ORIGINAL ARTICLE

Expression of Mucin 4 in leukoplakia and oral squamous cell carcinoma: An immunohistochemical study

Sangeetha Narashiman, Malathi Narasimhan, Ganesh Venkatraman[1]

Department of Oral and Maxillofacial Pathology, Faculty of Dental Sciences, [1]Human Genetics, Sri Ramachandra University, Porur, Chennai, Tamil Nadu, India

Address for correspondence:
Dr. Sangeetha Narashiman,
Department of Oral and Maxillofacial Pathology,
Faculty of Dental Sciences, Sri Ramachandra
University, No 1, Ramachandra Nagar,
Porur, Chennai - 600 116, Tamil Nadu, India.
E-mail: sangeetha_narashiman@yahoo.co.in

ABSTRACT

Background: Mucins are large glycosylated proteins that act as a selective molecular barrier on the epithelial surface and engage themselves in morphogenetic signal transduction pathways. MUC4 (Mucin 4) is a transmembrane mucin, that protects and lubricates the mucous membranes of the human body and involves itself in various cellular functions like growth, differentiation and signaling. An aberrant expression of MUC4 has been demonstrated in various human cancers. A thorough literature survey shows very few studies about MUC4 expression in normal and cancerous oral mucosa. **Aim:** Our study aimed at investigating the expression pattern of MUC4 in normal oral mucosa, oral leukoplakia and oral squamous cell carcinoma (OSCC) in an attempt to analyze its role played in oral carcinogenesis. **Materials and Methods:** Formalin-fixed paraffin-embedded tissues of five cases of normal tissue, 15 cases of leukoplakia, 10 cases of well-differentiated squamous cell carcinoma and 10 cases of moderately differentiated squamous cell carcinoma were retrieved from the archives of the department and MUC4 antigen was immunohistochemically localized. **Statistical Analysis:** The result was subjected to statistical analysis using Pearson's Chi-square test and an intergroup analysis was performed using one-way analysis of (ANOVA). **Results:** A total of 46.7% of leukoplakia and 70% of OSCC were stained positive with MUC4 antigen. Maximum intensity of staining was noted in well-differentiated OSCC. A steady increase in MUC4 staining was noted from normal oral tissues to leukoplakia to OSCC. **Conclusion**: The findings of the study suggest that MUC 4 plays a vital role in the pathogenesis of OSCC and can be regarded as a useful marker for oral dysplasia and OSCC.
Key words: MUC4, oral, leukoplakia, squamous cell carcinoma

INTRODUCTION

OSCC accounts for about 90% of all oral neoplasms. Despite advanced therapeutic approaches, the 5 year survival rate of patients with OSCC remains less than 50%.[1] Cancer research has provided us with a wide range of biological and chemical agents that play a vital role in the molecular pathogenesis of OSCC. However, we lack a reliable molecular marker that predicts both early diagnosis and prognosis of this devastating disease. Oral leukoplakia is a potentially malignant disorder that presents as a white patch that cannot be characterized as

any another definable disease. The malignant transformation rate of leukoplakia into OSCC is accounted to be about 16.64%.[2] Discovery of a biomarker that predicts the malignant transformation of leukoplakia into OSCC is the need of the hour.

Mucins are high molecular weight glycoproteins that play a major role in cell growth, differentiation and cell signaling. Based on their structural properties mucins are classified into gel forming, membrane bound/transmembrane and soluble mucins.[3] MUC4 is a membrane-bound mucin that was first isolated from human tracheobranchial complementary DNA library by Moniaux *et al*. in the year 1999.[4]

The gene for MUC4 is located at 3q29. The MUC4 protein is composed of a transmembrane segment and a cytoplasmic tail. Proteolytic cleavage of MUC4 results in two subunits MUC4 α and MUC4 β. The transmembrane segment of MUC4 anchors it to the cell surface and bears an epidermal growth factor domain that acts as a ligand for the tyrosine kinase receptor

