ORIGINAL ARTICLE

Association of Mucin-4 Expression In Anneroth Grades of Oral Squamous Cell Carcinoma

Naila Umer[1], Afifa Ehsan[2], Ali Raza[3], Rabia Masood[4], Saima Chaudhry[5]

ABSTRACT

Objective: To evaluate the expression of Mucin-4 amongst separate histological categories of oral squamous cell carcinoma patients at a tertiary care hospital in Pakistan.

Study Design: It was a cross-sectional descriptive study.

Place and Duration of Study: It was carried out from July 01, 2021, to December 31, 2021, at the Department of Oral Pathology, University of Health Sciences, Lahore, Pakistan.

Materials and Methods: The study comprised fifty cases of oral squamous cell carcinoma randomly taken from the archives of Sheikh Zayed Hospital and Federal Postgraduate Medical Institute, Lahore as per the selection criteria agreed upon. The association of mucin-4 expression with different histological grades (based on the Anneroth grading system) was analyzed in this study. Data were entered into SPSS 20 for statistical analysis. Age was represented as mean ± standard deviation while gender distribution and tumor grades were presented as frequencies and percentages.

Results: Out of a total of fifty retrieved samples, thirty were analyzed (twenty were excluded based on the exclusion criteria). Of these thirty samples, 16 (53.3%) were male and 14 (46.6%) were female. Consistent with the Anneroth grading method, grade I comprised 11 (36%), grade II comprised 13 (43.3%), and grade III comprised six cases (20%) respectively. Overall positive expression of Mucin-4 was found to be 73% and a significant relation was found to exist between the intensity score and the total score of MUC-4 with Anneroth grades of Oral Squamous Cell Carcinoma with a decrease in expression and an intensification in tumor grade.

Conclusion: A decrease in the expression of Mucin-4 was noted with an increase in the tumor grade when the expression of Mucin-4 was evaluated amongst separate histological categories of oral squamous cell carcinoma patients.

Key Words: *Immunohistochemistry, MUC4, Mucin 4, Oral Squamous Cell Carcinoma, Squamous Cell Carcinoma.*

Introduction

Mucins are high-level molecular weight multifunctional glycoproteins expressed by innumerable epithelia of oculo-rhino-otolaryngeal tracts, respiratory tracts, reproductive tracts, and gastrointestinal tracts. Being a foremost component of mucus, it aids to hydrate the epithelia, lubricates, and shields them from injurious microbes.[1-2] Distinctive silhouette of mucin expression in the epithelium of different organs is depicted and a modification in this expression is accompanied by cancer expansion promoting cell growth, differentiation, adhesion, and invasion.[3-5]

Atypical expression of mucins in numerous cancers has been accepted and numerous mucins are recognized as therapeutic agents, diagnostic and prognostic markers, and appealing therapeutic targets.[4-5] MUC4, one of the transmembrane mucins has lately emerged as an expedient biomarker. Modification in manifestation, as well as glycosylation, is documented concerning several epithelial malignancies for instance the esophagus, breast, lung, pancreas, and cervix.[6]

In cancerous cells, biochemical alterations of MUC4 in concert with changes in cell polarity permit its interaction with proteins that are otherwise cytoarchitecturally segregated.[7] MUC4 enacts its function in tumor advancement secondarily through the anti-adhesion mechanism or directly through the ErbB2 signaling pathway.[7] MUC4 is furthermore localized in the oral squamous epithelium and its

[1] *Department of Oral Pathology, Faryal Dental College, Lahore*
[2] *Department of Oral Biology, Faryal Dental College, Lahore*
[3,5] *Department of Oral Pathology, University of Health Sciences, Lahore*
[4] *Department of Oral Pathology, Islamic International Dental College, Islamabad*

Correspondence:
Dr. Afifa Ehsan,
Associate Professor & HOD Oral Biology,
Faryal Dental College, Lahore.
E-mail: afifaehsan@gmail.com

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character in malignant alteration is under research.[8-12] The present study aimed to assess the countenance of Mucin 4 in oral squamous cell carcinoma patients at a tertiary care hospital in Pakistan as it can be a beneficial diagnostic and prognostic marker.

