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RESEARCH ARTICLE

COMPARISON OF GLYCOPROTEIN LEVELS IN ORAL CARCINOMA BY SPECTROPHOTOMETRIC ESTIMATION USING HISTOCHEMICAL STAINS

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ABSTRACT

Histological assessment of surgical margins as negative lacks sensitivity in identifying cells with a cancerous genotype but no pathologic phenotype. Aberrant glycosylation in tumor cells results in loss of cell surface glycoprotein and sialic acid. Studies have shown a modified expression of such tumor-related genes and cell molecules in histologically negative margins. 20 cases of margins and its corresponding lesional tissue were stained using Periodic-acid Schiff's (PAS) reagent and Alcian Blue (AB) and their optical densities and stain remaining per micro-gram of the tissue were analyzed using spectrophotometer. T-test showed a significant correlation (p=0.06) between the absolute PAS stain and AB stain remaining per micro-gram of the tissue. Non-parametric Mann-Whitney U test showed that the difference in PAS and AB staining was found to be significantly associated with males (p=0.01), moderate to poor histological differentiation of cells (p=0.012) and positive lymph nodes (p=0.004). No significant difference was seen between the clinic-pathological parameters and amount of stain remaining. Thus, the routinely used histochemical stains can be used as an adjunct to predict the prognosis and diagnosis of oral carcinomas.

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INTRODUCTION

Tumor markers are naturally occurring or modified molecules which can be measured in serum, plasma, and other body fluids like saliva and tissue sample (Vora *et al.,* 2012). The components of cell and tissue like carbohydrates, proteins and lipids are being studied by various authors. Glycoproteins are protein-carbohydrate complexes found on the outer surface of cell membrane, the terminal epitopes of which contain amino sugars and hexoses, these play an important role in cell-cell interactions, cell adhesion and malignant transformation; serving as differentiation and developmental markers (Taneja *et al.*, 2009; Rajpura *et al.*, 2005; Dabelsteen *et al.*, 1991). The expression of these carbohydrate antigens are significantly altered in tumors, particularly those arising from epithelial tissues by aberrant glycosylations resulting in increased branching sites for incorporation of sialic acids (Rajpura *et al.*, 2005; Dabelsteen *et al.*, 1991). Identification and monitoring of these intermediate markers allow clinicians to measure increased risk for development of cancer. These markers are useful for the purpose of screening, prognosis, diagnosis and even to treat early stages of oral carcinogenesis through potentially malignant diseases (Vora *et al.,* 2012; Hemalatha *et al.,* 2013). Elevated levels of serum sialic acid and glycoproteins have been reported in various lesions like potentially malignant disorders and oral cancer, melanoma, colorectal cancer, etc using various methods (Taneja *et al.*, 2009). Quantitative estimation of the glycoprotein tropins of pituitary hormones has been done using spectrophotometry (Fand and Thorell, 1962). Periodic acid-Schiff's reagent and Alcian Blue are routinely used histochemical stains for demonstration of glycoproteins and sialic acid. In light of the previous studies, we aim to demonstrate and quantify the role of Sialic acid present on the epithelial cell membrane glycoporoteins as potential diagnostic marker for oral cancer by using routine histochemical stain.

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MATERIALS AND METHODS

Source of data: This retrospective study involved a sample size comprising of histopathologically diagnosed biopsy tissue

specimen of 20 cases of radical neck dissection (RND), from the archives of Department of Oral Pathology were selected by random sampling.

Method of collection of data

Lesional tissue proper (LP) and negative margin (M), from each case of RND were selected as a study group. The study samples consisted of primary cases of OSCC, with an exception of one recurrent case. The surgical margins comprised of apparently normal mucosa clinically, and reported as 'histologically negative' after microscopic examination. The lesional tissue proper and negative margins, which did not contain (or contained minimal) muscle tissue and mucous salivary acini were preferred over those containing them to minimize the error of excess stain uptake.