ErbB2, which functions like a growth factor. The cytoplasmic tail composes of serine and tyrosine residues that participate in signal transduction pathways.[3] The MUC4-ErbB2 complex of the cell membrane participates in various cellular functions like cell adhesion, growth, repair, replacement, signaling and immunologic responses. Expression of MUC4 in various normal epithelia such as upper aerodigestive tract, gastrointestinal tract, endocervix, vagina, cornea and conjunctiva and secretory epithelium of salivary gland, lacrimal gland and breast have been demonstrated by various authors. MUC4 has also been detected in body fluids like blood, saliva, tears and breast milk.[5] An aberrant expression of MUC4 in various human cancers including breast, lung, pancreas, salivary gland and squamous cell carcinoma of the oral cavity, esophagus and cervix[6-11] has highlighted its role in the pathogenesis of cancer. The cancer cells use mucin for cell proliferation, survival, invasion, metastatic growth and protection against innate immunity.[12] This study aimed to evaluate the expression of MUC4 in oral leukoplakia, OSCC and normal oral tissues.

MATERIALS AND METHODS

The study involved formalin-fixed paraffin-embedded tissue sections of histopathologically diagnosed cases of leukoplakia (*n* = 15) and OSCC (*n* = 20) from the archives of Department of Oral Pathology. History of all these patients with details of age, sex, site, related habits and duration was also retrieved from the medical records department. Normal tissues (*n* = 5) from volunteers with no oral lesions and related oral habits were obtained and processed in the same way as the pathological specimens. An additional tissue section was taken from all the cases and stained with hematoxylin and eosin for comparative purpose.

Immunohistochemistry

All 4 μm sections of the tissues were cut and transferred to APES coated slides and incubated overnight at room temperature. After warming in a slide warmer for 15 min the sections were deparaffinized in three changes of fresh xylene each for 5 min followed by dehydration in a series of absolute alcohol each for 5 min. Endogenous peroxidases were blocked with peroxide block (Biogenex life sciences Pvt Ltd) for 15 min at room temperature and washed in distilled water followed by citrate buffer (pH 6.0) wash for 10 min. Antigen retrieval was undertaken with a help of pressure cooker. The sections were immersed in citrate buffer solution and placed into the pressure cooker and heated for 15 min. The cooker was allowed to cool to room temperature by placing it under running tap water and later the slides were washed with distilled water for 5 min. With an intention to block endogenous biotin, the sections were incubated with a blocking agent (Biogenex life sciences Pvt Ltd) for 15 min. Excess power block solution was drained and the sections were incubated with primary antiMUC4 monoclonal antibody for 1 hr and later thoroughly washed with citrate

buffer. For further enhancement of the staining, the sections were then incubated with antimouse secondary antibody (super enhancer) for 30 min followed by two consecutive buffer washes; each for 5 min. Horse radish peroxide (HRP) was added to the sections and incubated for 30 min. The chromogen diaminobenzidine (DAB) was prepared just prior to use by mixing one drop of chromogen to 1 ml of buffer in a mixing vial and later added over the sections. After 5 min, the sections were washed in buffer followed by water and counterstained with Harris hematoxylin, air dried, cleared and mounted with dibutylpthalate xylene. Lung adenocarcinoma and normal colon were used as positive control.

