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RESEARCH ARTICLE

EXPRESSION OF METALLOTHIONEIN IN ORAL LEUKOPLAKIA

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ABSTRACT

Background and Objectives: Leukoplakia is the most common potentially malignant lesion of the oral cavity. As the histological study of oral leukoplakia cannot predict precisely the malignant transformation of this lesion and metallothionein (MT) is a protein that has been associated with carcinogenesis, this study could be auxiliary in this histological assessment of this lesion. The present study was done to evaluate the immunoexpression of metallothionein in oral leukoplakia (OL) and to correlate with histological grade and clinical localization.

Methods: 30 diagnosed cases of oral leukoplakia along with 10 cases of normal oral mucosa were taken for the study. Oral leukoplakia was graded as: hyperkeratosis without dysplasia change (8 cases), mild dysplasia (13 cases), moderate dysplasia (6 cases) and severe dysplasia (4 cases). Immunohistochemistry for the metallothionein was performed and the Pearson Chi-Square test was used in statistical analysis.

Results: In normal oral mucosa MT expression is restricted only to basal and parabasal cells with a mosaic cytoplasmic-nuclear expression pattern, whereas in dysplastic lesions an additional focus in the spinous layer was noted.

Interpretation and Conclusion: Metallothionein expression was highest in severe dysplasia with the lowest expression found in cases of Hyperkeratosis without dysplasia. The expression in mild dysplasia and moderate dysplasia was more or less equal. Thus, this study suggests that Metallothionein overexpression can be one of the useful diagnostic marker for predicting the potential behavior of oral Leukoplakia turning into oral squamous cell carcinoma. (OSCC)

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INTRODUCTION

The health of an individual has its origin in healthy cells. The living cell is a dynamic mass of activity which reflects dysfunction when affected by disease. For any disorder to be confirmed, examination should be done at the cellular level which further modifies its structure and function in response to changing demands and stresses. The individual cellular changes are referred to as 'atypia' and the general disturbance in the epithelium is designated as 'dysplasia'. (Nidhi Sharma *et al*., 2010) The term leukoplakia is used to describe certain white patches in the mouth. (Axéll *et al*., 1996) The lesion is defined by World Health Organization (1997) as "a predominantly white lesion of oral mucosa that cannot be characterized as any other definable lesion". (Axéll *et al*., 1996; Mishra *et al*., 2005) The disease is widely studied due to the risk of malignant transformation. The annual malignant transformation rate of oral leukoplakia is proximal to 0.8-1%. (Johann *et al*., 2003) Worldwide, oral cancer is one of the most prevalent disease and tenth most common cause of death. As the hallmark of cancer is excessive proliferation of cells, this increase is helpful in understanding and grading their biological behavior. Oral cancer lesions are usually preceded by potentially malignant lesions and conditions. However, the clinical aspects and histological study of these cannot predict precisely the rate of malignant transformation. (Pontes *et al*., 2009; Warnakulasuriya, 2000; Chattopadhyay *et al*., 2002) Many markers pertaining to the malignancy, have been developed in the recent years and Metallothionein (MT) is one amongst them. Metallothioneins are ubiquitous proteins or polypeptides that have high affinity for heavy metal ions including Cd, Cu and Zn. It is known to participate in metal homeostasis and detoxification, protection against reactive forms of oxygen, intracellular repair process, growth and differentiaton. (Kägi and Schäffer, 1988) Since not many studies have been done to evaluate the expression of metallothionein in dysplasia, this study was undertaken to evaluate the histological assessment of dysplasia and to use MT as an adjunct in the histological grading.

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Aims and Objectives

1. To evaluate the metallothionein immunoexpression in oral leukoplakia.
2. To compare the metallothionein expression among the different histological grades of leukoplakia.
3. To correlate the metallothionein expression with clinical localization.

