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ORIGINAL ARTICLE

Histochemical Study Of Salivary Mucins In Normal And Neoplastic Salivary Glands

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ABSTRACT

Background:
Salivary glands include major and minor salivary glands which are important components of the oral cavity and theirsecretions directly bathe the tissues. The biochemical and histochemical analysis of saliva revealed the presence of mucins as the main component, which showed sugar moieties and amino acids. The histochemical properties of mucins show the presence of glycoproteins andproteoglycans which differ in their chemical and structural natures. Mucins are altered in their normal and pathological states. Using special stains like Periodic Acid Schiff Reagent [**PAS**], Alcian Blue [**AB**], Aldehyde Fuchsin [**AF**], Mucicarmine [**MC**] they can be categorized into acidic, neutral, sulpho and sialomucins.

Aims:
The present study was carried out to assess different staining patterns of mucins in normal salivary glands and their neoplastic counterparts, to interpret type of mucin and change in their expression in normal and neoplastic counterparts.

Methods and Materials:
The study comprised of 19 salivary gland neoplasms which include 9 pleomorphic adenoma [**PA**], 4 adenoid cystic carcinoma [**ADCC**] and 6 mucoepidermoid carcinoma[**MEC**] and 10 normal salivary glands[1 parotid, 7 sub mandibular, 2 sub lingual] which were analyzed with special stains.

Results:
The results showed varied heterogeneity of mucin expression in mucous acini and change in mucin expression from benign to malignancy.

Summary and Conclusion:
The study concluded in considering the probable role of mucins in tumorigenesis and also its usage as an adjunct to diagnosis.

Key words: mucins, salivary glands, histochemistry, special stains.

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INTRODUCTION

Secretory cells having different distribution and morphological organizations are present with respect to the oral cavity.

They are seen from the simplest diploblastic animals to the mammals. The mammals, at the macroscopic levels, possess well developed major salivary glands viz the parotid, submaxillary and the sublingual pairs of glands. The glands are derived from the extradermal or endodermal lining of the primitive oral cavity.

Saliva is the combined product of all the major and minor salivary glands. It varies qualitatively and quantitatively under normal and pathological conditions. Saliva primarily serves to moisten and lubricate food and it also assists in mastication, solubilization and partial digestion. Because of its peculiar physiochemical and immune properties, saliva maintains the homeostasis of the oral cavity and is responsible for the integrity of the oral structures. From the foregoing brief account regarding salivary glands and saliva, various studies on the nature of

secretion were carried out in experimental animals with little emphasis to the salivary glands and their neoplasms in human beings. The biochemical analysis of saliva which was carried out in the early era, revealed "mucins" as the main content, which comprised of glycoproteins and oligosaccharides which were attached to sugar moieties and aminoacids. Further studies pointed out the differences in the histochemical properties of the salivary mucins of different mammalian glands and in the human salivary glands.[1],[2] Mucins are complex carbohydrates which are secreted by different types of epithelial cells and the glandular tissues of the oral cavity, the alimentary tract and the respiratory tract. Mucins reflect in their composition, the change in the functional state of the mucosa, both in the healthy and diseased states.[2]Although there have been many efforts to isolate and chemically characterize mucins, some lacunae still persist in many areas. The understanding of both the nature and the significance of mucin changes in foetal development may be potentially useful in the recognition of early neoplastic changes in adults. This has been a proven fact in the tumours of the gastrointestinal tract[2] Not many studies focus on the correlation of foetal mucins with adult content with respect to the salivary glands.

The histochemical evaluation of mucins which was elaborated by major sublingual and submandibular glands, reveal that a heterogenous population of mucosubstances exist.[3] Heterogeneity is prevalent between cells within a single acinar cluster and from acinus to acinus.[3],[4] The nature of the cartilage like substance in mixed salivary gland tumours is still widely debated and different authors have suggested that both cellular and mucinous components have an epithelial or mesenchymal origin.

Histochemically, the mucins are classified into neutral mucins and acidic mucins [which include sulpho and sialo mucins]. Many reviews on their histochemical classification and identification have been put forward to explain the intricacies of mucins.[4],(5),[6],[7],[8],[9] The simplest, yet a lucid method to identify mucins by routine light microscopy was employed in the present study.[4],[6],[9]

This study was undertaken in an endeavour to analyze the nature of the chondroid elements of mixed tumours and to study the changes in mucin expression in normal and neoplastic tissues by using special stains like PAS, AB at pH 1 and 2.5, Mucicarmine, the AB-PAS combination and the AF and AF-AB combination. A small attempt was made to draw a map of the mucins which were present in diseases and to find a trait which could be an aid to their morphological diagnosis, apart from its usage as an ancillary method in diagnosis.

METHODOLOGY

Tissue blocks were obtained from the department's archives. A sample size of 10 normal and 10 neoplastic salivary glands, which included 9 PA, 4 ADCC and 6 MEC were taken. Normal salivary glands were obtained from the fresh cadavers and from the radical neck dissection cases which were received in the department. Normal colon and appendix samples were taken from the cadavers as controls. Due to the rarity of tumour prevalence in the geographical area where the study was done, it was not subjected to statistics.