Interpretation of staining

The MUC4 antibody stained the membrane and cytoplasm brown against a blue background in the positive cells. The staining pattern in colon carcinoma was used as the standard to interpret the study sections [Figure 1]. The MUC4 staining was graded as mild (<25% of the cells stained positive), moderate (25 to 50% of the cells stained positive) and intense (>50% of the cells stained positive). All IHC-stained slides along with the corresponding H and E sections were analyzed by two pathologists. The staining pattern along with the patient demographics was statistically analyzed
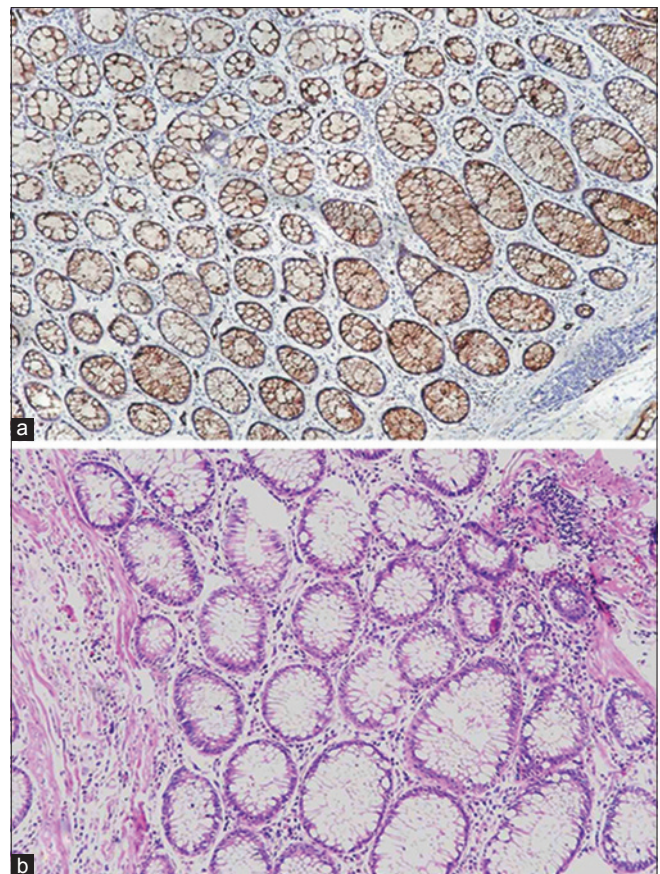
Figure 1: (a) Photomicrograph of the section shows membrane staining of MUC4 in colon tissue (control) (IHC stain, ×200). (b) The corresponding H&E section (×400)

using SPSS software. Pearson Chi-square test was performed to analyze the expression pattern of MUC4 with various disease parameters and one way ANOVA was performed for intergroup comparison.

OBSERVATION AND RESULTS

The study comprised of 15 leukoplakia cases, 10 well differentiated OSCC (WDOSCC) and 10 moderately differentiated OSCC (MDOSCC) cases. The study population comprised 28 males and seven females with a mean age of 50 years [Table 1]. The mean age of the subjects involved in the study was 49.7. The mean age of cases in the positive group was 51.8 yrs and the mean age of cases in the negative group

Table 1: Depicting the age, sex and site distribution of the study samples

Cases	Gender	Age	Site
Leukoplakia	Male	58	Tongue
Leukoplakia	Male	76	Tongue
Leukoplakia	Male	33	Buccal mucosa
Leukoplakia	Male	51	Commissure
Leukoplakia	Male	53	Buccal mucosa
Leukoplakia	Male	47	Commissure
Leukoplakia	Male	50	Commissure
Leukoplakia	Female	52	Buccal mucosa
Leukoplakia	Male	25	Buccal mucosa
Leukoplakia	Male	40	Buccal mucosa
Leukoplakia	Male	46	Tongue
Leukoplakia	Female	32	Buccal mucosa
Leukoplakia	Male	45	Buccal mucosa
Leukoplakia	Male	27	Buccal mucosa
Leukoplakia	Male	62	Lips
WDSCC	Male	75	Buccal mucosa
WDSCC	Male	69	Alveolar ridge
WDSCC	Male	40	Buccal mucosa
WDSCC	Male	52	Buccal mucosa
WDSCC	Male	60	Floor of the mouth
WDSCC	Female	65	Buccal mucosa
WDSCC	Male	27	Buccal mucosa
WDSCC	Female	63	Buccal mucosa
WDSCC	Male	40	Buccal mucosa
WDSCC	Female	56	Buccal mucosa
MDSCC	Male	53	Tongue
MDSCC	Female	40	Alveolar ridge
MDSCC	Male	49	Buccal mucosa
MDSCC	Male	48	Buccal mucosa
MDSCC	Male	50	Tongue
MDSCC	Male	68	Palate
MDSCC	Female	35	Buccal mucosa
MDSCC	Male	42	Tongue
MDSCC	Male	43	Buccal mucosa
MDSCC	Male	69	Alveolar ridge