MATERIALS AND METHODS

The material for the study included 41 formalin-fixed, paraffin-embedded tissue blocks retrieved from the Department of Oral and Maxillofacial Pathology, Sri Sai College of Dental Surgery, Vikarabad. All these cases were diagnosed by routine hematoxylin and eosin staining. These were subjected to immunohistochemical staining for metallothionein. The criteria of the WHO (2005) for the histological grading of Leukoplakia were used. The histological degree of epithelial dysplasia was based on the proportion of the height of the epithelial layer that presents the dysplastic changes. The antibodies and reagents used for immunohistochemical technique were obtained from ABCAM Company (UK) and SCKTEK LAB (USA) ready to use kit which consist of

1. Primary antibody – mouse antihuman Metallothionein
2. Secondary antibody – antimouse IgG
3. Peroxidase Block
4. Conjugate – Horse Radish Peroxidase
5. Chromogen substrate – Diaminobenzidine tetra hydrochloride (DAB)

Sectioning

4 micron thick sections were taken onto poly-L-lysine adhesive coated slide and incubated for 3 hour at 50-60 degrees centigrade in a slide warmer for proper adhesion of the section to the slide.

Evaluation of the staining for MT

Assessment of MT positive cells was performed using double headed light microscope at 10x and 40x. The criteria used to define MT antigen positive cells were brown: mosaic staining in dysplastic cells, within the nucleus and cytoplasm.

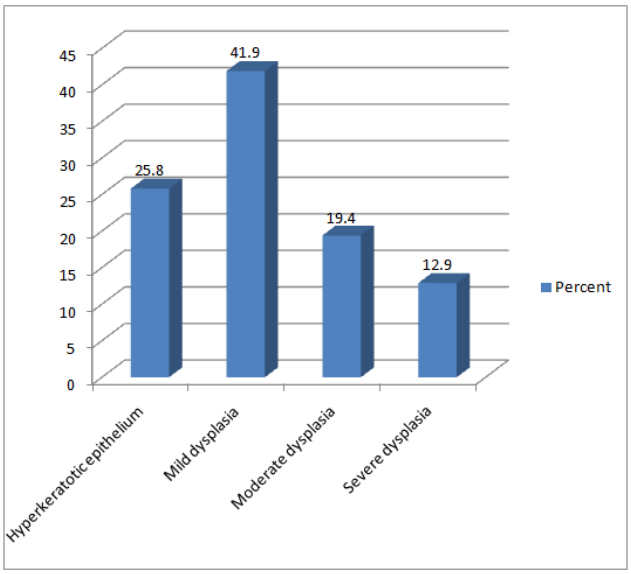
RESULTS AND OBSERVATIONS

The study group comprised of 31 tissue samples of OL and 10 samples of normal oral mucosa which served as controls. In normal oral mucosa MT expression was restricted only to basal and parabasal cells with a mosaic cytoplasmic-nuclear expression pattern, whereas in cases of leukoplakia additional foci in the spinous layer were noted. The data obtained from the study was compiled, tabulated and subjected to statistical analysis.

The results which were obtained are presented in the following manner: Cases of OL were graded histologically into hyperkeratosis without dysplastic changes (8 cases), mild dysplasia (13 cases), moderate dysplasia (6 cases) and severe dysplasia (4 cases). The histological grading among study group is given in (Table 1 and Graph 1).

Table 1. Histological grading among study group

Histological diagnosis	Number of cases (41)	Percent
Hyperkeratotic epithelium	8	25.8
Mild dysplasia	13	41.9
Moderate dysplasia	6	19.4
Severe dysplasia	4	12.9

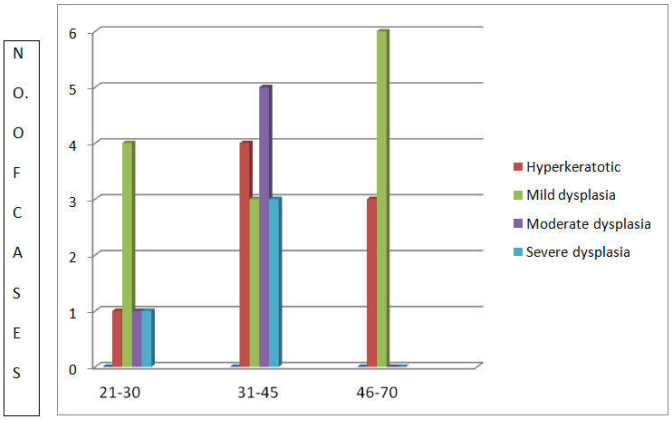


Graph 1. Histological grading among study group

Table 2. Age distribution among study group

Group	Hyperkeratotic epithelium	Mild dysplasia	Moderate dysplasia	Severe dysplasia	*P value
21-30	1	4	1	1	
31-45	4	3	5	3	
46-70	3	6	0	0	0.168
Total	8	13	6	4	