Materials and Methods

The present cross-sectional descriptive analysis was carried out at the Department of Oral Pathology, University of Health Sciences, Lahore from July 01, 2021, to December 31, 2021. Ethical endorsement of the study was acquired through the Ethical Review Board of the University of Health Sciences, Lahore (Letter#: UHS/Education/126-16/039).

A total of 50 cases of Formalin-fixed paraffin blocks from diagnosed patients of Oral Squamous Cell Carcinoma (OSCC) were recovered from the archives of Sheikh Zayed Hospital, Postgraduate Medical Institute, Lahore. Blocks with inadequate data and any sort of damage were excluded from the study. The H & E slides were prepared and graded using the Multiparameter (Anneroth's) grading system. Also, 20 cases in which the stage of invasion was not found were excluded as Anneroth grading couldn't be done on them.

For immunohistochemistry with anti MUC4 antibody, 4 µm dense tissue segments were chosen on Poly-L-lysine covered. The segments were dehydrated at 60^{0} Ċ for 50 minutes in warm air. Following dewaxing, in xylene sections, they were positioned in scored alcohol. Antigen repossession was done through placing slides in a Coplin jar filled with antigen retrieval solutions. Jars were then positioned inside a warm water bath at 95 °C for 30 minutes. Then the slides were set aside to lower the temperature and evaporative damages were substituted by newly prepared phosphate-buffered saline (PBS).

Afterward, the slides were incubated for 15 minutes with 1-2 drops of H_2O_2. Additional 1-2 drops of protein blocker were placed atop slides which were again incubated for another 10 minutes. Subsequently, they were then washed 3 times with PBS using a washer bottle. Primary Antibody incubation was done for 2 hours with anti-MUC 4 antibody (code ab52263; Abcam, USA) reduced near the strength of 5µg/ml followed with Biotinylated Secondary Antibody Incubation for 30 minutes.

Following washing with PBS, slides remained incubated for 10 minutes with Substrate Chromogenic Solution (DAB) and then counterstained with hematoxylin. Slides were then mounted employing dibutyl phthalate polystyrene xylene (DPX). Human pancreatic cancer tissue was chosen as an affirmative control whereas excluding the principal antibody stage in the peroxidase-labeled streptavidin-biotin procedure delivered the negative control for MUC4.

Quantification:[14]

MUC4 expression transpired to be appraised based upon the extent and intensity of immunolabelling in the tumor's cell membrane as well as cytoplasm. The complete score for every single case was determined by the accumulation of the proportion score (PS) along with the intensity score (IS) of that case. Scoring criteria are given in Table I.[14]

Data were entered into SPSS 20 for statistical analysis. Age was represented as mean ± standard deviation (SD) and the significance level remained at $P \leq 0.05$. Gender distribution and tumor grades were presented as frequencies and percentages.

Table I: Scoring Criteria of MUC4 Expression

Intensity Score (IS)	Proportion Score (PS)	Total Score (TS): TS = IS + PS
0 = No Staining	0 = < 5% Immunoreactive	Score 0 = Negative
1 = Mild	1 = 6 - 32% Immunoreactive	Score 1 - 2 = Weak Positive +1
2 = Moderate	2 = 33% - 66% Immunoreactive	Score 3 - 4 = Moderate Positive +2
3 = Intense	3 = > 66% Immunoreactive	Score 5 - 6 = Strong Positive +3

Results

Our study comprised 50 cases of OSCC out of which 20 were excluded based on the exclusion criteria and the rest were analyzed. In the 30 cases that underwent analysis, the average age range of the patients was found to be 53 ± 3.77 years with the majority of them being male (53.3%) while only 46.6% were observed to be females. The most common site of involvement in this study was found to be the tongue and buccal mucosa while gingiva, lower lip, palate, and tonsil were less common. Grade 2 comprised a total of 43.3% cases which were recorded to be the maximum followed by grade 1

(36%) and grade 3 (20%) (Table II).

Out of the total, 73% of cases were found to be positive for MUC-4 expression while the rest were negative (Figure 1). Most of the tumors showed moderate intensity highlighting 33 - 66% of tumors. A significant relation ($p \leq 0.05$) was established between intensity and total score of MUC-4 with Anneroth grades of OSCC with a decrease in expression and an intensification in tumor grade (Table III).