Method of performing the study

A slide for each sample of lesion proper and its margin of a RND were taken for Periodic acid-Schiff's (PAS) and Alcian Blue [8GX, Certified, HIMEDIA] (AB) staining. Prior to staining, clean glass slide used for each tissue sample was weighed using an electronic weighing scale and the weight was noted for each slide without the tissue (W_0). The tissue was then sectioned and taken on the corresponding weighed slide. Slides were kept on the slide warmer for 10 minutes, followed by immersing it in xylene for another 10 minutes and allowed to air dry. After complete evaporation of xylene, the slides containing the tissue sections were weighed again (W_1). On subtracting the two measured weights (W_1-W_0), the weight of the tissue (W_t) on the slide could be achieved. The slides were then immersed in absolute alcohol for 5 minutes and washed with distilled water to carry out further staining procedure. Polypropylene tray of microscope slide was used for staining individual slides. For PAS staining, each slide of the study group was immersed in periodic acid for 5 minutes, followed by distilled water wash, further followed by layering of Schiff's reagent (3ml) on the slide for 20 minutes using micro-pipettes (1000 micro-liter). After the completion of procedure, stains were collected in test-tubes using the same micro-pipettes and their optical density was measured at wavelength 610nm on spectrophotometer Shimadzu UV 1700 immediately. Since freshly prepared Schiff's reagent is near to colorless, the optical density (OD_{PAS}) of the stain remaining after the completion of staining procedure was found to be in the range of 0.004 – 0.020. This observed value of optical density was divided by the calculated value of the tissue weight (W_t) to estimate the stain remaining after stain uptake by per micro-gram of the tissue ($S_{PAS\ -\ LP}$ and $S_{PAS\ -\ M}$). For Alcian Blue staining, 30mg percent stain at 2.5pH was layered over the slide for 15 minutes after the completion of the above mentioned initial steps. Similar to PAS stain, the optical density (OD_{AB}) of the stain remaining was measured at 650nm after completion of the procedure. The observed values were found to be in the range of 0.8-1.2 for lesion proper and margins. Similar to the above mentioned, the optical density value was divided by the calculated weight of tissue (W_t) to estimate the stain remaining after stain uptake by per micro-gram of tissue ($S_{AB\ -\ LP}$ and $S_{AB\ -\ M}$). The difference between the stain remaining after staining of lesion proper and margins for PAS and AB was recorded by subtracting the $S_{PAS\ (AB)\ -\ Lesion\ proper}$ from $S_{PAS\ (AB)\ -\ Margin}$ of the same sample. Based on the behavior of the result obtained from study and control group, values were assessed by Mann-Whitney U non-parametric tests, T-test and one-way ANOVA test.

RESULTS

The clinical and pathological data of the 20 study samples of this study are presented in Table 1. The demographic data of the present study sample consisted of predominantly males except 4 cases of females with majority samples falling in the age group more than 45 years with 5 cases occurring in patients with less than 45 years of age. Tobacco habit, either in the form of chewing or smoking or a combination of the two, was present in 12 patients, while 3 samples had the habit of betel quid chewing, either solely or in combination with tobacco chewing. 1 patient had the habit of alcohol drinking alone and the remaining 4 patients denied any habit history. The site of the tumor involved the buccal mucosa alone or with involvement of one or more of its surrounding tissues of retromolar trigone, gingivo-buccal sulcus, alveolus and the lip. 3 cases showed involvement of posterior mandible, tongue (recurrence case) and retromolar trigone region alone. All the lesions showed an exophytic type of growth pattern except for 8 cases showing an endophytic growth. On histological examination, except for 7 cases, all cases were graded as well-differentiated squamous cell carcinomas. The surgical margins were reported as negative by microscopic examination, except case 13 and case 20 with positive surgical margins. 7 cases showed lymph node positivity, while only 1 case showed submandibular gland involvement with 1 case showing involvement of submandibular as well as parotid gland.

Table 1 shows that in the same study sample, the S_{LP} and S_M show the difference in stain remaining with respect to PAS as well as AB in spite of subjecting both the tissues to stains for an equal amount of time. Table 2 shows that in spite of a larger tissue volume of lesion proper as compared to the margins, 8 cases showed lesser PAS uptake by lesional tissue as compared to margins while the same observation was made in 7 cases stained by AB. The details of the values of stain (PAS and AB) remaining per micro-gram of the tissue (lesion proper as well as margin) have been tabulated in Table 2. Their difference calculated after the completion of the procedure is obtained by subtracting the stain remaining of lesion tissue from the stain remaining of margin (assuming that the margins are normal with no changes at the molecular level i.e. the levels of glycoproteins are normal as compared to the levels of glycoproteins in the lesional tissue, which may be lost due to various processes as mentioned in the discussion). The more amount of stain remaining indicates that the particular tissue has taken up less stain and vice-versa.