Pearson chi-square test, p>0.05 (not significant)



Graph 2. Age distribution among study group

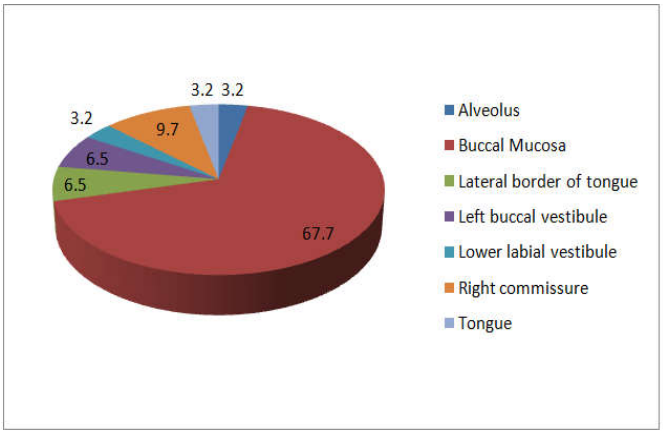
Table 3. Site distribution among study group

Site of biopsy	Number of cases	Percent
Alveolus	1	3.2
Buccal Mucosa	21	67.7
Lateral border of tongue	2	6.5
Left buccal vestibule	2	6.5
Lower labial vestibule	1	3.2
Right commissure	3	9.7
Tongue	1	3.2

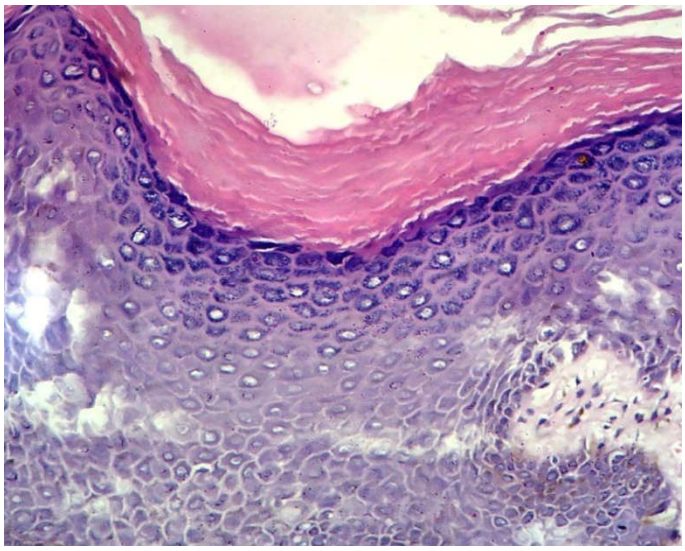
Table 4. Interobserver variability of metallothionein expression among different histological grades of leukoplakia and normal mucosa

	Types	1+	2+	3+	Total	*p value	Intraclass Correlation coefficient
Observer 1	Normal Mucosa	10	0	0	10		
	Hyperkeratotic epithelium	6	2	0	8		
	Mild dysplasia	7	3	3	13	0.021	0.94
	Moderate dysplasia	3	2	1	6		
	Severe dysplasia	0	0	4	4		
	Total	26	7	8	41		
	Types	1+	2+	3+	Total		
Observer 2	Hyperkeratotic epithelium	6	2	0	8		
	Mild dysplasia	6	4	3	13	0.026	
	Moderate dysplasia	1	3	2	6		
	Severe dysplasia	0	0	4	4		
	Total	23	9	9	41		

