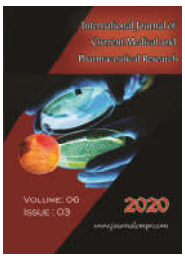

Research Article

ASSESSMENT OF MOLECULAR CHANGES IN LUEKOPLAKIA WITH EPITHELIAL DYSPLASIA USING SYNDECAN-1 ANTIGEN

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ABSTRACT

Aim: This study aimed to evaluate the syndecan-1 expression in Leukoplakia (LPK) according to grades of epithelial dysplasia. **Materials and Methods:** Twenty samples of leukoplakia were selected from the archives of the institution and further divided into three groups: Group 1 (LPK with mild epithelial dysplasia), Group 2 (LPK with moderate epithelial dysplasia) and Group 3 (LPK with severe epithelial dysplasia). The slides stained with syndecan-1 antigen were examined under light microscope. **Results:** A statistically non significant ($p>0.05$) difference was noted when staining intensity of syndecan-1 in stratum basale were compared between LPK with mild dysplasia (L1) and LPK with moderate dysplasia (L2) ($p=0.889$), L1 and leukoplakia with severe dysplasia (L3) ($p=0.212$) also between L2 and L3 ($p=0.134$). A statistically non significant difference was noted when staining intensity of syndecan-1 in stratum spinosum were compared between L1 and L2 ($p=0.854$), L1 and L3 ($p=0.299$), also between L2 and L3 the difference was not statistically significant ($p=0.321$). A statistically non significant difference was noted when intensity of syndecan-1 expression in superficial layers of the epithelium were compared between L1 and L2 ($p=0.285$), L1 and L3 ($p=0.398$), also when L2 and L3 were compared, the difference was not statistically significant ($p=1.00$). **Conclusion:** The expression of syndecan-1 antigen in leukoplakia according to grades of epithelial dysplasia is less reliable to rule out the malignant potential.

INTRODUCTION

Oral leukoplakia (LPK) was first time defined by a World Health Organisation (WHO) in the year 1978 as a white patch or plaque which can not otherwise be characterized clinically or pathologically as any other disease[1].Histopathologically leukoplakia is characterized by hyperkeratosis and may or may not be associated with epithelial dysplasia. When it is associated with epithelial dysplasia, it is having more potential for progression into Oral Squamous Cell Carcinoma (OSCC). According to WHO 1978 classification oral epithelial dysplaia is classified as mild, moderate and severe.[2] It is observed that OSCC develops in a stepwise manner initiated with non dysplastic lesion which progresses to increasing grades of epithelial dysplasia and transform into OSCC.[3] In India, OSCC represents about 30% malignancies, and five year survival rate after adequate treatment is only 50%.[4,5] It is the oral LPK which is the most common potentially malignant disorder which progresses to oral cancer, having malignant transformation rate of about 0.13 % to 10 % in India.[1,4] The malignant transformation rate of oral LPK further increases when associated with epithelial dysplasia and it is about 36%.[6] Although Histopathological examination is routinely practiced to rule out the grade of epithelial dysplasia but changes occur first at molecular level which cannot be appreciated under the microscope.[7] These changes occurs in growth properties, morphology and antigens etc.[8] To detect these specific antigens by antigen antibody interaction, Immunohistochemistry (IHC) is used on formalin fixed and paraffin embedded tissues. syndecan-1 is expressed predominantly in epithelium.[9] It is important to regulate the cell to cell and cell to extracellular matrix interactions, down regulation of which is associated with loss of polarity of the cells.[10]

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Survival rates of OSCC can be increased if it is detected at an early stage by differentiating Potentially Malignant Disorders (PMD) into high risk and low risk categories.[11] By considering these facts, The present study was formulated to analyse the syndecan-1 expression in Leukoplakia and its correlation with grades of epithelial dysplasia.

MATERIALS AND METHODS

Twenty samples of optimally formalin fixed and paraffin embedded tissues of Leukoplakia were selected from the archives of the institution. Diagnosis of Hyperkeratosis was confirmed by light microscopic examination of Hematoxylin and Eosin (H & E) stained slides. After confirmation of diagnosis all these samples were grouped into three categories depending upon the grade of epithelial dysplasia. LPK with mild epithelial dysplasia (n=7), LPK with moderate epithelial dysplasia (n=8) and LPK with severe epithelial dysplasia (n=5).

Reagents used

Ready to use mouse monoclonal antibody syndecan–1 antigen :- 1 bottle (7 ml) Product code: RTU-CD138-MI15 was used. Polymer detection kit – The Novolink ^{TM} min. polymer detection kit was used. In this kit all the solutions were ready to use except for the 3,3-diaminobenzidine (DAB), whose working solution was prepared every time using DAB chromogen and substrate buffer.