Example in Case 5:

$S_{PAS\ -\ LP}$ = 0.638297872, $S_{PAS\ -\ M}$ = 0.531914894; this suggests that the amount of stain remaining is more for lesional tissue than the margin and therefore, stain up take by per micro-gram of the lesional tissue is less as compared to the margin.
$(S_{PAS\ -\ M}) - (S_{PAS\ -\ LP})$ = - 0.106382979 , where the negative sign symbolizes that lesional tissue has taken up less stain compared to the margin for the same study sample.

Table 1. Represents the clinic-pathological parameters and the staining differences (PAS and AB) between lesional tissue and margins of 20 cases

CASE No.	AGE/G	HABIT- TYPE / FREQUENCY / DURATION	SITE	TYPE OF LESION	GRADE	MARGINS	LYMPH NODE	DIFFERENCE BETWEEN STAIN REMAINING (PAS)	DIFFERENCE BETWEEN STAIN REMAINING (AB)
1.	70/M	TC- 5 to 6 times/day/7-8 yrs	BM	Exophytic	W-SCC	Negative	Negative	0.051593671	14.31492843
2.	54/M	TS- 8 to 10 times/day/10 yrs	RMT	Endophytic	M-SCC	Negative	Positive (IB)	0.187134503	6.134969325
3.	44/M	5-6 pkts/10 yrs	BM	Exophytic	W-SCC	Negative	Negative	-0.090725806	-1.142857143
4.	50/F	Nil	BM + Lip	Exophytic	W-SCC	Negative	Negative	-0.054945055	-5.142857143
5.	55/F	TC+BQ- 3 to 4 times/day/20 yrs	BM + RMT	Exophytic	W-SCC	Negative	Positive (IB, SBG)	-0.106382979	6.285714286
6.	51/M	TC- 3 to 4 times/day/15-20 yrs	BM + RMT	Endophytic	W-SCC	Negative	Negative	0.087470449	1.714285714
7.	38/M	Gutka 15 pkts/day/2 yrs	BM + GB	Endophytic	W-SCC	Negative	Negative	0.64821077	-3.428571429
8.	31/M	Alcohol	BM	Endophytic	M-SCC	Negative	Negative	0.155837929	24
9.	55/F	TC-10 times/day/15-17 yrs, TS-20/day/8 yrs	BM	Endophytic	SCC	Negative	Negative	0.034340659	-12
10.	75/M	Nil	RMT + alveolus + GB	Exophytic	M-SCC	Negative	No lymph node/ neck dissection	-0.088751496	2.857142857
11.	26/M	TC- 7-8 times a day/ 5 yrs	BM + GB	Exophytic	M-SCC	Negative	Positive (IA)	-1.279646937	-1.714285714
12.	70/M	Tobacco- 6-8 times a day/ 30 yrs	Posterior maxilla	Exophytic	W-SCC (Verrucous type)	Negative	Negative	-0.240305281	-7.428571429
13.	47/M	Nil	BM	Endophytic	M-SCC	Positive (Inferior -skin)	Positive (IA, IB, IIA, IIB, III, SBG, PG)	-0.263075478	1.142857143
14.	65/M	TC which he quit 1-1/2 months back	Recurrence tongue	Exophytic	W-SCC	Negative	Negative	-0.013157895	4
15.	43/M	TC- 7-8 times a day/10years	BM	Endophytic	W-SCC	Negative	Positive (IIA)	0.838095238	1.142857143
16.	49/M	BQ- 10-12 times a day/ 22 yrs	BM	Exophytic	W-SCC	Negative	Positive (I)	0.707142857	3.428571429
17.	49/M	TC- 10 packets/ day since 27 yrs	BM	Exophytic	W-SCC	Negative	Negative	1.481314433	9.714285714
18.	52/F	Nil	BM + GB	Exophytic	M-SCC	Negative	Negative	0.936734694	1.142857143
19.	50/M	Beedi 1pkt/day / 20 yrs	BM	Exophytic	W-SCC	Negative	Negative	1.005370569	-1.142857143
20.	65/M	BQ- 1pkt/day/25yrs	GB + RMT + Alveolus	Endophytic	W-SCC	Positive (Lingual margin)	Positive (III)	0.952427184	13.14285714