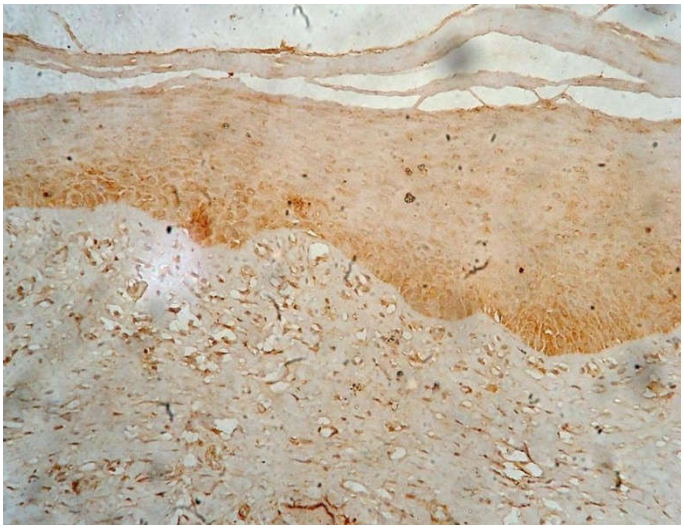
Pearson chi-square test, $p<0.05$ (significant)



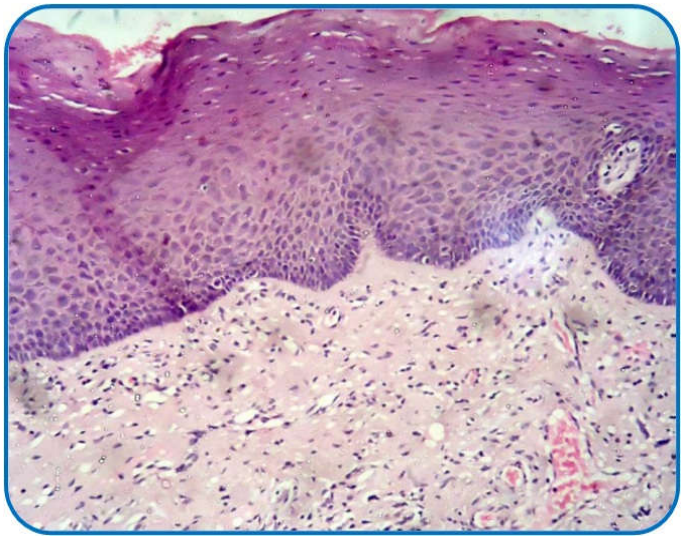
Pie Chart 1. Site distribution among study group