Preparation of Solutions

Antigen retrieval solution: To prepare 0.001M of Ehylene diamine tetra acetic acid (EDTA) buffer solution, 0.37gm of EDTA salt is added to 1000ml of distilled water and pH of 8.0 is achieved by adding sodium hydroxide solution drop by drop.

Phosphate buffer saline (PBS) (pH 7.4): 0.45 gm of sodium dihydrogen phosphate dihydrate, 1.69 gm of disodium hydrogen phosphate dihydrate and 8gm of sodium chloride is added to 1000ml of distilled water.

DAB: 5µl of DAB chromogen with 100 µl of DAB substrate buffer (polymer) in a test tube. All these solutions were prepared few minutes before its use every time.

Immunohistochemical procedure

1. Three micron sections each of 20 samples of leukoplakia were prepared on semi automatic rotary microtome (LEICA RM2245) and mounted on pre-coated slides.
2. After drying at room temperature, sections were incubated at 60^0C for 1 hour
3. The sections were deparaffinised as routine
4. The sections were cleared in xylene and rehydrated through descending grades of alcohol
5. Antigen retrieval procedure was carried out using conventional pressure cooker, for this, equal number of slides were kept in two coplin jars filled with antigen retrieval solution Ehylene diamine tetra acetic acid (EDTA) which were placed in pressure cooker 1/4th filled with pre-heated tap water. The pressure cooker was then sealed and heated at 100^0C for one hour. Constant temperature was maintained by using temperature adjustable hot plate.
6. For every cycle of antigen retrieval, one section of normal oral mucosa (associated with impacted third molar during surgical removal) was used as a positive control and treated similarly as the test sample.
7. After one hour, the cooker was depressurized and cooled under running tap water. The lid was removed and coplin jars taken out from the pressure cooker, the sections were allowed to cool at room temperature in the coplin jar
8. The cooled sections were washed with Phosphate buffer saline (PBS) for 5 min. the excess was wiped using absorbent wipes. The slides transferred to humidifying chamber for immunohistochemical staining.
9. The sections were treated with peroxidase block for 5 min. followed by PBS wash twice for 5 min. each, the excess was wiped with absorbent wipes.
10. The sections were treated with protein block for 5 min. followed by PBS wash twice for 5 min. each.
11. The sections were incubated for 60 min. at room temperature with primary antibody syndecan-1 followed by PBS wash twice for 5 min. A negative control was performed by omitting the step of primary antibody during the staining.
12. The Sections were subjected to post primary for 30 min. and PBS wash twice for 5 min. each.
13. The sections were subjected to Polymer Horse radish peroxidase reagent for 30 min. and PBS wash twice for 5 min. each.
14. The sections were subjected to DAB for 5 min. and washed with distilled water for 5 min.
15. The slides were counterstained with Hematoxylin, washed with water, dehydrated through alcohol and cleared in xylene and mounted with Dibutylpthalate polyesterene xylene (DPX)

During the whole immunohistochemical staining procedure, all the tissue sections were not allowed to dry.

Evaluation of tissue sections

The tissue sections were observed under light microscope. syndecan-1 is the cell membrane marker on the oral epithelial cells. Presence of brown coloured end product at the site of target antigen was considered as positive. The evaluation of staining was done according to the scoring criteria as described by Kamat S S *et al.*[12] Intensity of staining was recorded as, Negative (0; no color), Weak (1; light brown color), Moderate (2; dark brown colour) and Intense (3; very dark brown colour). Observations were made on the basis of intensity of staining in the three different layers of epithelium like, Stratum basale, Stratum spinosum, and Superficial layer. Intensity of staining was recorded as negative, weak, moderate, and intense by directly comparing with positive control and they were assigned numerical values of 0, 1, 2, and 3, respectively, for the purpose of statistical analysis.

Statistical Analysis

Statistical analysis of the observed values for syndecan-1 expression in leukoplakia were done by Mann Whitney U test. Inter-observer reliability was also tested by Cohen's Kappa test. All the observers were found to be reliable.