G= Gender; M= Male; F= Female; TC= Tobacco chewing; TS= Tobacco smoking; BQ= Betel quid; BM= Buccal mucosa; RMT= Retromolar trigone; GB= Gingivobuccal sulcus; W-SCC= Well differentiated SCC; M-SCC= Moderately differentiated SCC; SBG= Submandibular gland; PG= Parotid gland.

Table 2. Represents the staining details of PAS and AB of lesional tissue and margins of 20 cases

CASE No	TISSUE	STAIN (PAS) REMAINING PER MICRO-G OF TISSUE	STAIN (AB) REMAINING PER MICRO-G OF TISSUE	DIFFERENCE BETWEEN STAIN REMAINING (PAS)	DIFFERENCE BETWEEN STAIN REMAINING (AB)
1.	Lesion proper	0.510204082	- 16.87116564	0.051593671	14.31492843
	Margin	0.561797753	- 2.556237219		
2.	Lesion proper	0.444444444	11.24744376	0.187134503	6.134969325
	Margin	0.631578947	5.112474438		
3.	Lesion proper	0.520833333	13.71428571	0.090725806	1.142857143
	Margin	0.430107527	12.57142857		
4.	Lesion proper	0.714285714	17.71428571	- 0.054945055	- 5.142857143
	Margin	0.659340659	12.57142857		
5.	Lesion proper	0.638297872	6.857142857	- 0.106382979	6.285714286
	Margin	0.531914894	13.14285714		
6.	Lesion proper	0.444444444	13.71428571	0.087470449	1.714285714
	Margin	0.531914894	15.42857143		
7.	Lesion proper	0.003963143	10.85714286	0.64821077	- 3.428571429
	Margin	0.652173913	7.428571429		
8.	Lesion proper	0.309278351	11.42857143	0.155837929	24
	Margin	0.465116279	35.42857143		
9.	Lesion proper	0.625	16.57142857	0.034340659	-12
	Margin	0.659340659	4.571428571		
10.	Lesion proper	1.28440367	17.71428571	- 0.088751496	2.857142857
	Margin	1.195652174	20.57142857		
11.	Lesion proper	1.401869159	13.71428571	- 1.279646937	- 1.714285714
	Margin	0.122222222	12		
12.	Lesion proper	1.386138614	17.71428571	0.240305281	7.428571429
	Margin	1.145833333	10.28571429		
13.	Lesion proper	1.553398058	12	- 0.263075478	1.142857143
	Margin	1.290322581	13.14285714		
14.	Lesion proper	1.263157895	9.142857143	- 0.013157895	4
	Margin	1.25	13.14285714		
15.	Lesion proper	1.5625	12	0.838095238	1.142857143
	Margin	1.30952381	13.14285714		
16.	Lesion proper	2.02020202	23.42857143	0.707142857	3.428571429
	Margin	1.428571429	26.85714286		
17.	Lesion proper	1.32231405	14.28571429	1.481314433	9.714285714
	Margin	1.958762887	24		
18.	Lesion proper	1.960784314	21.71428571	0.936734694	1.142857143
	Margin	1.836734694	22.85714286		
19.	Lesion proper	1.826923077	18.85714286	1.005370569	- 1.142857143
	Margin	1.836734694	17.71428571		
20.	Lesion proper	1.834862385	13.71428571	0.952427184	13.14285714
	Margin	1.747572816	26.85714286		