Table II: Demographic Data & Anneroth Tumor Grades of OSCC

Age	**21 - 40 years**		**41 - 60 years**		**61 - 80 years**		**Total**
	06 (20%)		18 (60%)		06 (20%)		N = 30
Gender	**Male**			**Female**			
	16 (53.3%)			14 (46.6%)			
Site	**Tongue**	**Buccal mucosa**	**Gingiva**	**Lower lip**	**Palate**	**Tonsil**	
	11	11	3	2	2	1	
Anneroth Grade	**Grade 1 (≥ 15)**		**Grade 2 (16 - 18)**		**Grade 3 (≥ 19)**		
	11 (36%)		13 (43.3%)		6 (20%)		

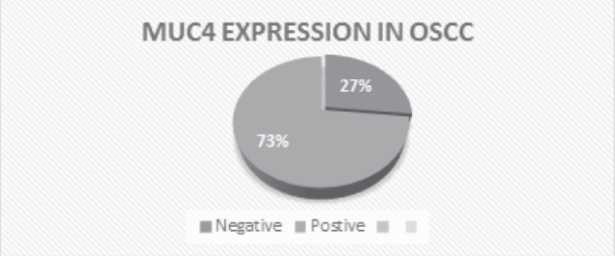

Fig. 1: Mucin-4 Expression in Oral Squamous Cell Carcinoma (OSCC)

Table III- Intensity, Proportion & Total Score of Mucin-4

MUCIN-4 STAINING	**Anneroth's Histological Grades of OSCC**				
	Grade 1	**Grade 2**	**Grade 3**	**Total**	***P* - value**
Intensity Score (IS)					
No staining	1	3	3	7	$p = 0.004$ df = 1
Mild	1	4	3	8	
Moderate	5	6	0	11	
Severe	4	0	0	4	
Proportion Score (PS)					
< 5%	1	3	3	7	$p = 0.072$ df = 1
< 33%	1	2	3	6	
33 - 66%	6	4	0	10	
> 66%	3	4	0	7	
Total Score (IS + PS)					
Negative	1	3	3	7	$p = 0.02$ df = 1
Weak Positive	0	1	3	4	
Moderate Positive	4	7	0	11	
Strong Positive	6	2	0	8	

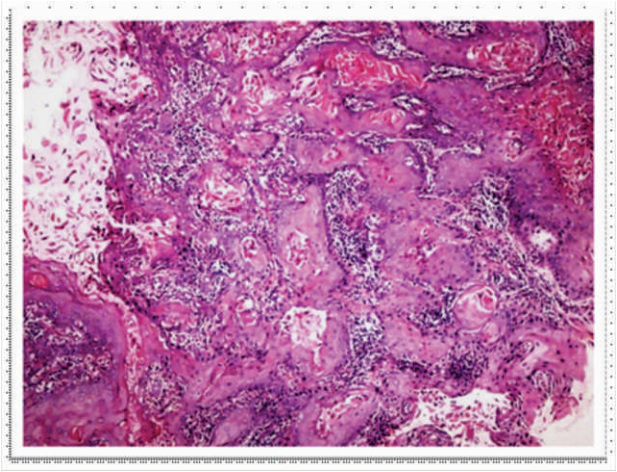

Fig. 2: Well Differentiated Oral Squamous Cell Carcinoma (Grade1) (H&E; 10x10 X)

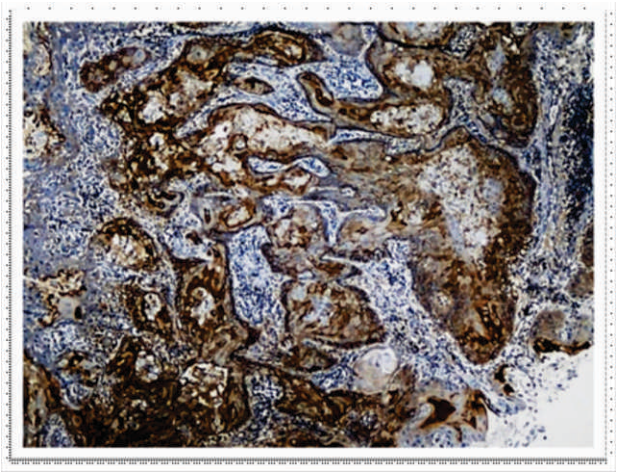

Fig. 3: Strong Cytoplasmic Expression In Well-Differentiated OSCC (Grade 1) (MUC4 IHC; 10 X 10 X)

