DOI: https://doi.org/10.47391/JPMA.217

Immunohistochemical expression of MUC4 in different grades of Head and Neck Squamous cell carcinoma

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Abstract

Objective: To determine immunohistochemical expression of Mucin 4 in head and neck squamous cell carcinoma and its different histological grades among patients reporting to various tertiary care hospitals in an urban setting.

Method: The descriptive study was conducted at the Department of Oral Pathology / Morbid Anatomy and Histopathology, University of Health Sciences, Lahore, Pakistan, from January to July 2017, and comprised cases of head and neck squamous cell carcinoma. Histological diagnosis and grading was done for each case. Haematoxylin and eosin stain followed by immunohistochemistry was done. Relation of Mucin 4 expression with tumour types was explored. SPSS 20 was used for statistical analysis.

Result: Of the 63 samples, 40(63.5%) were from male patients. The overall mean age of the patients was 53±3.77 years. Mucin 4 expression was positive in 47(74.6%) cases. Of them, 16(34%) had grade 1 tumour, 28(59.6%) had grade 2 and 3(6.4%) had grade 3 tumour. tumourThere was a significant relation (p=0.03) between tumour grades and intensity of Mucin 4 expression. tumour

Conclusion: Upregulation of Mucin 4in tumour tissue with no expression in normal epithelium was found, and loss of Mucin 4 expression with increase in tumour grade was noted.

Key Words: Mucin 4, MUC4, Squamous cell carcinoma of head and neck, HNSCC, Immunohistochemistry.

Introduction

Head and neck squamous cell carcinoma (HNSCC) constitutes up to 90% of cases among all cancers affecting the head and neck region.[1] It is the 6th leading cancer in the world with overall high global incidence and mortality causing over 550,000 new cases and around 300,000 deaths each year.[2] In Pakistan, collective data report of cancer registry 1994-2013 by Shaukat Khanum Memorial Cancer Hospital and Research Centre (SKMCH&RC) has ranked HNSCC to be the third most common tumour in all age groups and the second most common in adults affecting both genders equally.[3] Also, four years (2004-2008) of aggregated data from five leading cancer hospitals of Pakistan has revealed HNSCC to be the second most common cancer, accounting for 9.9% of all cases.[4]

The overall high incidence and mortality rate can be attributed to incomplete understanding of molecular pathways and lack of reliable and validated biomarkers that predict the early diagnosis and prognosis of this alarming disease. Though molecular characterisation of this disease has facilitated the understanding of the molecular mechanisms contributing to the development of HNSCC and recognition of different molecular biomarkers, like p53, hypoxia-induced factors, interleukins (ILs), melanoma-associated antigen (MAGE), microsatellite instability (MSI), matrix metalloproteinases (MMPs), among others, but there are issues of specificity, sensitivity and clinical validation with some of these biomarkers.[5] Therefore, extrication of cellular pathways involved pathogenesis of HNSCC

and searching of new diagnostic and prognostic biomarkers is still the need of the hour.[6]

Mucins are glycosylated proteins expressed by various epithelial structures and involved in distinct functions, such as cell differentiation, cell adhesion and cell signalling. Changes in their glycosylation pattern are associated with development and progression of malignant diseases, and, therefore, mucins are analysed as potential markers for diagnosis and progression of epithelial malignancies.[7] Mucin 4 (MUC4), a trans-membrane mucin, has recently appeared as a useful biomarker. It plays its role in tumour progression indirectly through anti-adhesion mechanism or directly through ErbB2(Receptor Tyrosine Kinase 2) signalling pathway.[8] Variation in its expression and glycosylation has been reported in various epithelial malignancies, like breast, lung, pancreas, oesophagus and cervix.[9]

MUC4 is also localised in head and neck squamous epithelium and its role in malignant transformation is being researched. Three studies have so far investigated the role of MUC4 in carcinogenesis of squamous cell carcinoma (SCC); one in Japan[10] in which 61(40.6%) cases showed over-expression of MUC4, one in India[11] in which MUC4 over-expression was observed in 14(70%) cases, and the third in the United States[12] which reported that knockdown of MUC4 gene inhibited cell proliferation both in vitro and in vivo through induction of senescence programming pathways.

The over-expression varied in the three studies, highlighting variations among different populations. Further studies need to be carried out in high prevalence populations so as to add to the literature and validate previous findings. The current study was planned to determine the immunohistochemical (IHC) expression of MUC4 in HNSCC and its different histological grades among patients reporting to various tertiary care hospitals of an urban centre.