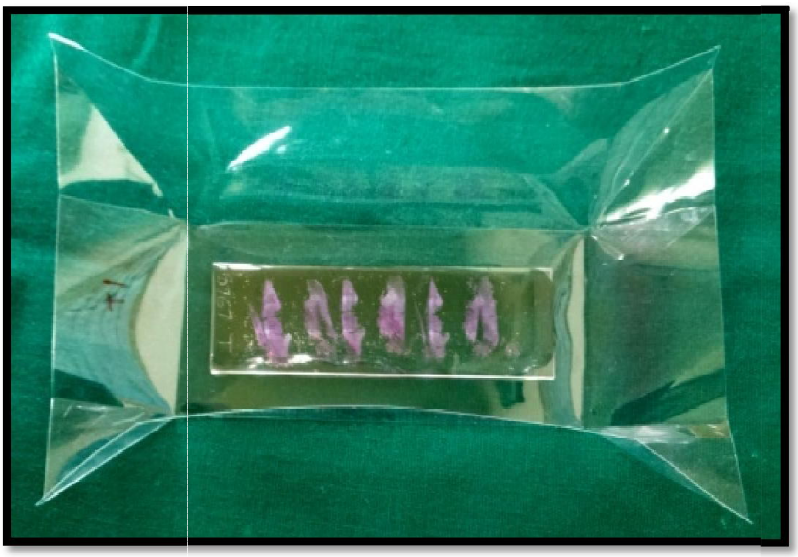

Fig. 1. Polypropylene tray containing slide stained by Periodic acid Schiff stain

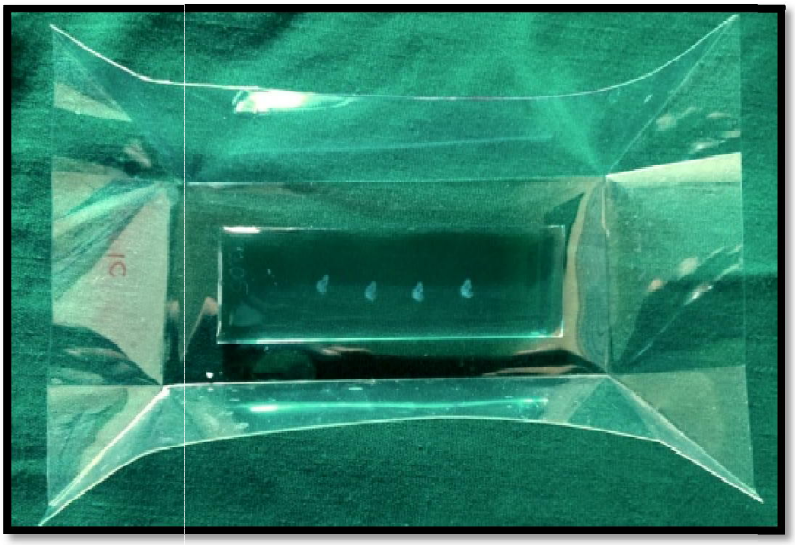

Fig. 2. Polypropylene tray containing slide stained by Alcian Blue stain

Fig. 3. Spectrophotometer Shimadzu UV 1700 giving the reading of optical density of stain remaining after completion of AB staining procedure as 0.837 at 650 nm wavelength

$S_{\underline{AB} - LP}$ = 6.857142857, $S_{\underline{AB} - M}$ = 13.14285714; these values indicate that the amount of stain remaining for margin is more and therefore, the uptake of stain by per micro-gram of margin is less when compared to the lesional tissue.

$(S_{AB - M}) - (S_{AB - LP})$ = 6.285714286, where the positive value denotes that margin has a lesser stain uptake compared to the lesional tissue for the same study same.

All the clinic-histopathological parameters and the observed and calculated values were subjected to the Shapiro-Wilk test of normality, and further subjected to non-parametric Mann-Whitney U test, T-test and one-way ANOVA test for statistical analysis of various parameters. Each clinic-pathological parameter was tested with the difference in the stain remaining per micro-gram of the tissue (PAS and AB) by non-parametric Mann-Whitney U test which showed no significance difference. T-test showed a significant correlation of p=0.06 between the absolute PAS stain and AB stain remaining per micro-gram of the tissue. Non-parametric Mann-Whitney U test showed that the difference in PAS and AB staining was found to be significantly associated with males (p=0.01), moderate to poor histological differentiation of cells (p=0.012) and positive lymph nodes (p=0.004).