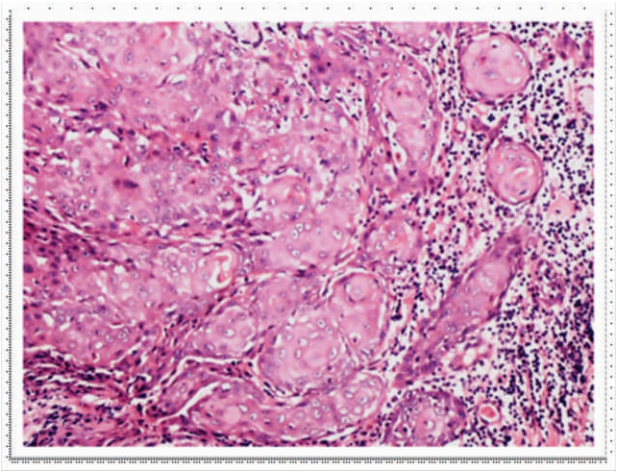

Fig. 4: Moderately Differentiated OSCC (Grade 2) (H&E; 10 x 10 X)

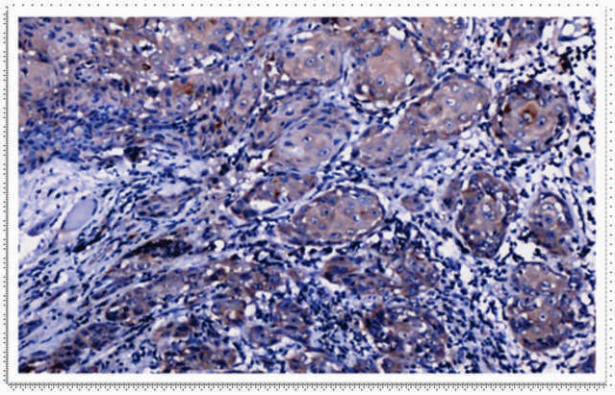

Fig. 5: Moderately Strong Cytoplasmic Expression In Moderately Differentiated OSCC (Grade 2) (MUC4 IHC; 10 x 10 X)

Discussion

Oral Squamous cell carcinoma remains nevertheless one of the greatest widespread tumors globally and the second most common tumor in Pakistan.[15-19] It is of immense significance to be a consistent diagnostic and prognostic biomarker for cancer patients to be able to make available indispensable information for attaining a clinical conclusion. Lately, mucins have been contemplated as prospective biomarkers in cancer diagnosis attributable to their distinctive representation in cancer patients as contrasted to the normal one. Among them, MUC4 is considered a promising one. [15-18]

In the current study expression of MUC-4 was observed in 73% of oral squamous cell carcinoma cases with the majority of tumors presenting a moderate intensity of the marker staining 33 - 66% of tumor cells. This positive ratio was analogous to the investigations carried out by Narashiman et al. and Kohli et al. while variance was found in one study performed in the population of Japan where the proportion of positive expression was found to be 40 %.[10-11] This variance can be attributed to dissimilarities in the population targeted where the incidence of OSCC is low. The rest of the studies have targeted the South Asian population with an elevated incidence rate of oral squamous cell carcinoma.[12-14]

Studies conducted by Hamada et al, Narashiman et al., and Kohli et al. have categorized OSCC only based on the degree of tumor differentiation/ keratinization and did not take into consideration any other parameters contemplated in Anneroth's grading systems. [8,10-11] No study has so far compared Anneroth's tumor grading system for OSCC regarding MUC4 expression. In the current study, the Anneroth grade of OSCC when associated with MUC4 expression revealed statistically significant results. A reduction in expression in conjunction with an intensification in tumor ranking was observed.