Histochemical analysis:

Staining was performed by using Harris Hematoxylin and Eosin [S.D. Fine chemicals Ltd., Mumbai, India] for the H and E sections. MC staining was carried out by using the Southgates Mucincarmine Method. The PAS technique was employed by using the Mac Manuis [1946] method. The combined AB- PAS technique for acid and neutral mucins by [Mowry 1956] was employed. The combined AF-AB method [Spicer and Meyer 1960] was used to identify sulphated mucins and sailomucins. All the chemicals which were required for the preparation of the stains, were obtained from S.D. Fine chemicals Ltd., Mumbai, India and Qualigens Fine chemicals Ltd., Mumbai, India.

Formalin fixed, paraffin embedded tissue blocks of the biopsied specimens which were obtained, were sectioned into pieces of 5 micron thickness by using a soft tissue microtome. Special staining techniques, exclusively for mucins, were used as prescribed by the standard methodology.[4],[5],[6],[9] The slides were reviewed for "different types of mucins", based on the staining patterns. All the slides were analyzed under a trinocular research microscope [Olympus BX 51, Japan] and the images were captured by using a 3-chip CCD Camera [Proview, Media Cybernetics, U.S.A] The results were purely descriptive in nature.

RESULTS

A total of 19 cases of neoplastic salivary gland tumors, which included 9 benign and 10 malignant tumours respectively i.e. 9 PA, 6 MEC and 4 ADCC along with 10 normal salivary glands (2 cases of sublingual, 1 case of parotid gland 7 cases of submandibular glands), were analyzed. The goblet cells of colon and appendix were taken as controls in the study. The sections were made at 5 µ thickness, fixed to slides and were subjected to a battery of special stains including the routine H and E staining. The serous cells were negative for all stains, although they showed strong positivity for Alcian blue. The color matching index was not similar to the standard control colon and appendix which was taken, which showed lighter intensity. As the serous acini were MC and PAS negative, the presence of mucins was

ruled out with the preliminary stains. Hence, no further analysis was done to study the composition of the serous acini. The mucous acini of the submandibular and the sublingual salivary glands showed perfect heterogeneity in various areas of same acini, of different acini and between the lobules of the acini [Table/Fig.1]. This was in concordance with the standard controls which were taken, thus indicating the presence of a mixture of sialomucins and sulphomucins in different areas of the mucous acini. The serous cells however could contain acid mucopolysaccharides and other highly sulfated substances (Table/Fig.1).

The PA sections showed a strong positivity for PAS and MC, which was revealed by strong magenta and red coloured staining. The myoepithelial cells were negative to all stains. The chondroid areas were highly acidophilic, indicating rich acid mucopolysaccharides. Strong AF positivity was seen in the lumen and the chondromyxoid areas, thus revealing the presence of sulphomucins.

[Table/Fig 1]: Normal salivary gland with colon as control

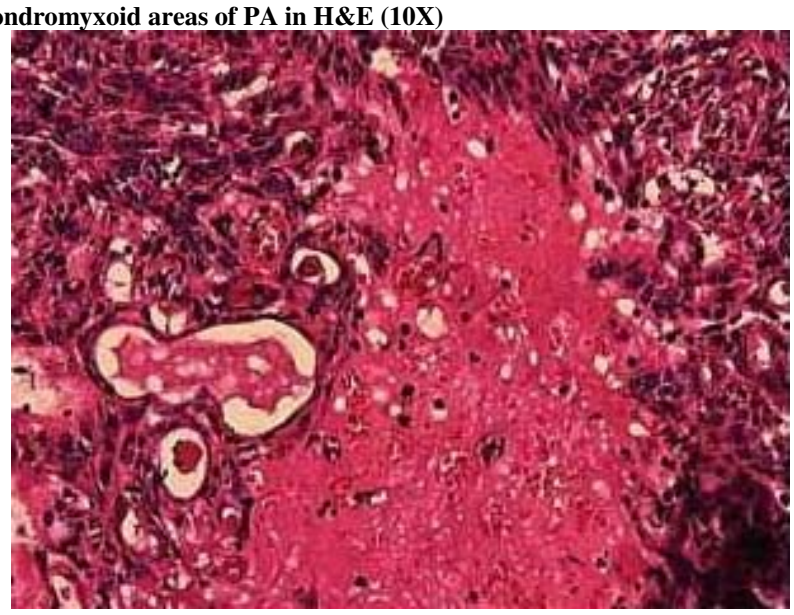
STAIN	CONTROL	SEROUS	MUCOUS	IMPRESSION	
				SEROUS	MUCOUS
MC	Red	Negative	Red	No mucins	Mucins
PAS	Magenta	Negative	Magenta	No mucins	Neutral Mucins
AB at pH 1 and pH 2.5	Blue	Blue	Blue	Acid mucopolysaccharides	Acidic mucins
AF	Purple	-nil-	Purple	-nil-	Varied heterogeneity of sulphated mucins
ABPAS	Both positive with mixture of pink and blue stains	-nil-	Both positive with mixture of pink and blue stains	-nil-	Varied heterogeneity of neutral and acidic mucins.
AF-	Both	-nil-	Both	-nil-	Varied

The AB-PAS staining showed strong AB positivity in the chondromyxoid areas, thus indicating the presence of acid mucins and occasional PAS positivity in the lumen indicated the presence of neutral mucins. AF-AB staining showed an exceptionally strong reaction to AB, thus indicating a dominant presence of sialomucins. This gave the impression that the presence of sialomucins and neutral mucins was most commonly there in the benign tumour, PA [Table/Fig 2] to [Table/Fig 7].