DISCUSSION

The oral epithelium shows marked regional histological variations as well as in the synthesis of their cell surface molecules like proteins and carbohydrates (Dabelsteen *et al.,* 1991). These specific molecules confer them with certain characteristic functions that may modulate the immunological and inflammatory reactions (Dabelsteen *et al.,* 1991). The cell surface carbohydrates, present on all eukaryotic cells in the form of oligosaccharides, may be products of the cell itself or may comprise of glycoproteins and glycolipids secreted by other cells and gradually adsorbed on the cell surface (Dabelsteen *et al.,* 1991). These glycoconjugates contain the same principal monosaccharides in the form of hexoses (galactose, mannose), amino-sugars (glucose, glucosamine or galactosamine) or fucose, which are linked by different glycosidic linkages and different sequences (Dabelsteen *et al.,* 1991). Gene and protein expression as well as post-transitional modifications (PTMs) determine the complex glycoconjugate phenotype (Kuzmanov *et al.,* 2013). Glycoproteins, in contrast to glycolipids, contain multiple oligosaccharide chains resulting in an antigenic and heterogenous structure (Dabelsteen *et al.,* 1991; Hakomori, 1985). The extensive molecular microheterogeneity is the result of increased complexity of protein glycosylation process (Kuzmanov *et al.,* 2013). Phosphorylation and glycosylation modify the specific structural and functional molecules like growth factors, their receptors, cytoskeletal systems, transporters and surface membrane and pericellular components involved in cellular function (Hakomori, 1985). Phosphorylation modulates protein function directly, whereas glycosylation governs the conformation, localization and organization of functional and structural proteins (Hakomori, 1985). Glycosylation also plays an important role in protein folding and trafficking, cell-cell and cell-matrix interaction, cellular differentiation, fertilization and immune response (Kuzmanov *et al.,* 2013). These glycan structures vary based on differences in tissue, cell type and disease state (Kuzmanov *et al.,* 2013). Unlike DNA, RNA or protein synthesis which are template-based processes, glycosylation is based on (i) the balance achieved by the activity levels of the v-arious processing enzymes and the different glycan attachments (ii) their involvement in trimming (iii) addition of monosaccharides to it and (iv) on the availability of precursor monosaccharide molecules which are dependent on nutrient resources and expression of metabolic enzymes responsible for their synthesis and interconversion (Kuzmanov *et al.,* 2013).

The stages of development, differentiation and oncogenesis show changes in glycosylation as quickly and dramatically as the process of phosphorylation (Hakomori, 1985). Essentially, all carbohydrate chains undergo rapid, dramatic changes during development and differentiation in embryogenesis and pre-implantation as well as implantation changes (Hakomori, 1985). Thus, aberrant glycosylation in tumorigenesis may be representative of retrogenic expression of carbohydrate synthesis to a certain stage of embryogenesis and fetal development (Hakomori, 1985). The initial observation was made in 1969 which showed presence of high molecular weight membrane glycoproteins in transformed mouse fibroblasts compared to their normal counterpart progenitor cells, called the Warren-Blick-Buck phenomenon (Kuzmanov *et al.,* 2013). Thus, a common characteristic of oncologic malignancies were established to be aberrant glycosylations (Kuzmanov *et al.,* 2013).

Approximately, over a hundred specific glycosyltransferases catalyze the synthesis of carbohydrates by a stepwise chain elongation (Dabelsteen *et al.,* 1991). These glycosyltransferases are highly specific with respect to sugar donor, substrate and changes in their degree of activation (Dabelsteen and Clausen, 1987). Thus, the availability of substrate, donor and their organization results in changes in glycosylation and hence, in the expression of carbohydrate antigen (Dabelsteen and Clausen, 1987). The disturbances in the expression and activity levels of different glycosyltransferases and glycosidases along the secretory pathway in the endoplasmic reticulum and golgi apparatus of cancer cells most often arise the changes in glycan structures in tumors (Kuzmanov *et al.,* 2013). Aberrant glycosylation may occur due to either i) the suppression of the glycosyltransferases resulting in an incomplete or blocked synthesis and thus, causing accumulation of the precursor molecules or ii) neosynthesis of the tumor-associated carbohydrate marker through activation of new glycosyltransferase that is characteristic of a specific type of a tumor cell (Hakomori, 1985). This increased activity or expression of transferases leads to an increased glycan branching and density of oligosaccharide chains of glycoproteins with an increased tumor growth and metastasis (Kuzmanov *et al.,* 2013). These modified glycoconjugates are found to be released in circulation from the surface of malignant cells through increased turnover, secretion and/or shedding processes (Baxi *et al.,* 1991). The distribution of the complex and heterogeneous structures are stable and reproducible under physiologic conditions (Kuzmanov *et al.,* 2013). As malignant transformation sets in, there in under-

expression, over-expression or neo-expression of glycan moieties resulting in disturbance of balance and thus expanding the degree of pre-existing microheterogeneity of individual proteins (Kuzmanov *et al.,* 2013).