WDSCC: Well-differentiated squamous cell carcinoma,
MDSCC: Moderately differentiated squamous cell carcinoma

was 46.6 which were statistically significant with a *P* value of 0.01. However, no significance was noted in MUC4 expression with regard to related habits and lymph node status of the study group.
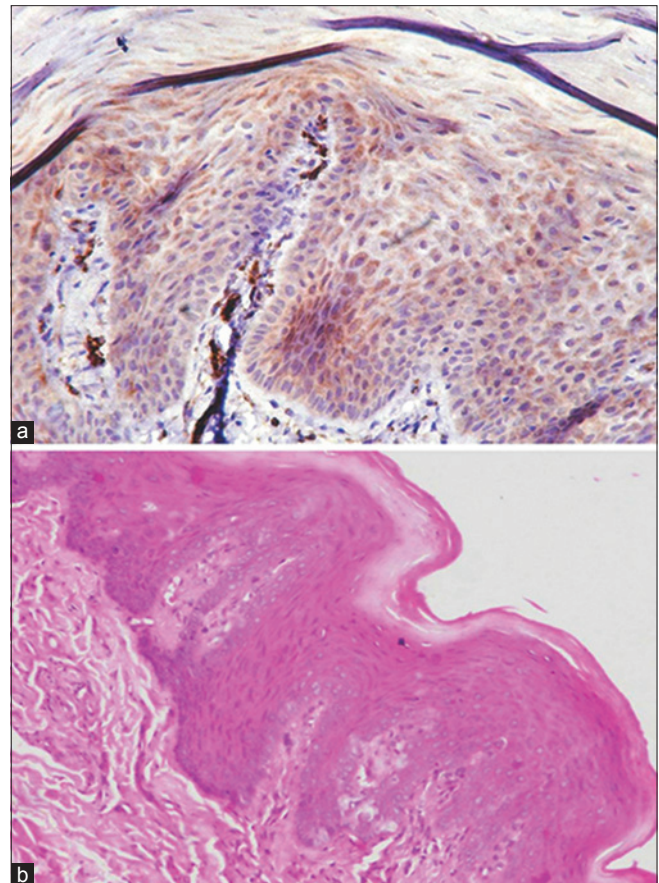
Seven out of 15 cases of leukoplakia stained positive with MUC4 antibody [Figure 2]. In all, nine out of 10 cases of WDOSCC [Figure 3] and five out of 10 MDOSCC [Figure 4] showed MUC4 positivity. The staining pattern among different groups of the study subjects was statistically significant ($\chi^2 = 11.395$, DF = 3, *P* = 0.010). Among the positive samples, five cases of leukoplakia and five cases of WDOSCC showed only cytoplasmic staining, whereas two cases of leukoplakia, five cases of WDOSCC and all the cases of MDOSCC exhibited both cytoplasmic and membrane staining [Figure 5]. The staining pattern of MUC4 differed markedly with the cases of leukoplakia. Cases with mild dysplasia showed MUC4 expression restricted to the basal and suprabasal layers whereas the cases with moderate to severe dysplasia demonstrated staining that extended into the granular layer of the epithelium. In contrast to this all the WDOSCC sections showed full thickness staining [Figure 6] of the epithelium.