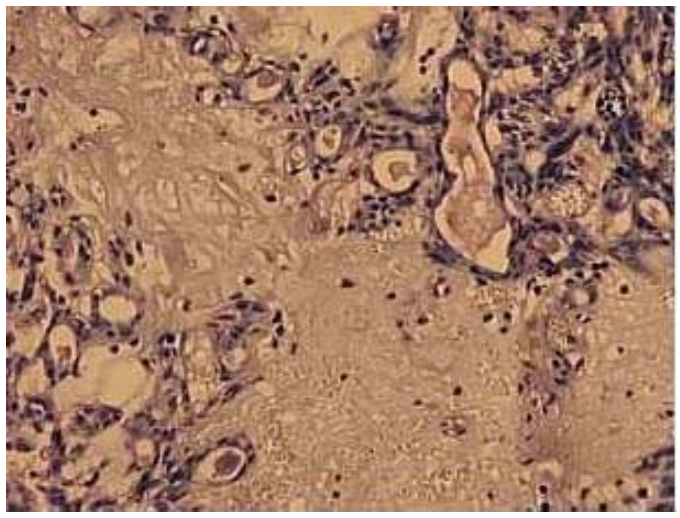
[Table/Fig 2]: Pleomorphic adenoma

STAIN	TUMOR DUCT LUMINA	CHRONDROMYXOID AREAS	IMPRESSION
MC	Red	Red	Mucins
PAS	Magenta	Magenta	Neutral / Acidic mucins
AB at pH1 and 2.5	Blue	Blue	Acidic mucins
AF	Purple	Purple	Sulphated Mucins
AB-PAS	AB- positive PAS- faintly positive [appearing blue and magenta mixture]	AB- positive [appearing varying intensities of blue] PAS- Negative	Acidic Mucins with few traces of neutral mucins in the lumen
AF-AB	AF- Negative AB- Positive [appearing blue]		Sialomucins

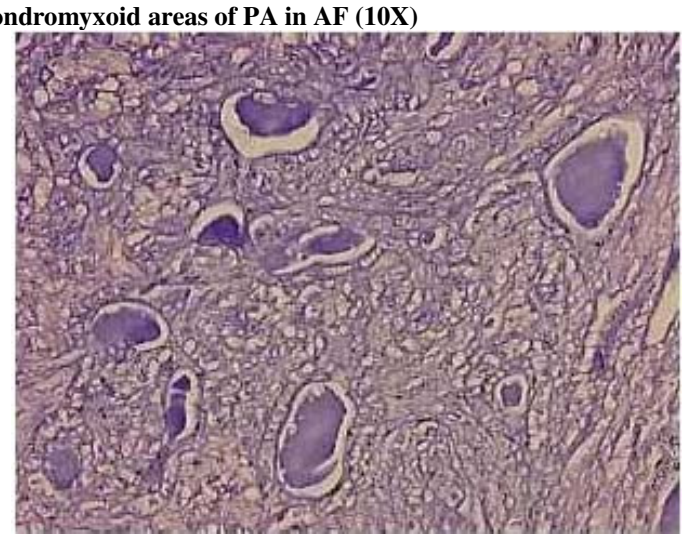
[Table/Fig 3]: Photomicrograph showing ductal lumen and chondromyxoid areas of PA in H&E (10X)



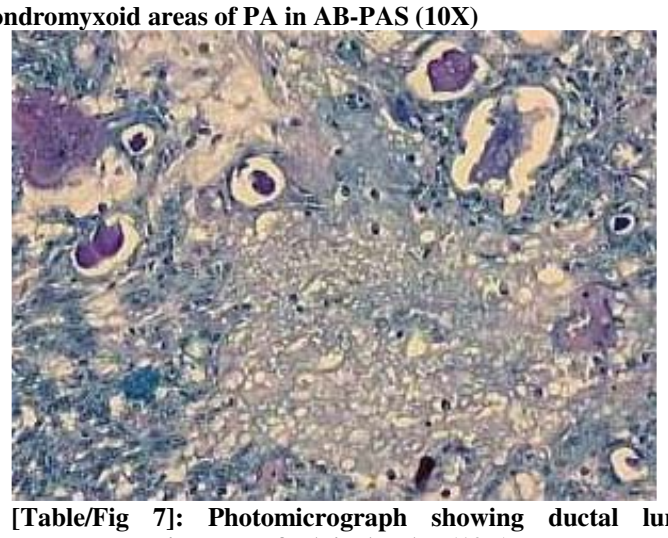
[Table/Fig 4]: Photomicrograph showing ductal lumen and chondromyxoid areas of PA in MC (10X)