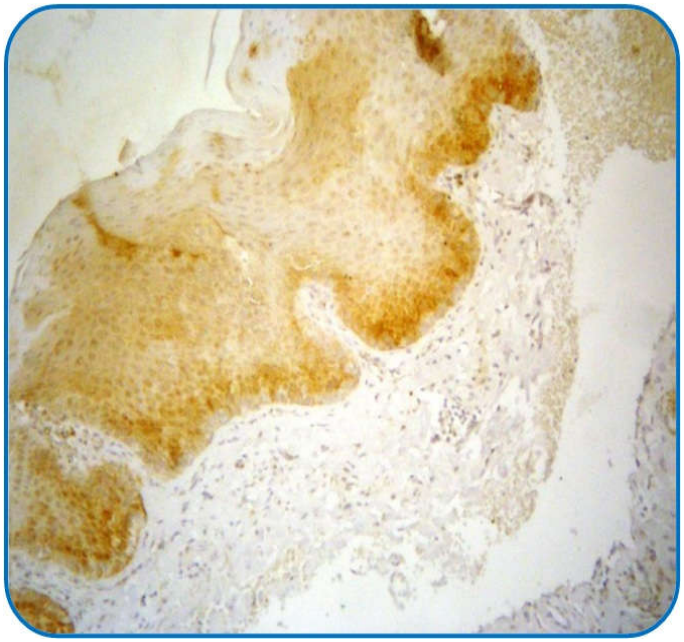
Pic. 1. Hyperkeratotic Epi H&E



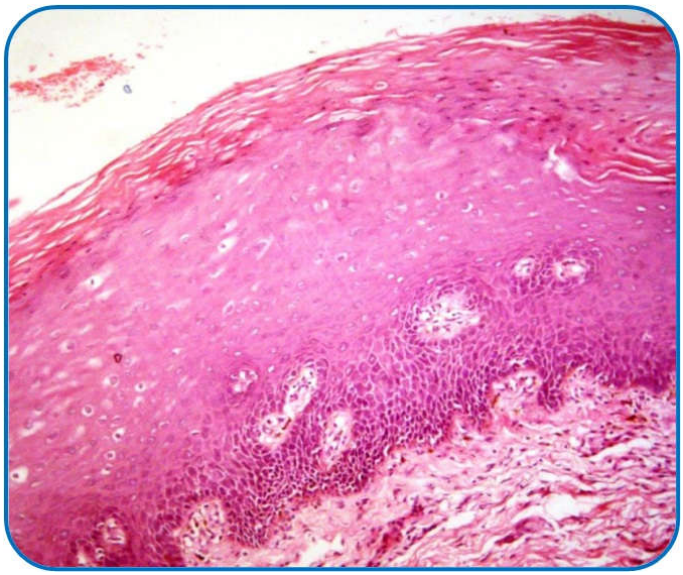
Pic. 2. Mild Expression



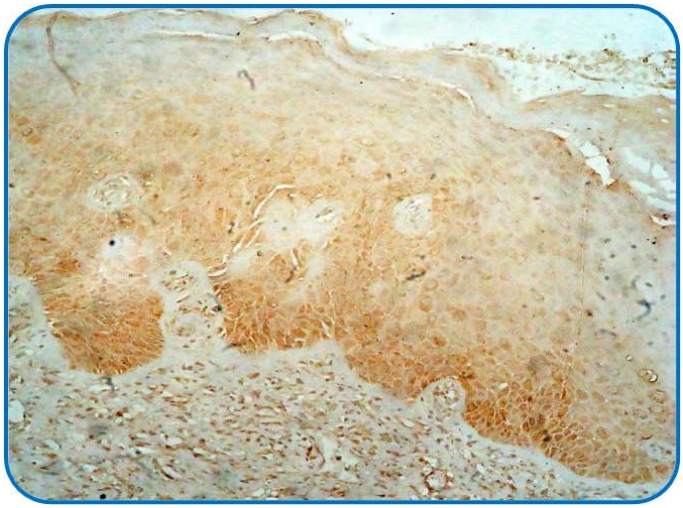
Pic. 3. Mild Dysplasia H&E



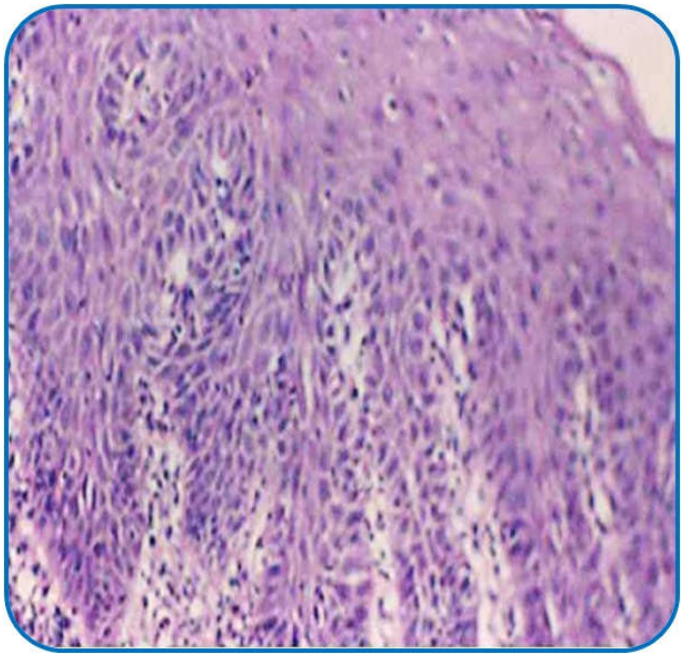
Pic. 4. Moderate Expression



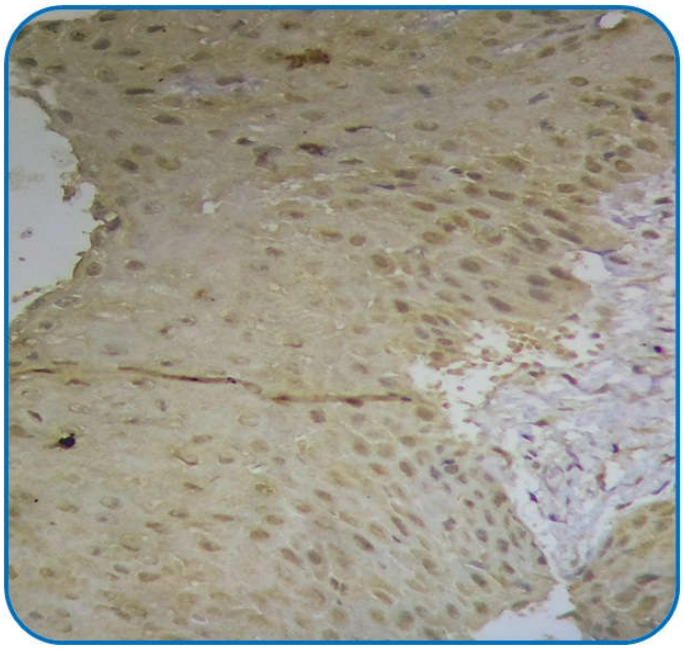
Pic. 5. Moderate dysplasia H&E



Pic. 6. Strong expression



Pic. 7. Severe Dysplasia H&E



Pic. 8. Strong expression 40 x

The patient's age ranged from 21-70 years of age. They were divided into three groups as 21-30 years (Group A), 31-45 years (Group B) and 46-70 years (Group C). The age wise distribution of study group is given in (Table 2 & Graph 2). The study group included (n=31) cases of OL which were diagnosed clinically at different sites. 21 cases were seen in buccal mucosa, 3 cases in commissure, 2 cases in lateral border of tongue, 1 case in tongue, 1 case in alveolus and 3 cases were seen in vestibular (labial/buccal) region. The Site distribution of cases of OL is tabulated in (Table 3 and Pie chart 1). A total of 41 sections in which (n=31) cases of oral Leukoplakia and (n=10) cases of normal mucosa were examined and compared for the immunohistochemical expression and distribution pattern of MT. In the group Hyperkeratosis without dysplasia (n=8), 6 cases showed a weak expression with a