Gene expression or metabolism in tumor tissues, have been found to change significantly before any histopathological manifestations, including the over-expression of oncogenes or the malfunction of cancer-related molecules (Li *et al.,* 2015). However, along with the tumor tissues, even the adjacent histologically negative margins have shown modified gene expression or metabolism (de Carvalho *et al.,* 2012; Li *et al.,* 2015). This can be attributed to the concept of field cancerization. Slaughter *et al.,* in 1953, observed that the dysplastic epithelium adjacent to invasive oral carcinomas accounted for the relatively increased high incidence of second primary tumors in patients treated with OSCC. Thus, field cancerization occurs due to prolonged and diffuse exposure of the entire area of exposed epithelium to carcinogenic insult in the form of tobacco products and alcohol, resulting in accumulation of genetic alterations of oncogenes and tumor suppressor genes, finally increasing the risk of development of malignant lesion (Hassan *et al.,* 2012). This results in formation of multiple primary tumors occurring either synchronously or metachronously (Hassan *et al.,* 2012). The second primary tumors are associated with a lower survival rate than the original tumor (Day *et al.*, 1999).

This survival rate is also determined by various prognostic factors such as the demographic factors, site and stage of the tumor, its grading, depth of tumor invasion, tumor thickness, nature of invasive front, involvement of the surgical margins and lymph node metastasis. The five-year survival rate of the patients has remained at approximately 55% for the past 30 years, local recurrence being one of the primary reasons for treatment failure (Li *et al.,* 2015). Although the adjacent tissues seem apparently normal, they are contiguous with tumors and could be precancerous tissue that is undergoing malignant progression (Li *et al.,* 2015). The genotypic alterations, without any phenotypic changes, have been proved by many authors using PCR and immunohistochemical studies (de Carvalho *et al.,* 2012; Nathan *et al.,* 2002; van Houten *et al.,* 2004; Goldenberg *et al.,* 2004; Li *et al.,* 2015) . Various tumor genes like *PTHLH, EPCAM, MMP9, LGALS, MET, P16, MGMT, TP53* and molecules like MMP9, p53, p21, eIF4E, telomerase activity have been studied in surgically negative margins to observe their molecular-margin positivity and thus, the identification of patients with a higher risk of developing second primary tumors as well as local recurrences (de Carvalho *et al.,* 2012; Nathan *et al.,* 2002; van Houten *et al.,* 2004; Goldenberg *et al.,* 2004; Li *et al.,* 2015; High *et al.,* 1996). The demographic factors like age and gender have a conflicting view on the prognosis of OSCC. Few authors believe that the patients above 45 years of age and female patients have poor prognosis (Massano *et al.,* 2006). Tobacco use in any form and betel quid is a potential risk factor for the development of oral cancer; use of tobacco with betel quid and/or alcohol has a synergistic effect (Massano *et al.,* 2006; Warnakulasuriya, 2014; Vargas-Ferreira *et al.,* 2012; Liu *et al.,* 2010; Shenoi *et al.,* 2015). An increased frequency and duration of habits have a poor prognosis (Garg *et al.,* 2013).

The anatomic site of oral cancer also influences the prognosis of patients, owing to its lymphatic and vascular regional variations. Thus, tongue and maxilla show a poor prognosis compared to the lips and buccal mucosa (Massona *et al.,* 2006). Also, the accessibility to the local extension of tumor, its evaluation and management affects the outcome of patient (Massona *et al.,* 2006). Authors are found to have a conflicting view regarding the prognosis of exophytic and endophytic lesions. Lower histologic differentiation of tumor cells is associated with poor prognosis (Massona *et al.,* 2006; Warnakulasuriya, 2014). Patients with nodal metastases show significantly lower survival rate than patients with disease-free nodes (Woolgar *et al.,* 1995; Massona *et al.,* 2006; Warnakulasuriya, 2014; Liu *et al.,* 2010).