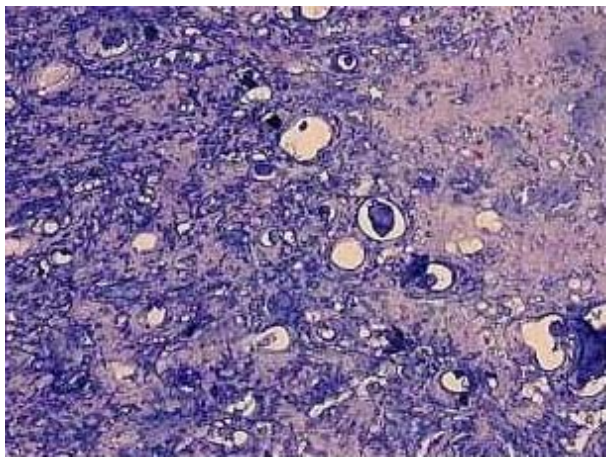
[Table/Fig 5]: Photomicrograph showing ductal lumen and chondromyxoid areas of PA in AF (10X)



[Table/Fig 6]: Photomicrograph showing ductal lumen and chondromyxoid areas of PA in AB-PAS (10X)



[Table/Fig 7]: Photomicrograph showing ductal lumen and chondromyxoid areas of PA in AF-AB (10X)

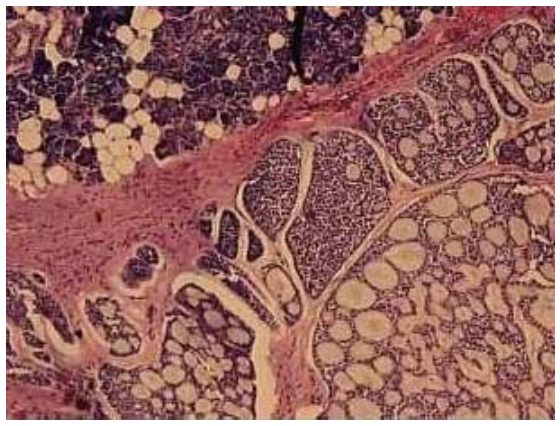


The pseudo cystic spaces and hyaline areas of ADCC showed uniform positivity with PAS and MC, thus indicating the presence of neutral / acidic mucins. Intense acidophilia was seen with AB at different pH levels. Sulphated mucins were dominant in both the areas with AF usage. AB– PAS staining yielded no staining with PAS and moderate staining with AB, thus indicating the presence of more acidic mucins than those of the neutral type. AF- AB staining showed the presence of sulpho and sialomucins [Table /Fig 8] to [Table/Fig 13]

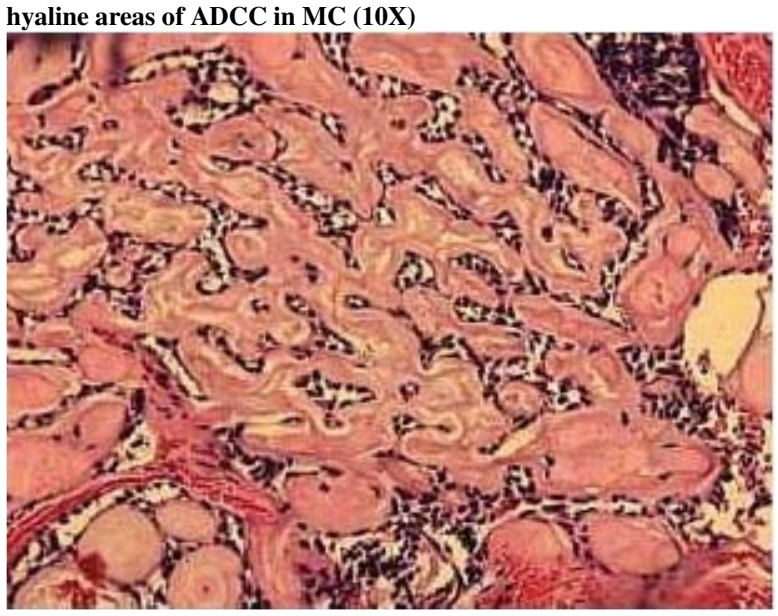
[Table/Fig 8]: Adenoid Cystic Carcinoma

STAIN	PSEUDOCYSTIC AREAS	HYALINE AREAS	IMPRESSION
MC	Red	Red	Mucins
PAS	Magenta	Magenta	Neutral/ Acidic mucins
AB at pH1 and 2.5	Blue	Blue	Acidic mucins
AF	Purple	Purple	Sulphated mucins
AB – PAS	AB – Positive [appearing blue] PAS – Negative		Acidic mucins
AF– AB	Both AF- AB positive appearing as mixture of blue, purple and violet		Sulpho and Sialomucins