Figure 2: (a) Photomicrograph of the section shows moderate cytoplasmic staining from basal to spinous layer of epithelium in leukoplakia (IHC stain, ×400). (b) The corresponding H&E, section (×400)
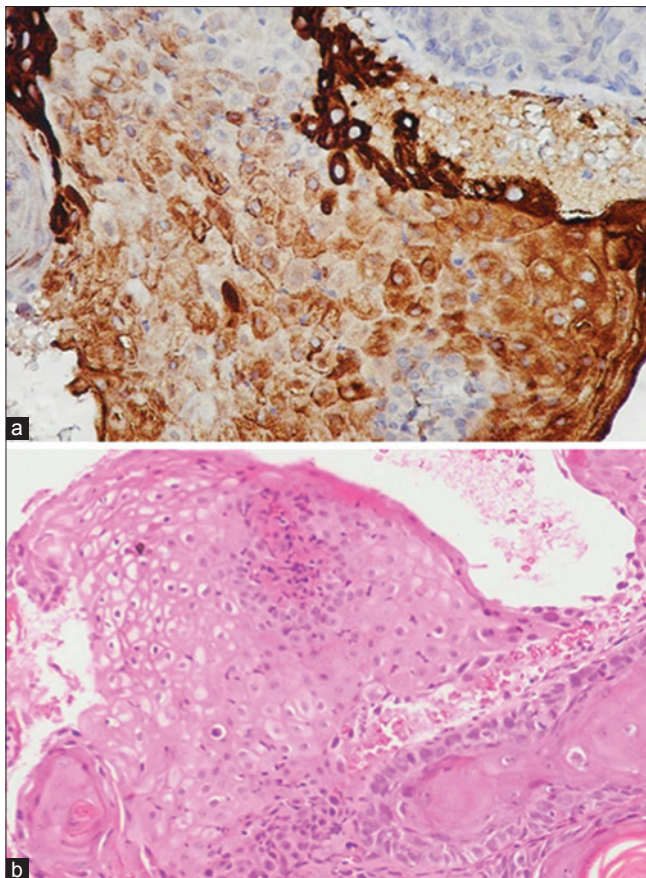
Figure 3: (a) Photomicrograph of the sections shows intense membrane and cytoplasmic staining of MUC4 in the epithelium of well-differentiated squamous cell carcinoma (IHC stain, ×400). (b) The corresponding H&E, section (×200)
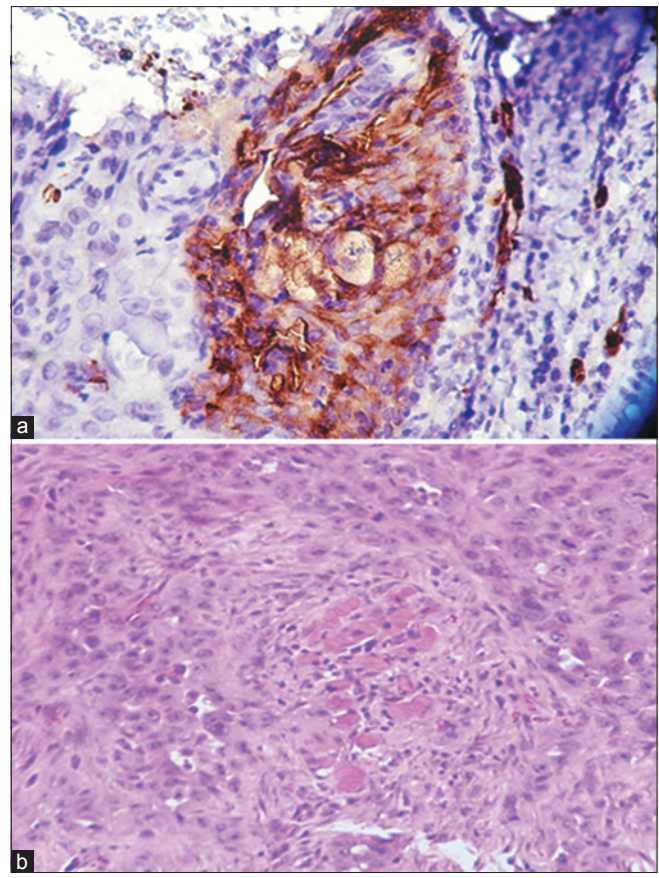
Figure 4: (a) Photomicrograph of the section shows membrane and cytoplasmic staining of MUC4 in moderately differentiated squamous cell carcinoma (IHC stain, ×400). (b) The corresponding H&E, section (×200)
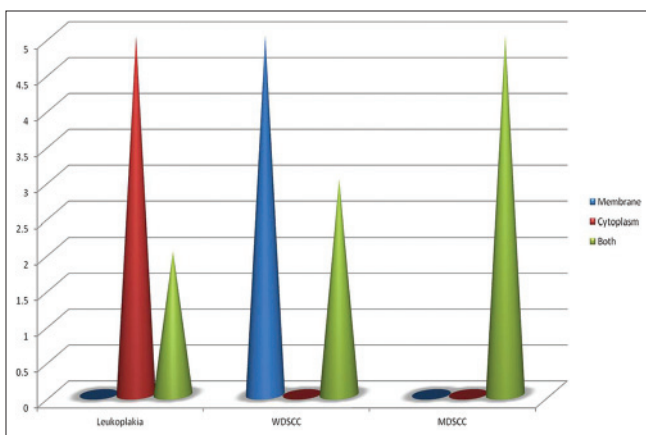
Figure 5: Bar diagram depicting the type of staining in the MUC4-positive study samples