When margins take up more stain than lesional tissue, does it indicate that

1. Margins are normal?
2. Margins are turning dysplastic?
3. Lesion is in an advanced stage?

When margins take up less stain,

1. Are margins turning neoplastic?
2. Lesion is an early stage?

On comparison of the difference between stain remaining (PAS and AB) with the dissimilar clinic-pathological parameters, we could make the following correlations-

Margins

Case 13 shows a conflicting stain uptake result such that an increased PAS uptake by margin, in contrast to a lesser AB uptake by the same margin was observed. On comparison with the clinic-pathological features, we observed that the patient was a 47year old male with no habit history who presented with an endophytic growth of the buccal mucosa and histopathologically, a moderately differentiated SCC with single positive margin and multiple positive nodes along with submandibular and parotid gland involvement was present. This clinic-pathological picture suggests that the prognosis of the patient may be poor and thus, the lesion may be, definitely, in an advanced stage with a high probability of margins undergoing genetic or molecular changes towards neoplasia. Do the dissimilarity in the staining values of PAS and AB, as mentioned above, may suggest that PAS may be a more specific for the lesional tissue (in this case, the lesion is in an advanced stage histopathologically) while AB may be a more definite stain for margins (since the probability of a second primary or recurrence in this case, on the basis of the correlation with the clinic-pathological parameters, is likely)?

Case 20 shows a decreased PAS as well as AB uptake by margins. The patient was a 65year male with a habit history of betel quid chewing since 25years who developed an endophytic growth of the gingivobuccal sulcus involving the retromolar trigone and the alveolus. Histopathologically a diagnosis of well-differentiated SCC with a single positive margin and node was given. The decreased stain uptake by margins may support the hypothesis that the modified

glycoproteins tend to be released in the circulation by turn-over, secretion or shedding process, thus mounting the likelihood for the neoplastic transformation of the margins.

Lymph node

Case 2 represents a 54year male with tobacco smoking habit since 10years who developed an endophytic lesion of the retromolar trigone and was diagnosed with moderately differentiated SCC with negative margins but a single positive node. The staining characteristic showed lesser PAS and AB uptake by margins. A similar observation of staining characteristics was made in Case 15 and 16. On comparing the values of difference in AB stain remaining (Table 2), we observed that the difference between all the above mentioned 3 cases varied with maximum stain uptake in Case 15, followed by Case 16 with least stain uptake by Case 2. Thus, in view of the clinic-pathological factors, Case 2 may show a higher possibility of recurrence or second primary tumors as compared to the other two cases. Also, though the remaining two cases have almost similar clinical and pathological factors, the difference in the stain uptake may be attributes to the habit history, its duration and the type of lesion present.

The staining values of Case 5, a 55year old female with a combined habit history of tobacco chewing as well as betel quid consumption since 20years who presented with an exophytic growth of buccal mucosa and retromolar trigone and diagnosed with a well-differentiated SCC with negative margin and a single postitive node and submandibular gland involvement, suggests that lesional tissue shows a decreased PAS uptake while AB uptake is lesser for margins. The habit duration along with involvement of the node as well as submandibular gland may be credited to the lesser AB uptake by margins and hence, a larger prospect for malignant transformation of margins leading to recurrence or development of new tumors.

Case 11 showed a lesser PAS as well as AB uptake by lesional tissue, in spite a moderately differentiated SCC of buccal mucosa and gingivobuccal sulcus and a single positive node. This may be correlated with the demographic factors being a young male having tobacco chewing habit for 5years and the lesion presenting as an exophytic growth.

Conclusion

The variations in the difference in staining values between PAS and AB for the same tissue (either lesional tissue or margin) suggest that certain molecular changes are definitely occurring. Also, the variations in the staining by PAS and AB between the lesional tissue and margins may suggest the genotypic as well as phenotypic changes. Further studies are required to follow-up the patients so as to answer the queries regarding which stain shows better prognostic significance, or whether a particular stain describes the changes taking place in a particular tissue more accurately compared to the other (AB correlation with respect to the growth pattern and margins as observed in this study), or their significance with the likelihood of recurrences, thus aiding in prohibiting the morbidity and mortalilty of patients with carcinoma.

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