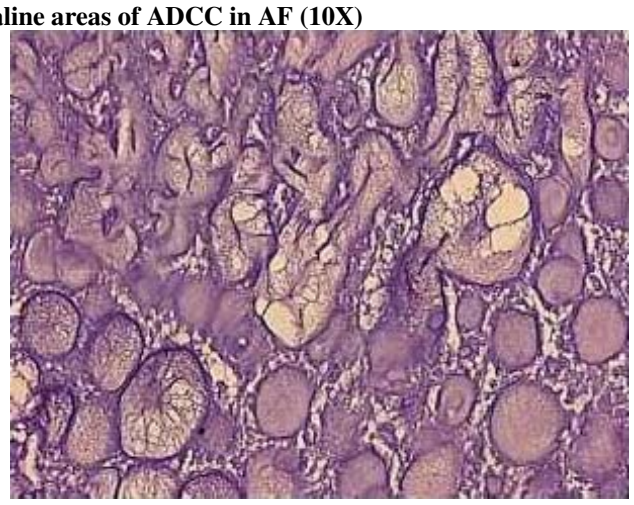
[Table/Fig 9: Photomicrograph showing pseudocystic areas and hyaline areas of ADCC in H&E along with submandibular salivary gland (low power).



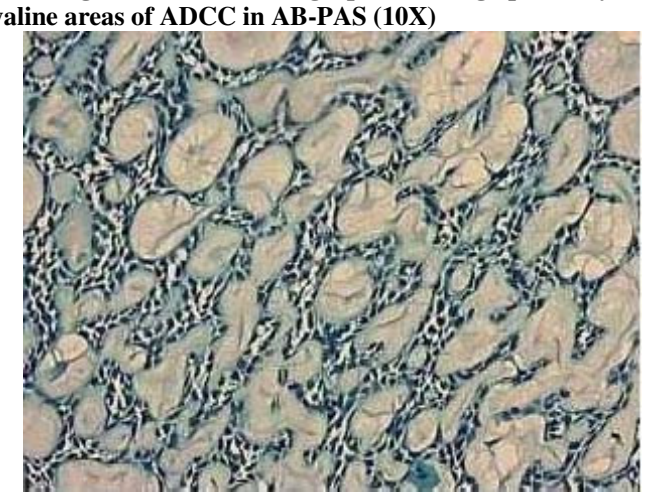
[Table/Fig 10]: Photomicrograph showing pseudocystic areas and hyaline areas of ADCC in MC (10X)



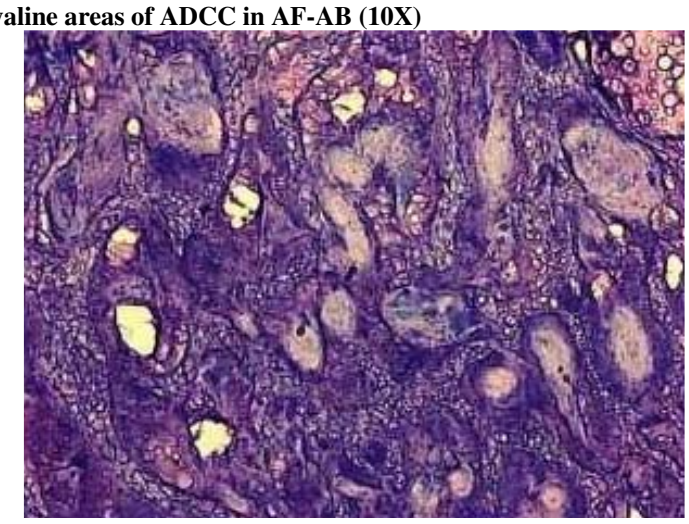
[Table/Fig 11]: Photomicrograph showing pseudocystic areas and hyaline areas of ADCC in AF (10X)



[Table/Fig 12]: Photomicrograph showing pseudocystic areas and hyaline areas of ADCC in AB-PAS (10X)



[Table/Fig 13] Photomicrograph showing pseudocystic areas and hyaline areas of ADCC in AF-AB (10X)

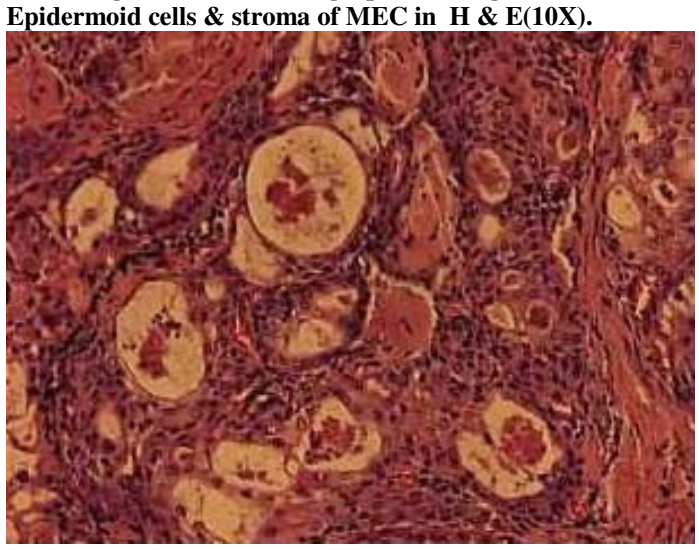


The epidermoid cells of MEC were negative to all stains. The mucous cells and lumen showed positivity to MC, PAS and AB at different pH levels, thus indicating the presence of sulphomucins. Both these areas showed a predominance of acidic mucins with AB-PAS staining and mixture of sulphomucins and sialomucins when AF-AB staining was employed. However, the stroma showed sialomucin dominance when AF- AB staining was employed and sulphated mucins when AF staining alone was done [Table /Fig 14] to [Table/ Fig 19].