Materials and Methods

The descriptive study was conducted at the Department of Oral Pathology / Morbid Anatomy and Histopathology, University of Health Sciences (UHS), Lahore, Pakistan, from January to July 2017. The ethical approval of the study was taken by Ethical Review Committee of University of Health Sciences.

The sample size was calculated based on anticipated expression of MUC4 to be 78%[12] with a confidence level of 95% and a finite population of 80 HNSCC cases per year.

Formalin-embedded paraffin (FFPE) blocks of patients with diagnosis of primary HNSCC regardless of gender and age were obtained from UHS, Postgraduate Medical Institute (PMI), Lahore, and Sheikh Zayed Hospital, Lahore. Damaged blocks with insufficient clinical data and inadequate tissue sample were excluded. Age and gender of the patients along with the site of tumour involvement were recorded.

Tissue sections of 4µm thickness were cut from the blocks and taken on to the frosted glass slides. The sections were processed for haematoxylin and eosin (H&E) staining. After the confirmation of histological diagnosis the tissues were graded according to Bryne's histological grading criteria.[13]

IHC was done according to the protocol described by Bancroft and Gamble.[14] Briefly, 4µm thick tissue sections were cut with the help of rotary microtome and taken on Poly-L-lysine-coated slides for IHC staining with anti-MUC4 8G7 (abcam) antibody. Tissue sections of HNSCC along with positive control in the shape of pancreatic cancer tissue were taken on glass slides. The sections were dried at 60^{0}Ċ for 50 minutes in a hot-air oven followed by de-waxing in xylene and rehydration in graded alcohol. The slides were placed in Coplin jar along with antigen retrieval citrate solution which was prepared by dissolving 2.94g of sodium citrate in 1000ml of distilled water; potential of hydrogen (pH) was adjusted at 6.0 and 250µl of Tween 20 was added for final use. The jars were then placed in hot-water bath for 30 minutes at 95ºC. Slides were allowed to

cool and evaporative losses were replaced by fresh phosphate buffered saline (PBS). Endogenous peroxidase activity was blocked by incubating slides with1-2 drops of hydrogen peroxide (H_2O_2) for 15 mins followed by PBS washes. Next, 1-2 drops of protein blocker were put on the slide and incubated for 10 minutes. Again, thorough washes with PBS were done. Slides were then incubated with primary antibody, anti-MUC4 antibody (code ab52263; Abcam, USA) which was diluted to the concentration of 5µg/ml, as suggested by the manufacturer, for 2 hours. Next, 1-2 drops of yellow-coloured biotinylated secondary antibody reagent were placed for 30 minutes followed by 1-2 drops of red coloured streptavidin peroxidase reagent for 10min. After being washed 3 times with PBS, diaminobenzidine (DAB) was allowed to react with tissue sections for 10 min for visualisation, and the slides were rinsed with tap water counterstained by haematoxylin and mounted using dibutylphthalate polystyrene xylene (DPX). Human pancreatic cancer tissue was taken as positive control, while omitting the primary antibody step in peroxidase-labelled streptavidin-biotin technique provided the negative control for MUC4.

MUC4 expression was evaluated on the basis of extent and intensity of immune-labelling in tumour cell membrane and cytoplasm. Total score (TS) for each case was calculated by adding the proportion score (PS) and intensity score (IS).[15]

Data was analysed using SPSS 20. Age was presented as mean ± standard deviation (SD). Gender distribution, tumour grades, morphological parameters, MUC4 staining intensity, proportion and TS were presented as frequencies and percentages. Association between MUC4 expression and clinicopathological variables were computed using Chi square test of independence, and $p \leq 0.05$ was taken as significant. Multinomial regression was applied to assess the role of tumour grade in MUC4 staining pattern and to assess the odds of staining in relevance to grades of tumour.

Results

Of the 63 samples, 40(63.5%) were from male patients. The overall mean age of the patients was 53±3.77 years. MUC4 expression was positive in 47(74.6%) cases. Of them, 16(34%) had grade 1 tumour, 28(59.6%) had grade 2 and 3(6.4%) had grade 3 tumour. Maximum cases were from the oral cavity followed by neck, face and head (Table).

IHC with MUC4 was performed on all the 63(100%) cases, and staining reaction in different grades of HNSCC were noted (Figures 1-2).

Association of MUC4 expression with age, gender and tumour site was non-significant ($p>0.05$). Significant association was observed between IS and tumour grade ($p<0.05$), indicating a decrease in intensity with increasing grade. A significant inverse association was found between TS and tumour grade ($p<0.05$), indicating a loss of MUC4 expression with increasing tumour grade.