Allred scoring system was utilized in our study encompassing both intensity (IS) and proposition score (PS) as contrasted to the rest of the studies which only considered PS. When the association of MUC4 expression was equated with a grade of tumors a strong to moderate expression was observed in grade 1 tumors, mild to moderate in grade 2 while grade 3 tumors revealed weak/mild expression only. A noteworthy relationship was established concerning the expression of MUC4 and intensity in addition to total score (IS + PS). When tumor grade correlation in conjunction with positive and negative expression of MUC 4 was assessed, no substantial relation was obtained. This finding was analogous to the results of studies carried out by Hamada et al. They correlated various grades of SCC with positive and negative MUC4 expression instead of a difference in the positivity that is from weak to strongly positive, while our findings were significant as regards this aspect.[8]

A lessening in MUC 4 expression in moderately as well as poorly differentiated SCC could possibly be accredited to the deficit of differentiation of squamous cells as contrasted to well-differentiated SCC. Formerly many investigations carried out upon the function of MUC4 in SCC have revealed the aforementioned's association in conjunction with tumor differentiation. Philipe Guillem in 2000 reported the association of MUC4 gene expression with squamous differentiation, as he discerned the strongest hybridization signals in well-differentiated esophageal SCC.[20] Correspondingly, a study done by Kathpalia to determine cellular pathways in cutaneous SCC found MUC 4 m RNA upregulation nearly threefold in well-differentiated SCC.[20-23]

Conclusion

In conclusion, a decrease in the expression of Mucin-4 was seen with an increase in the tumor grade when the expression of Mucin-4 was assessed amongst separate histological categories of oral squamous cell carcinoma patients. The findings of our study also reveal upregulation (73%) of MUC4 appearing in

tumor tissue alongside negative expression in the regular epithelium. Loss of MUC4 expression along with an intensification in tumor scoring is observed. Given that the differential expression of MUC4 is observed with higher expression in grade 1 tumors in contrast to grade 3, so it provides supportive evidence to incorporate it as a marker for tumor cell differentiation.

Strength of Study

Given the differential expression of MUC4, it provides supportive evidence to incorporate it as a marker for tumor cell differentiation.

Limitations of Study

The study was conducted on a smaller scale with less sample size. Further studies should be conducted with larger sample sizes including a larger number and a wider spectrum of participants from different hospitals in various areas of the country.

Future Recommendations

MUC4 can demonstrate to be a beneficial diagnostic and prognostic marker. Furthermore, additional epidemiological studies should be carried out in the future on a greater scale to extend these discoveries and find a correlation between MUC4 expression with tumor stage, treatment modalities, and patient outcome.