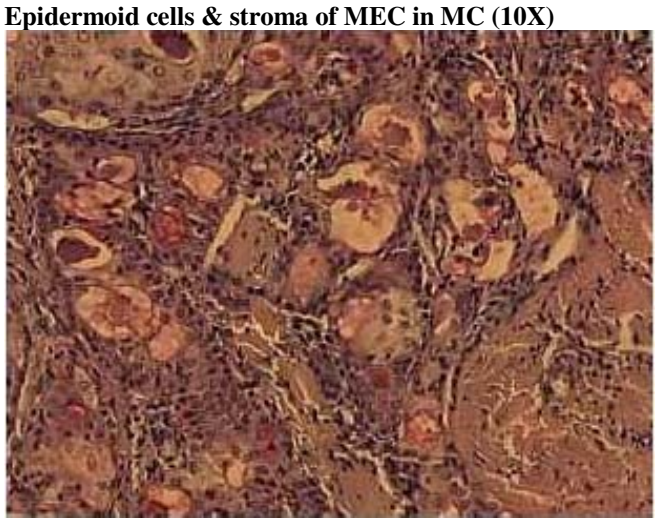
[Table/Fig 14]: Mucoepdermoid Carcinoma

STAIN	EPIDERMOID CELLS	MUCOUS CELLS	LUMEN	STROMA	IMPRESSION
MC	Negative	Red	Red	Red	No mucin in Epidermoid cells
PAS	-nil-	Magenta	Magenta	Magenta	Presence of mucins
AB at pH1 and 2.5	-nil-	Blue at varying intensities			Acidic mucins
AF	-nil-	Purple			Sulphated mucins
AB- PAS	-nil-	AB- positive [appearing blue] PAS - Negative			Acidic mucins
AF – AB	-nil-	Both AF- AB positive appearing as mixture of blue, purple and violet			Sulpho and Sialomucins

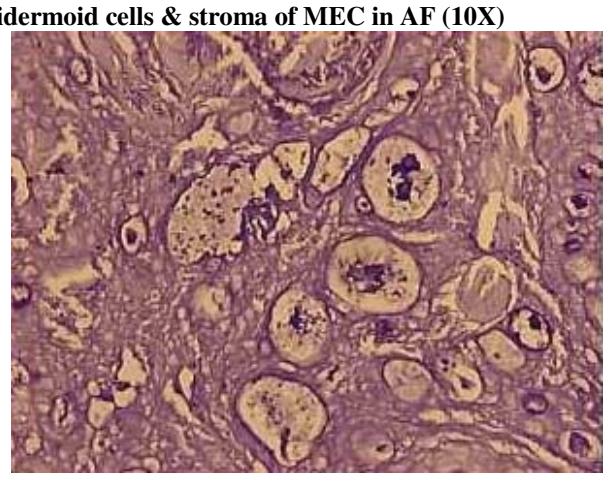
[Table/Fig 15]: Photomicrograph showing Mucous cells, Lumen, Epidermoid cells & stroma of MEC in H & E(10X).



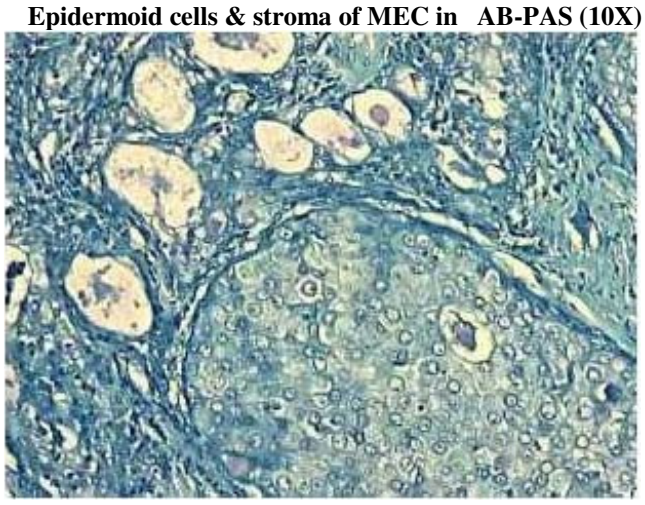
[Table/Fig 16]: Photomicrograph showing Mucous cells, Lumen, Epidermoid cells & stroma of MEC in MC (10X)