By taking grade 3 tumours as reference, there was significant difference in staining pattern of grade 1 (odds ratio [OR] = 3.9; 95% confidence interval [CI] = 1.074-6.723; p=0.007) and grade 2 (OR=3.1; 95%CI=0.418-5.782p=0.023) tumours, meaning that all grade 1 HNSCCs were approximately 4 times more likely to be stained with MUC4 antibody compared to grade 3 tumours. In case of grade 2, CI was <1 so the odds were slightly weaker for a significant difference between MUC4 staining patterns of grade 2 and grade 3 HNSCCs ($p>0.05$).

Discussion

Mucins have been considered potential biomarkers in cancer prognosis due to their unique expression in cancer patients compared to healthy individuals. Among them, the role of MUC4 in carcinogenesis has been proved by its aberrant expression in various tumours. In the present study, 75% HNSCCs showed positive MUC4 expression with almost no expression in attached benign stratified squamous epithelium. Expression was mainly cytoplasmic with

maximum cases showing moderate intensity and >66% stained tumour cells. In some cases, MUC4 expression in adjacent dysplastic epithelium was also noted. These results are in line with Macha et al. and Narashiman et al. who reported positive expression in 78% and 70% of HNSCC tissue samples.[11, 12] However, the current results are different from Hamada et al.[10] who found MUC4 positivity in 40% HNSCC samples . This can be due to the difference in population targeted as in our study and in the study carried out by Narashiman et al.[11], southeast Asian population was targeted where there is high incidence of HNSCC, while Hamada et al.[10] targeted northeast Asian population of Japan with comparatively low tumour incidence.[16]

In the current study, no association was observed between age and MUC4 expression. Comparable results were seen in earlier studies.[10,12] On the contrary, a study carried in the USA on cutaneous SCC observed a significant relation between MUC4 expression and age of the patients, with MUC4-positive patients being older than MUC4-negative patients, suggesting that longer years of sun exposure may induce MUC4 in subset of cutaneous SCC.[17] Different studies conducted in Japan, USA and India showed that MUC4 expression had no association with gender[10-12] and the findings of the current study matched that result. MUC4 expression was also independent of site involved by HNSCC as change in site did not have any effect on molecular pathogenesis of HNSCC. Likewise, a USA study[12] also did not find any association between the two. Even when oral cases of SCC were analysed separately, no association was seen. This was in concordance with results of a study done in Japan[10] on cases of oral squamous cell carcinoma (OSCC). On the contrary, a study in India observed significant site-dependent MUC4 positivity[11]. This difference can be explained by the fact that sites compared included not only the ones affected by OSCC, but also the sites presenting with leukoplakia whoch could confound the results.

To our knowledge, the current study is the first to have compared MUC4 expression in different grades of HNSCC. When the pattern of intensity, proportion of positively stained cells and degree of positivity were observed with biomarker, tumour grades were seen to be significantly associated with MUC4 expression, showing a decrease in expression with increase in grade. Grade 1 exhibited moderate to strong positivity for MUC4, grade 2 exhibited weak to moderate positivity, and grade 3 showed only weak positivity. These findings were in concordance with studies done in cutaneous SCC and OSCC.[11, 17] They reported a strong to moderate expression in well-differentiated and moderately differentiated SCC, and weak positive expression in poorly differentiated SCC. When tumour grade association with positive and negative expression of MUC4 was evaluated, no significant relation was found in the current study. This finding was similar to earlier results [10, 12].

A decrease in MUC4 expression in moderately and poorly differentiated SCC may be attributed to loss of differentiation of squamous cells compared to well-differentiated SCC. Previous studies on the role of MUC4 in SCC have shown its relationship with tumour differentiation[18,19]. There is no universal scoring system to analyse IHC data, combinative semi-quantitative approach like Allred score, Immunoreactive score and H-score are considered to be gold standards for IHC estimation and data presentation although individual scoring systems for particular IHC marker may be the best viable way to answer the special scientific question[20] Proportion of positively stained cells in our samples did not show any association with different grades of SCC.

Hamada et al. took >5% immunopositive cells as a criterion of MUC4 positive cases, and Narashiman et al. also analysed the data on the basis of proportion of immunopositive cells only[10, 11] Proportion scores help quantify the data, but taking into account only the proportion scores (PS), results due to lack of information regarding subtle differences in protein expression level are evaluated as intensity score.[21] Both of the studies[10,11] did not compare the PS

with histological tumour grades. While in the current study, Allred scoring approach was used and significant relation was seen between TS and tumour grades. Also, IS when compared separately with tumour grades gave significant results. Macha et al. used a combined scoring system but did not compare IS and PS individually with tumour grades. [12] To the best of our knowledge, there is no published data regarding comparison of different grades of HNSCC with MUC4 intensity and proportion.