REFERENCES

1. Bansil R, Turner BS. Mucin structure, aggregation, physiological functions and biomedical applications. Current opinion in colloid & interface science. 2006 Jun 1;11(2-3):164-70.
2. Pinzón Martín S, Seeberger PH, Varón Silva D. Mucins and pathogenic mucin-like molecules are immunomodulators during infection and targets for diagnostics and vaccines. Frontiers in chemistry. 2019 Oct 22;7:710.
3. Krishn SR. 2016.Secretory mucin MUC5AC in gastrointestinal malignancies.*Doctoral Dissertations*.University Of Nebraska Medical Center.Digtal Commons database.
4. Kufe DW. Mucins in cancer: function, prognosis and therapy. Nature Reviews Cancer. 2009 Dec;9(12):874-85.
5. Hollingsworth MA, Swanson BJ. Mucins in cancer: protection and control of the cell surface. Nature Reviews Cancer. 2004 Jan;4(1):45-60.
6. Chakraborty S, Jain M, Sasson AR, Batra SK. MUC4 as a diagnostic marker in cancer. Expert opinion on medical diagnostics. 2008 Aug 1;2(8):891-910.
7. Singh AP, Chaturvedi P, Batra SK. Emerging roles of MUC4 in cancer: a novel target for diagnosis and therapy. Cancer research. 2007 Jan 15;67(2):433-6.
8. Hamada T, Wakamatsu T, Miyahara M, Nagata S, Nomura M, Kamikawa Y, Yamada N, Batra SK, Yonezawa S, Sugihara K. MUC4: a novel prognostic factor of oral squamous cell carcinoma. International journal of cancer. 2012 Apr 15;130(8):1768-76.
9. Macha MA, Rachagani S, Pai P, Gupta S, Lydiatt WM, Smith RB, Johansson SL, Lele SM, Kakar SS, Lee JH, Meza J. MUC4 regulates cellular senescence in head and neck squamous cell carcinoma through p16/Rb pathway. Oncogene. 2015 Mar;34(13):1698-708.
10. Narashiman S, Narasimhan M, Venkatraman G. Expression of Mucin 4 in leukoplakia and oral squamous cell carcinoma: An immunohistochemical study. Journal of oral and maxillofacial pathology: JOMFP. 2014 Jan;18(1):25.
11. Kohli M, Shivam AK, Ahuja P, Dutta J. Mucin-4: A novel marker for oral cancer. Journal of Oral and Maxillofacial Pathology: JOMFP. 2019 Jan;23(1):49.
12. Rathee R, Devi A, Narwal A, Kamboj M, Singh S. Immunohistochemical Coexpression of MUC1 and MUC4 in Oral Leukoplakia and Oral Squamous Cell Carcinoma. Head and Neck Pathology. 2021 Sep;15(3):831-42.
13. Almangush A, Mäkitie AA, Triantafyllou A, de Bree R, Strojan P, Rinaldo A, Hernandez-Prera JC, Suárez C, Kowalski LP, Ferlito A, Leivo I. Staging and grading of oral squamous cell carcinoma: An update. Oral oncology. 2020 Aug 1;107:104799.
14. Kwon KY, Ro JY, Singhal N, Killen DE, Sienko A, Allen TC, Zander DS, Barrios R, Haque A, Cagle PT. MUC4 Expression in Non–Small Cell Lung Carcinomas: Relationship to Tumor Histology and Patient Survival. Archives of pathology & laboratory medicine. 2007 Apr;131(4):593-8.
15. Bray F, Ferlay J, Soerjomataram I, Siegel RL, Torre LA, Jemal A. Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. CA: a cancer journal for clinicians. 2018 Nov;68(6):394-424.
16. Anwar N, Pervez S, Chundriger Q, Awan S, Moatter T, Ali TS. Oral cancer: Clinicopathological features and associated risk factors in a high risk population presenting to a major tertiary care center in Pakistan. Plos one. 2020 Aug 6;15(8):e0236359.
17. Qureshi MA, Syed SA, Sharafat S. Lip and oral cavity cancers (C00-C06) from a mega city of Pakistan: Ten-year data from the Dow Cancer Registry. Journal of Taibah University Medical Sciences. 2021 Aug 1;16(4):624-7.
18. Malkani N, Kazmi S, Rashid MU. Epidemiological Assessment of Oral Cancer Burden in Pakistan. Cancer Investigation. 2021 Nov 26;39(10):842-53.
19. Khaleel ME, Raza A, Ehsan A, Masood R, Javed M. Clinicopathological spectrum of oral squamous cell carcinoma at a public sector health facility. Biomedica. 2015;31(1):21-6.
20. Guillem P, Billeret V, Buisine MP, Flejou JF, Lecomte-Houcke M, Degand P, Aubert JP, Triboulet JP, Porchet N. Mucin gene expression and cell differentiation in human normal, premalignant and malignant esophagus. International journal of cancer. 2000 Dec 15;88(6):856-61.
21. Kaur S, Momi N, Chakraborty S, Wagner DG, Horn AJ, Lele SM, Theodorescu D, Batra SK. Altered expression of transmembrane mucins, MUC1 and MUC4, in bladder cancer: pathological implications in diagnosis. PloS one.

2014 Mar 26;9(3):e92742.

22. Kathpalia VP, Mussak EN, Chow SS, Lam PH, Skelley N, Time M, Markelewicz Jr RJ, Kanduc D, Lomas L, Xiang Z, Sinha AA. Genome-wide transcriptional profiling in human squamous cell carcinoma of the skin identifies unique tumor-associated signatures. The Journal of dermatology. 2006 May;33(5):309-18.
23. Macha MA, Rachagani S, Pai P, Gupta S, Lydiatt WM, Smith RB, Johansson SL, Lele SM, Kakar SS, Lee JH, Meza J. MUC4 regulates cellular senescence in head and neck squamous cell carcinoma through p16/Rb pathway. Oncogene. 2015 Mar;34(13):1698-708.

CONFLICT OF INTEREST

Authors declared no conflicts of Interest.

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DATA SHARING STATMENT

The data that support the findings of this study are available from the corresponding author upon request.