score of 1+ and 2 cases showed moderate expression with a score of 2+. None of the cases showed strong expression and so none were scored as 3+. (Photo micrograph 1 & 2) In the group mild dysplasia (n=13 cases), 7 cases showed a weak expression and scored as 1+, 3 cases showed a moderate expression and scored as 2+ and 3 cases showed a strong expression and scored as 3+. (Photo micrograph 3 & 4) In the group moderate dysplasia (n=6), 3 cases showed a weak expression and scored as 1+, 2 cases showed a moderate expression and scored as 2+ and 1 case showed a strong expression and scored as 3+. (Photo micrograph 5 & 6) In the group severe dysplasia (n=4), all 4 cases showed a strong expression and scored as 3+. (Photomicrograph 7 & 8) Statistical analysis was carried out using Pearson chi square test to compare the MT expression among the study and control group and it was found to be stastically significant ($p<0.05$) as given in (Table 4 and Graph 3).

DISCUSSION

Oral Leukoplakia is a potentially malignant lesion of the oral mucosa and proceeds to OSCC in 16-62% of cases. (Johann *et al*., 2003) On the basis of the degree of dysplastic cells and the thickness of dysplastic epithelium, this lesion is graded as mild, moderate and severe forms. Till now, in various oral mucosa lesions, different isoforms of the MT were investigated.