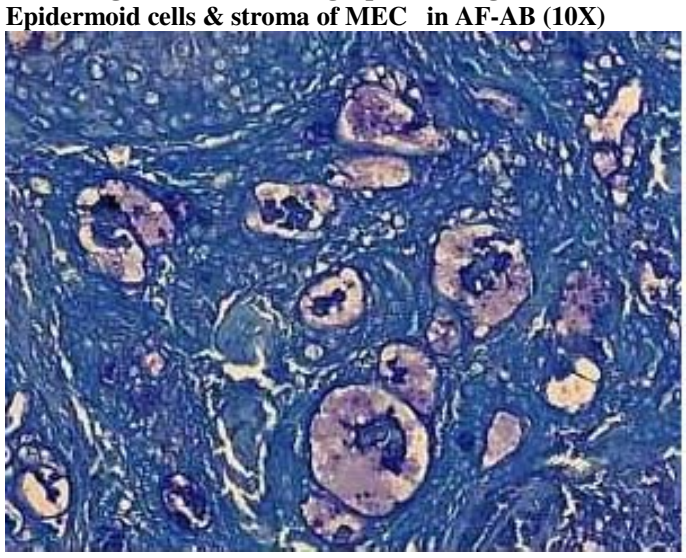
[Table/Fig 17]: Photomicrograph showing Mucous cells, Lumen, Epidermoid cells & stroma of MEC in AF (10X)



[Table/Fig 18]: Photomicrograph showing Mucous cells, Lumen, Epidermoid cells & stroma of MEC in AB-PAS (10X)



[Table/Fig 19]: Photomicrograph showing Mucous cells, Lumen, Epidermoid cells & stroma of MEC in AF-AB (10X)



The overall impression suggests the presence of sulphomucins and sialomucins in the mucous acini of the sublingual and the submandibular glands. The serous acini contain other sulfated substances in the salivary glands. The benign tumour, pleomorphic adenoma, showed a predominance of sialomucins, whereas the malignant tumours contained both sulphomucins and sialomucins.

DISCUSSION

The mucosubstances are complex molecules, in the sense that they contain both proteins and polysaccharide components which are linked together by strong covalent chemical bonds. The mucosubstances are conveniently divided into glycoproteins and proteoglycans on the basis of their structural characteristics. [3],[10]

The glycoproteins contain one or many carbohydrate side chains of relatively small size and gross analysis shows the polypeptide chain to be the major component of the molecule. The protein-polysaccharide complexes are very different from glycoproteins and they are called as proteoglycans. They are now called as GAG and always seem to occur in the form of PG in which several polysaccharide chains are covalently linked to a polypeptide backbone.[3],[10]

The histochemical differentiation of the polysaccharide protein complex in normal and pathological tissues is of ever-increasing importance in the investigation of normal and diseased processes.[2],[11],[12],[13],[14]

Mucins are composed of a number of chemical substances which differ chemically, depending on the cell from which they are derived e.g., Epithelial mucins which include neutral mucins and acidic mucins contain sulpho and sialomucins. The connective tissue mucins show acid mucosubstances like chondroitin sulphates, keratin sulphates, hyaluronic acids and dermatan sulphates. By using different stains along with their combinations, these mucins can be categorized accordingly.[6], [7], [8],[9].

Histochemistry of the salivary gland tumours depicts the presence of the mucosubstances, but does not comment on the refined and detailed constituents of those mucosubstances, nor does it emphasize on the prognosis or longetievity of those neoplastic diseases based on the mucin pattern which has been secreted. There have been no views on the different gradations of the tumour based on pure histochemical analysis.

Mucins are complex carbohydrates which are secreted by different types of epithelial cells and glandular tissues of the respiratory and the alimentary tracts. The nature of the mucins is altered in composition from the normal to the diseased state. [12], [13], [14]

In the present study, for normal salivary glands, the serous acini were negative for all stains except AB. The mucous cells of sublingual and submandibular glands (mixed) showed diverse heterogeneity in a single acinus and in between clusters of acini, thus indicating a mixture of both acid and neutral mucins coexisting in the same gland. The mucin content in the mucous acini was composed of sulphomucins and sialomucins.

Eversole (1972) studied the mucin histochemistry in the normal salivary glands. The mucous acini of the sublingual and the submandibular glands showed a varied heterogeneity with the AB-PAS and AF stains.[15]

The present study was in concordance with the above features, with respect to the mucous acini; but the serous acini showed strong AB positivity. The results obtained from the present study coincide with the highly sulphated groups in the serous acini (which may not be the mucins), thus rendering them strongly positive for AB. As the present study did not involve enzyme histochemistry, nothing could be commented upon the loss of alcianophilia with prior enzyme treatment. Hence, probably the strong alcianoplilia of the serous acini may be attributed to the lack of enzyme usage.[16]

In this study, the chondromyxoid areas of PA showed strong positivity with the AB, PAS and MC stains. This was in accordance with the study done by Azzopardi JG and Smith OD (1959) who conducted a survey on 100 cases of salivary gland tumours.[17] The authors did not use any combination techniques for further classification. The combined AF-AB staining indicated the presence of sialomucins in these areas in the present study.

The pseudocystic spaces and hyaline areas of ADCC were positive to PAS, AB at varying pH and AF, thus indicating the presence of sulphated mucins. The AF- AB staining showed the presence of sulpho and sialomucins.

Toida et al (1985) studied 13 cases of mucinous content of ADCC by using PAS, AB 2.5, AB-PAS and other immune enzyme analyses.[18] Their results coincided with the present study in showing strong AB at pH 2.5 positivity and faint PAS staining when the AB-PAS staining technique was employed. The histochemical analysis indicated that the glycosaminoglycans in the pseudocysts were composed mainly of chondroitin sulphate and heparan sulphate.