RESULTS

The slides were evaluated by the three observers who were blinded for the study and observations were statistically analysed. (Table-1), Fig.1 is showing the syndecan-1 expression in normal oral mucosa which was used as a positive

control, while Fig.2, Fig.3 and Fig.4 showing the syndecan-1 expression in leukoplakia according to the different grades of epithelial dysplasia. All the slides of normal oral mucosa were strongly positive for syndecan-1 expression. A statistically non significant ($p>0.05$) difference was noted when staining intensity of syndecan-1 in stratum basale were compared between LPK with mild dysplasia (L1) and LPK with moderate dysplasia (L2) ($p=0.889$), L1 and leukoplakia with severe dysplasia (L3) ($p=0.212$) also between L2 and L3 ($p=0.134$). A statistically non significant difference was noted when staining intensity of syndecan-1 in stratum spinosum were compared between L1 and L2 ($p=0.854$), L1 and L3 ($p=0.299$), also between L2 and L3 the difference was not statistically significant ($p=0.321$). A statistically non significant difference was noted when intensity of syndecan-1 expression in superficial layers of the epithelium were compared between L1 and L2 ($p=0.285$), L1 and L3 ($p=0.398$), also when L2 and L3 were compared, the difference was not statistically significant ($p=1.00$).

Table 1 Immunohistochemical expression of syndecan-1 in leukoplakia and its correlation with grades of epithelial dysplasia

Diagnosis	Staining intensity of syndecan -1											
	Stratum basale				Stratum spinosum				Superficial layer			
	N	W	M	I	N	W	M	I	N	W	M	I
L1 (n=7)	5	1	1	0	1	3	3	0	6	0	1	0
L2 (n=8)	5	3	0	0	2	2	3	1	8	0	0	0
L3 (n=5)	5	0	0	0	2	2	1	0	5	0	0	0
Correlation	Mann Whitney U test		p value		Mann Whitney U test		p value		Mann Whitney U test		p value	
L1 Vs L2	27.000		0.889		26.500		0.854		24.000		0.285	
L1 Vs L3	12.500		0.212		11.500		0.299		15.000		0.398	
L2 Vs L3	12.500		0.134		13.500		0.321		20.000		1.000	

(Abbreviations:- L1 = Leukoplakia With Mild Epithelial Dysplasia, L2 = Leukoplakia With Moderate Epithelial Dysplasia, L3 = Leukoplakia With Severe Epithelial Dysplasia; N = Negative, W = Weak, M = Moderate, I = Intense, Vs = Versus)

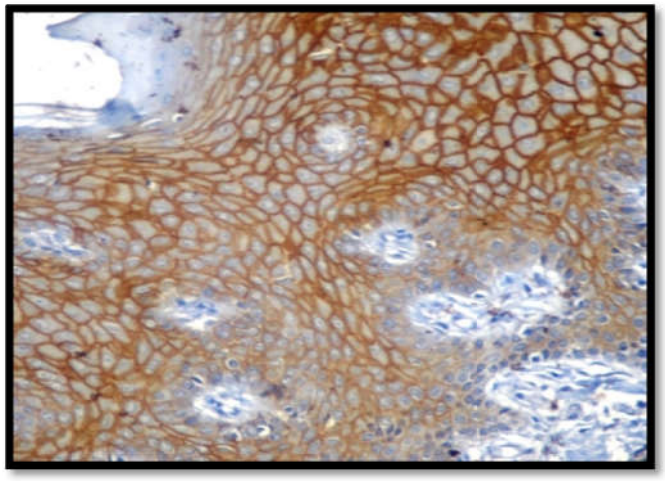

Fig 1 Normal oral mucosa, Positive control for syndecan-1(40X)

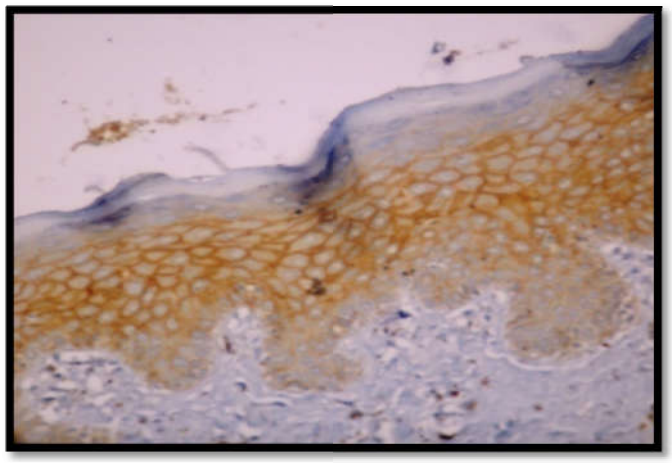

Fig 2 syndecan-1 expression in leukoplakia with mild dysplasia (40X)