A very high statistical significance (χ^2 = 58.190, DF = 18, $P = 0.000$) was noted with regard to the site-dependent positivity of MUC4 in the oral cavity. About 61.9% of lesions in the buccal mucosa, 50% in the tongue, 66.6% in the commissure and 33.3% of the lesions in the alveolar ridge [Figure 7] showed positive expression with MUC4 antibody. One case each from the floor of the mouth and palate also showed MUC4 positivity. All the normal tissues exhibited negative expression for MUC4 [Figure 8].

DISCUSSION

In India, OSCC is the most common cancer accounting for 12% of all cancers in men and 8% of all cancers in women.[13] Mucins are heavily glycosylated proteins that act as a molecular barrier and engage themselves in morphogenetic signal transduction pathways at the epithelial surface. MUC4 plays a vital role in the carcinogenesis, which has been proved by its aberrant expression in various human cancers.[14] The current study attempted to evaluate the expression of MUC4 in oral leukoplakia and OSCC by IHC method and also compared its expression in normal oral mucosa.

Though a couple of studies have reported the expression of MUC4 in OSCC earlier, ours is the first of its kind to analyze MUC4 expression in leukoplakia- a potentially malignant disorder of the oral cavity. Among the MUC4 positive cases of leukoplakia, a steady increase in MUC4 expression was noted from mild to moderate to severe dysplastic epithelium.
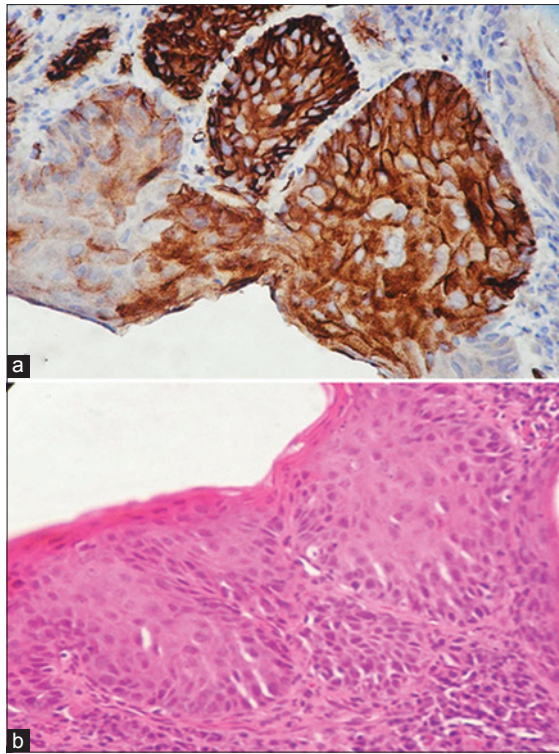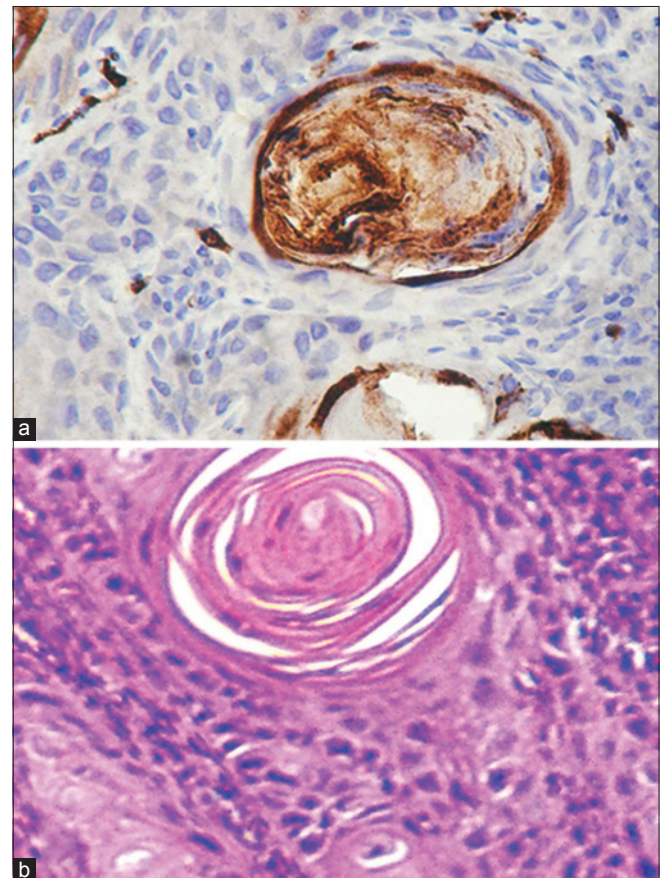
Figure 6: (a) Photomicrograph of the section shows intense full thickness MUC 4 staining of the epithelium of well-differentiated squamous cell carcinoma in a "Honeycomb" pattern (IHC stain, ×400). (b) The corresponding H&E, stain (×200)
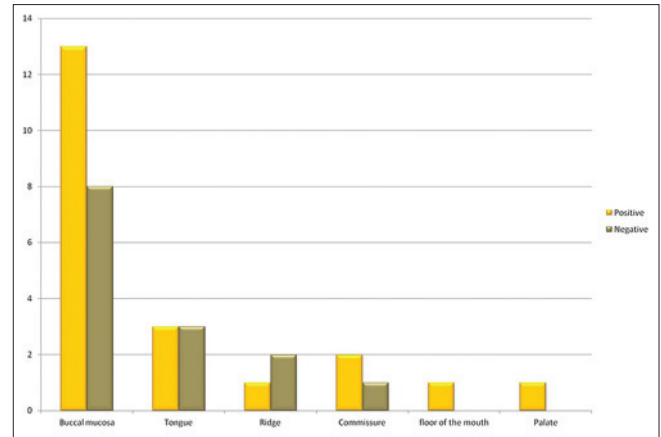
Figure 7: Bar diagram depicting the expression of MUC4 in the study sample according to site
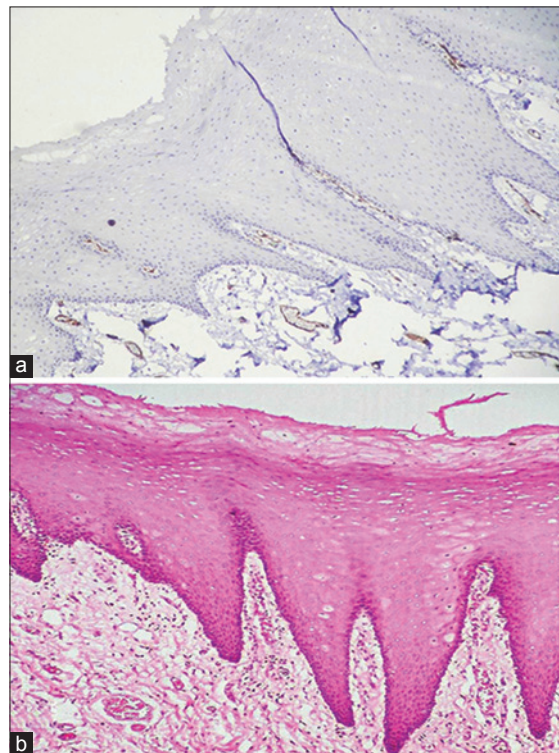
Figure 8: (a) Photomicrograph of the section shows negative expression of MUC4 in normal oral tissue (IHC stain, ×200). (b) The corresponding H&E section (×200)