Modifying the scoring system for this particular marker and taking into account only the IS may be sufficient as a reliable prediction of MUC4 expression as this alone showed decrease in expression with increasing tumour grade.

Conclusion

Upregulation of MUC4 in tumour tissue with no expression in normal epithelium was found, and loss of MUC4 expression with increase in tumour grade was noted. There was evidence to suggest the need to include MUC4 as a marker for tumour cell differentiation.

Disclaimer: None.

Conflict of interest: None.

Source of Funding: University of Health Sciences, Lahore, and the Pakistan Health Research Council (PHRC).

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Table: Intensity, proportion and total scores of Mucin 4 (MUC4) staining among Bryne's grades of Head and neck squamous cell carcinoma (HNSCC).

Age	MUC4 EXPRESSION (n=63)		
	Negative	**Positive**	**Level of significance (p-value)**
21-40 years	2(12.5%)	10 (21.3%)	*p*=0.43
41-60 years	11(68.8%)	24 (51.1%)	
61-80 years	3(18.8%)	13(27.7%)	
Total	16(100%)	47(100%)	
Gender			
Male	12 (75%)	28 (59.6%)	*p=0.61*
Female	4 (25%)	19 (40.4%)	
Total	16(100%)	47(100%)	
Site			
Head	0 (0%)	1 (2.1%)	*p =0.16*
Face	1(6.2%)	2(4.3%)	
Oral cavity	13(81.2%)	32(68.1%)	
Neck	2(12.5%)	12(25.5%)	
Total	16(100%)	47(100%)	
Grade			
Grade 1	3(18.8%)	16(34%)	*p=0.06*
Grade 2	9(56.2%)	28(59.6%)	
Grade 3	4(25%)	3(6.4%)	
Total	16(100%)	47(100%)	

p-value (level of significance)

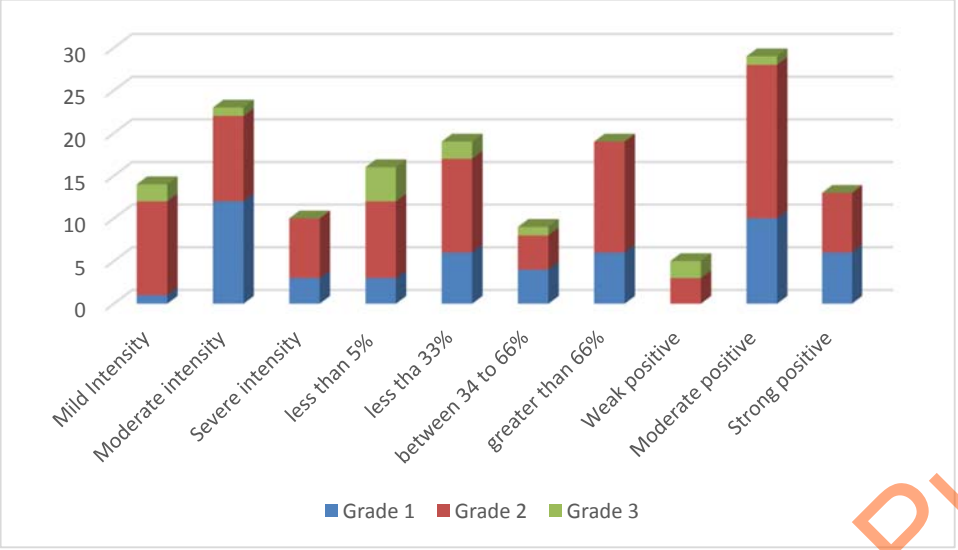

Figure 1: Intensity (mild, moderate, severe) proportion (<5%, <33%,34-66%,>66%) and total scores (weak positive, moderate positive, strong positive) of Mucin 4 (MUC4) staining among Bryne's grades of **Head and neck squamous cell carcinoma (**HNSCC).