(Dziegiel Piotr *et al*., 2004; Warnakulasuriya, 2000) Our study showed the expression of MT in both the nucleus and cytoplasm of squamous cells of basal and parabasal layers in normal mucosa. However our results differed from those of Sundelin *et al*. (1997), Johann *et al*. (2008) and Pontes *et al*. (2009) (Sundelin *et al*., 1997; Pontes *et al*., 2009; Johann *et al*., 2003) who reported expression of MT only in cytoplasm of squamous cells of basal and parabasal layers in samples of normal mucosa. Although MT has been characterized as a cytoplasmatic protein, it may cross the nuclear membrane by passive diffusion which is responsible for the expression of MT in the nuclear compartment of the cells. The significance of nuclei localization is a more effective biological protection against oxidative stress and genomic damage. In addition to its interference in genomic regulation and other proteins linked to DNA. When leukoplakia slides were analyzed it was observed that the expression of MT was not only restricted to the cytoplasm but was observed in both cytoplasmic and nuclear compartment of squamous cells in the basal and parabasal layers cells with an additional foci in the spinous layers. Similar findings have been reported by many authors namely Sundelin *et al*. (1997), Ioachim *et al*. (1999), Johann *et al*. (2008) and Pontes *et al*. (2009). (Ioachim *et al*., 1999; Sundelin *et al*., 1997; Pontes *et al*., 2009; Johann *et al*., 2003) Our study showed higher expression of MT in severe dysplasia with the lowest MT expression in hyperkeratosis without dysplasia and the expression in mild and moderate dysplasia was more or less equal. Our results are not in accordance with the results obtained by Johann *et al*. (2008), Pontes *et al*. (2009). (Johann *et al*., 2003; Mishra *et al*., 2005) who noted higher expression of MT was almost equal in cases of Moderate and severe dysplasias when compared with normal oral mucosa, hyperkeratosis and mild dysplasia and concluded that the moderate dysplasia is the hallmark point in the process of carcinogenesis.

Literature reveals that lesions without dysplasia and mild dysplastic conditions also showed malignant transformation but the reason for its transformation is not clear. Over expressions of MT in 3 cases of mild dysplasia were observed in our study. Literature reveals that over expression of MT may mean that the altered cells are more protected with more chances of survival, but it can also indicate alterations in genomic regulation and in other proteins linked to DNA, thus presenting its possible role in carcinogenesis. (Theocharis *et al*., 2002; Cardoso *et al*., 2002) Hence, even cases of mild dysplasia should not be overlooked and should be kept under observation. In the present study (n=31) cases of Leukoplakia, all cases showed a positive immunoreactivity. 16 cases showed a weaker expression and were scored as 1+, 7 cases showed a moderate expression and were scored as 2+ and 8 cases showed a strong expression and were scored as 3+. The over expression of MT is variable in different grades of Leukoplakia and this may be because of difference in phenotypic variation that are associated with dysplastic cells and might be worthy of a more meticulous investigation. Overexpression noted as early as in mild dysplasia gives a clue regarding the initiation or promotion of carcinogenesis. Thus, this study suggests that MT can be one of the useful diagnostic marker for predicting the potential behavior of oral leukoplakia turning into OSCC.

Summary and Conclusion

The present study was undertaken to observe MT immunoexpression in OL. 31 samples of OL and 10 samples of normal mucosa were taken as controls. Each specimen was sectioned in 4µ thickness and immunostained with MT antibody and viewed under light microscope. MT positive cells were brown with mosaic staining in within the nucleus and cytoplasm. Slides were graded as 1+ (weaker expression) when it involved basal and supra basal layer of epithelium and were graded as 2+ (moderate expression) when it involved up to middle third of epithelium and were graded as 3+ (strong expression) when it involved entire epithelium. p value < 0.05 was considered significant. Expression of MT in cases of normal mucosa was confined to basal and parabasal layers whereas in cases of OL, expression of MT was observed not only in the basal layer but also in the spinosum layer, suggesting that MT possibly because of its chelating properties may contribute to delaying cells entering apoptosis. Our study revealed MT expression in cases of severe dysplasia to be relatively more when compared with other cases like moderate dysplasia, mild dysplasia and Hyperkeratosis with no dysplasia. The over expression of MT in severe dysplasia may mean that the altered cells are more protected with more chances of survival, but it can also indicate alteration in genomic regulation and in other proteins linked to DNA, thus presenting its possible role in carcinogenesis. In conclusion, the results of this study suggest that MT over expression can be one of the useful diagnostic marker for predicting the potential behavior of oral leukoplakia turning into OSCC. Its over expression gives a clue regarding the initiation or promotion of carcinogenesis. However, a large sample is required to predict the over expression of MT in different histological grades of leukoplakia.

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