Barnes (2001) reviewed the staining pattern of ADCC by using PAS, MC, AB and AF staining on the pseudocystic spaces and concluded that apart from PAS, MC and AB positivity; these spaces also showed AF positivity.[19], [20] This particular aspect of AF was well appreciated in the present study, which also showed strong to moderate purple colour in the pseudocystic and hyaline areas of the tumour in all the study samples.

The mucous cells and the lumen of MEC showed positivity to MC, PAS andAF staining, thus indicating the presence of sulphated mucins. The AF- AB staining showed the presence of sulpho and sialomucins in these areas. The stroma revealed a dominance of sialomucins when the AF- AB staining was employed. The epidermoid cells were negative to all stains.

Lam (1993) assessed the mucin nature between the MEC and adenosquamous carcinoma of the oesophagus by using PAS, AB, Sialidase and HID. The results showed that the MEC of oesophagus showed high amounts of sialomucins. This particular feature may be helpful in distinguishing the MEC of oesophagus from the MEC of the salivary gland, which showed high amounts of sulpho and sialomucins.[21]

In an overall view, it can be said that the present study is in concordance with the various studies which were done on human normal salivary glands and their tumours.[17],[18],[19],[20] The varied heterogeneity of the mucous acini indicated a mixture of both sulpho and sialomucins.[15] However, strong alcinophilia of the serous acini was noted in the present study, which was not seen in the previous studies.[15],[16] The possibility of the alcinophilia is attributable to a high sulphate content, although enzyme usage can cause negative alcinophilia to be seen in serous cells. Enzyme usage renders negative alcinophilia to the serous cells. The benign tumours showed sialomucins predominantly and the malignant tumours showed sulpho and silaomucins. Comparative amounts of mucins in the foetus and in inflammatory, premalignant and malignant conditions were also studied in the various areas of the body. The grading of the tumours based on the mucin production was also noted in the gastrointestinal tract. [11],[12],[13],[14] In general, a two line inference which was drawn from the study on the gastrointestinal tumours, is that they generally show sulphomucin predominance and as the differentiation of the tumour increases, the sulphated mucin content decreases with significant increase in the neutral mucin content. [2],[11],[13],[14] Such interesting and valuable findings about the salivary gland and its tumours are not sufficiently being reviewed. An extensive research in this field with more number of tissue samples will throw more light on the mucin chemistry. May be the evidence that malignant transformation develops through the sequence of changes with gradual loss of cellular differentiation and the reappearance of the foetal phenotype, can be found.

In the present study, the benign tumours showed sialomucins and as the tumours attained the malignant stage, there was an addition of sulfomucins, rendering a mixture of sulfomucins and sialomucins. Whether the change in mucin expression causes the malignant transformation or whether malignancy causes the change in mucin expression, is an issue to ponder and evaluate. This question remains speculative and debatable. The malignant salivary gland tumours showed a mucin expression which was similar to that seen in the normal mucous acini, in having a mixture of both acid mucins, but the benign tumours expressed sialomucins predominantly.

The future of mucin research lies in establishing a better understanding of the mucins in salivary glands. Such research may throw light on the structural changes of glycoproteins in precancerous lesions, which may be a promising field for histochemists and molecular biologists in knowing the exact process of carcinogenesis.

Changes in the antigenesity of glycoproteins in different diseases will be a futuristic science for immunohistochemistry and histochemistry. The genetic determinants in the expression of glycotransferase during the biosynthesis of mucins by malignant cells will be an important topic for research in the future.

CONCLUSION

Studies on the mucin histochemistry of salivary gland tumours are very few and are not amply reviewed. The nature or content of these mucins are not well documented. The classification of salivary gland tumours on the nature of their mucin content has not been donetill date, unlike the classifications of gastrointestinal diseases.

The nature of mucins in the gastrointestinal tract and the colorectal regions are well documented and explained. The importance of the changes in mucin expression in various regions of the tract is a known fact. The studies in gastro intestine have opened many doors for the researchers to study tumour genesis at a molecular level.

Although the present study does not aim to classify or comment on the nature of the classification, an attempt to explore the mucins and to probably understand the mucin expression in normal to neoplastic tumours has been done. It can be concluded from the present study, that there is a change in mucin expression as the tumour progresses towards malignancy. However, due to the less prevalence of salivary gland tumours in the geographical area in which this study was done, the small

sample size that was studied could be the major limitation of this study. Further studies on a larger sample size may help in explaining the role of mucins in tumour pathogenesis.

The study of mucins in foetal glands and its relationship with tumorigenesis is not well established. The detailed mucin histochemistry in salivary gland tumours may give a clue about the pathophysiology of the disease, tumour differentiation, longetivity and prognostic values. The field of mucin histochemistry still remains to be further explored and and is open for research. Many hidden surprising findings might be traced regarding these tumours from their inception to a high grade malignancy. The wedding of mucin technology with immunology and lectin histochemistry is a major priority in future research. Mucin histochemistry acts as an adjunct to the routine H and E staining, but maybe helpful in assessing the malignant potentiality of tumours. A large number of tumours with various stages of malignancy and histological patterns is needed for a better understanding of the nature of mucins.

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