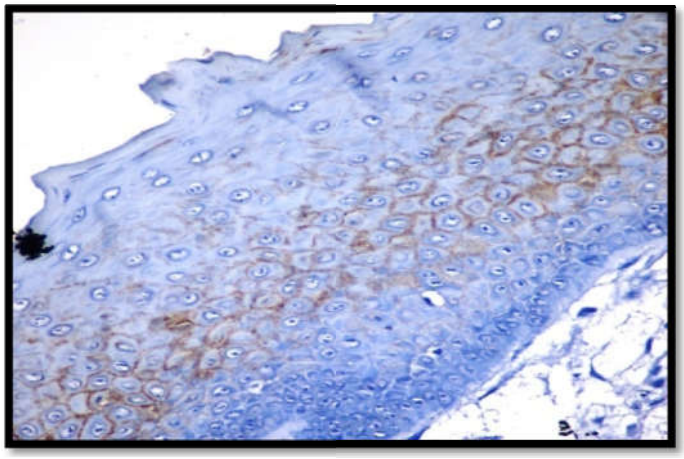

Fig 3 syndecan-1 expression in leukoplakia with moderate dysplasia (40X)

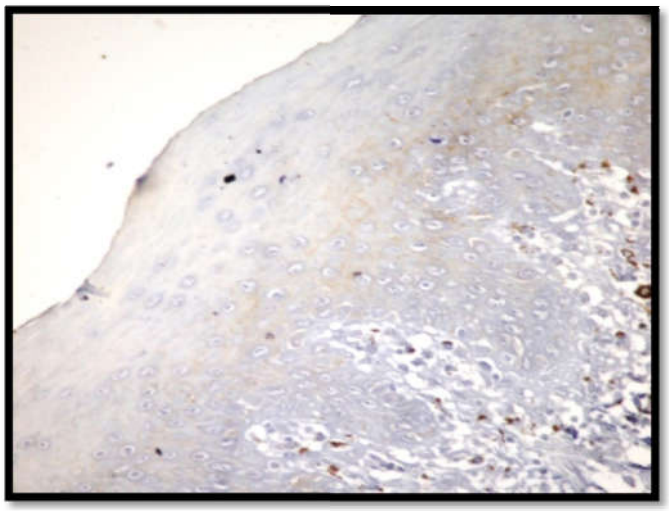

Fig 4 syndecan-1 expression in leukoplakia with severe dysplasia (40X)

DISCUSSION

Oral mucosa is made up of stratified squamous epithelium; the stratification is the result of cell proliferation and sequential differentiation.[13] Dysplastic features are characterized by cellular atypia and loss of normal stratification and maturation.[2]

Syndecans are cell surface proteoglycans with a long evolutionary history. No multicellular animal appears to be without at least one type, and in mammals, there are four types of syndecans.[14,15,16]Syndecans are type I membrane glycoproteins, having three major domains, ectodomain,

transmembrane and cytoplasmic.[16] The ability of syndecan-1 overexpression to inhibit cell growth and migration could be an explanation of the aggressive invasive behaviour of tomour cells lacking syndecan-1.[17,18] Knowing to these facts, use of syndecan-1 for predicting malignancy potential is recently established.

Results of the present study for the expression of syndecan -1 in leukoplakia group, were in accordance with Jackson L L et al.[19] who also found statistically non significant difference in the expression of syndecan-1 according to grades of epithelial dysplasia, according to the present study no statistically significant difference were noted for the expression of syndecan -1 in leukoplakia according to the grades of dysplasia still the expression was found to be reduced as the grade of dysplasia increased. But results of the present study were in contrast to the observations of Soukka T et al.[20] and Lakkam B et al.[21] which could be explained by the fact that, Soukka T et al. had not evaluated the expression of syndecan-1, according to the grades of dysplasia and comparison were done between normal, dysplastic and squamous cell carcinoma groups while Lakkam B et al. had observed the reduced expression of syndecan-1 in leukoplakia according to the grades of epithelial dysplasia, but there sample size was more while in case of this study the sample size was less to come to the definite conclusion.

Down regulation of the syndecan-1 from oral epithelial cells may progresses these cells to detach and invade.[22] It is also down regulated in OSCC.[20] From the results of the present study, it is clear that the syndecan-1 expression is down regulated as dysplastic changes increases from mild dysplasia to moderate and severe dysplasia, but in this study the difference was not statistically significant, hence further research with larger sample size is required to determine the significance of syndecan-1 in potentially malignant disorders and its correlation with increase in the grade of epithelial dysplasia.

SUMMARY AND CONCLUSION

syndecan-1 expression does not appear to be a key marker as a prognostic indicator in leukoplakia associated with epithelial dysplasia. The search for the suitable and more reliable marker is ongoing to grade the potentially malignant disorders into high risk and low risk categories. The expression of syndecan-1 antigen in leukoplakia according to grades of epithelial dysplasia is less reliable to rule out the malignant potential.

Ethical issues

Not applicable

Conflicts of interest

There is no conflicts of interest of any author regarding the preparation of the manuscript and publication of paper.

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