Figure 9: (a) Photomicrograph of the section shows MUC4 staining of keratin pearl in well-differentiated squamous cell carcinoma. (IHC stain, ×400). (b) The corresponding H&E section (×400)

Anna Lopez *et al.,* have reported a similar expression pattern of MUC4 in cervical dysplasia and also stated MUC4 as a useful molecular marker for malignant transformation of cervical dysplasia.[15] This relative overexpression of MUC4 with increasing grades of dysplastic epithelium attributes its tumor-promoting behavior.

The membrane and cytoplasm staining of MUC4 in the squamous cells might correspond to its transmembrane and cytoplasmic subunits, respectively. The human MUC4 transmembrane unit extends up to 2 μm above the cell membrane, which is the highest of all other cell membrane proteins. Thus it masks all the tumor antigens from their respective antibodies thereby facilitating immune surveillance of the tumor cells.[16] The transmembrane portion of human MUC4 also acts as a natural ligand for the growth factor receptor ErbB2.[9] Chu F *et al.*, demonstrated an overexpression of ErbB2 in OSCC.[17] An increased expression of ErbB2 along with its ligand MUC4 thereby feeds the squamous cells with continuous growth signals that are transmitted to the nucleus via the cytoplasmic tail of MUC4 imparting limitless replicating potential to the tumor cells. Bafna *et al.*, demonstrated an increased cellular proliferation and decreased apoptosis in MUC4 overexpressing mouse fibroblast cells. Through a series of studies they formulated (MUC4-ErbB2- Grb2/sos-Ras-Raf1-MEK-ERK1/2) pathway that caused oncogenic transformation in mouse fibroblasts cells and also established an increased expression of Cox 3 and ND1 genes that caused a depression in apoptosis.[18] The finding that an overexpression of MUC4 in OSCC cells compared with its normal and potentially malignant counterpart clearly suggests a similar role of MUC4 in the pathogenesis of OSCC. In the current study, MUC4 positivity in the OSCC samples was highly restricted to the well-differentiated areas and the keratin pearls [Figure 9] of the tumors. A decrease in positivity of MUC4 was noted in MDOSCC compared with WDOSCC cases. Studies conducted by Donald *et al.*, demonstrated a significant decrease in MUC4 expression with an increase in the histological grade of the OSCC thereby confirming the association of MUC4 with the well differentiated cells of squamous cell carcinoma.[5] This finding is consistent with the findings of the study conducted by Philippe G *et al.*, in esophageal squamous cell carcinoma using IHC and northern blot analysis.[19] The results of these studies prove the correlation of MUC4 with squamous cell differentiation. Donald *et al.*, reviewed various studies and postulated a strong association between MUC4 expression and cytodifferentiation. Thus a decrease in the expression of MUC4 in MDOSCC may be attributed to the inability of the less differentiated squamous cells to express MUC4 compared with that of the well differentiated cells of OSCC.

With MUC4 playing a diverse role in the pathogenesis of cancer, its over expression in OSCC has added a new forum for research in the onset and progression of oral cancer. The findings of this study have provided us with supportive evidence to add MUC4 as a novel marker for tumor cell differentiation and thus determine prognosis of OSCC.

CONCLUSION

Aberrancy in MUC4 expression is noted in various human cancers. The current IHC study demonstrated an overexpression of MUC4 in OSCC with no expression in the normal oral mucosa. The cellular expression of MUC4 showed a steady increase from dysplastic non-invasive lesions to invasive OSCC and was highest among the well-differentiated squamous cells of OSCC. With the above findings, it can be formulated that MUC 4 plays a vital role in the pathogenesis of OSCC and can be regarded as a useful marker for oral dysplasia and OSCC. Large-scale molecular studies can further help to establish MUC4 as a diagnostic and prognostic marker and also aid in formulating targeted therapy for OSCC.

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