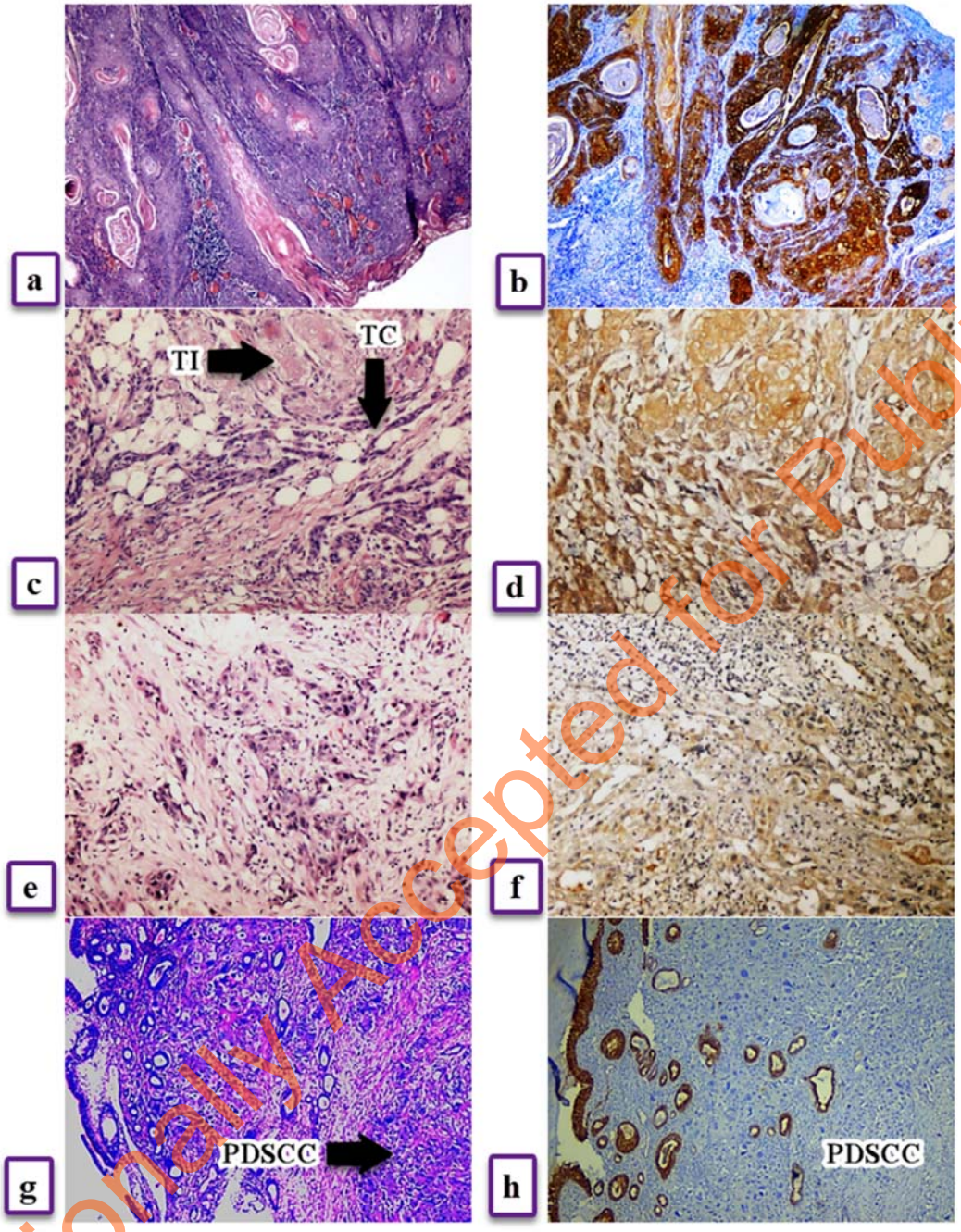

Figure 2: Photomicrographs of different grades of Head and Neck Squamous cell carcinoma (HNSCC) a) Well differentiated Squamous cell carcinoma (Grade1). (H&E;10x10X) (b) Strong cytoplasmic expression in grade 1(MUC4 IHC;10x10X) c) Moderately differentiated HNSCC (Grade 2) with tumor islands (TI) and tumor cords (TC) infiltrating into adipose tissue(AT) (H&E;10x10X) (d) Moderately intense cytoplasmic expression in grade 2 (MUC4 IHC;10x10X) (e) Poorly differentiated squamous cell carcinoma (Grade 3) (H&E;10x10X) (f) Mild cytoplasmic expression in grade 3. (MUC4 IHC; 10x10X) (g) Poorly differentiated squamous cell carcinoma (Grade 3) (H&E;10 X) (h) Strong cytoplasmic expression in pseudostratified ciliated columnar epithelium of nasal cavity (E) due presence of mucus in goblet cells with no expression in poorly differentiated squamous cell carcinoma (PDSCC) (MUC4 IHC; 10x10X).

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