DIFFERENTIAL DIAGNOSIS OF SALIVARY GLAND TUMORS: THE UTILITY OF IMMUNOHISTOCHEMICAL MARKERS IN ROUTINE PRACTICE

Dissertation submitted to

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towards the partial fulfillment for the degree of

MASTER OF DENTAL SURGERY

BRANCH – IV

ORAL PATHOLOGY & MICROBIOLOGY

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DECLARATION BY THE CANDIDATE

I hereby declare that this dissertation entitled **"Differential Diagnosis of Salivary Gland Tumors: The utility of immunohistochemical markers in routine practice"** is a bonafide and original research work done under the guidance of **Dr. I. Ponniah, MDS.,** Associate Professor, Department of Oral Pathology and Microbiology, Tamil Nadu Government Dental College and Hospital, Chennai - 600 003. I also declare that this work was done after careful and thorough analysis not amounting to any sort of plagiarisms or ethical deviations based on the retrospective records (1970 – 2008) of the Department of Oral Pathology and Microbiology.

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CERTIFICATE BY THE GUIDE

This is to certify that the dissertation entitled **"Differential Diagnosis of Salivary Gland Tumors: The utility of immunohistochemical markers in routine practice"** is a bonafide research work done by **Dr. V. ILAYARAJA** towards the partial fulfillment of the requirement for the degree of **MASTER OF DENTAL SURGERY** in the speciality of **ORAL PATHOLOGY AND MICROBIOLOGY (Branch IV),** under my constant supervision and critical evaluation.

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ENDORSEMENT BY THE PRINCIPAL / HEAD OF THE INSTITUTION

This is to certify that the dissertation entitled **"Differential Diagnosis of Salivary Gland Tumors: The utility of immunohistochemical markers in routine practice"** is a bonafide research work done by **Dr. V. ILAYARAJA** under the guidance of **Dr. I. Ponniah, MDS.,** Associate Professor, Department of Oral Pathology and Microbiology, Tamil Nadu Government Dental College and Hospital, Chennai – 600 003.

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DECLARATION

I **Dr. V.Ilayaraja**, do hereby declare that the dissertation titled **"Differential Diagnosis of Salivary Gland Tumors: The utility of immunohistochemical markers in routine practice"** was done based on the archival samples and records (Department of Oral Pathology, Tamil Nadu Government Dental College & Hospital, Chennai 600 003) in partial fulfillment of the requirements for the degree of **Master of Dental Surgery** in the speciality of **Oral Pathology & Microbiology (Branch IV)** during the course period 2006-2009 under the conceptualization and guidance of my dissertation guide**, Dr. I. Ponniah, MDS.**

I declare that no part of the dissertation will be utilized for gaining financial assistance for research or other promotions without obtaining prior permission from the Tamil Nadu Government Dental College & Hospital.

I also declare that no part of this work will be published either in the print or electronic media except with those who have been actively involved in this dissertation work and I firmly affirm that the right to preserve or publish this work rests solely with the prior permission of the Principal, Tamil Nadu Government Dental College & Hospital, Chennai 600 003, but with the vested right that I shall be cited as the author(s).

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DEDICATED TO GOD & MY FAMILY

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ABSTRACT

Objectives:

The purpose of this study is to determine whether selective immunohistochemical markers may aid in the differential diagnosis of morphologically difficult salivary gland tumors.

Study Design:

The records and archival paraffin blocks of the Department of Oral Pathology and Microbiology, Tamil Nadu Government Dental College and Hospital, Chennai, South India, served as a source of material for this study. About 20 salivary gland tumors [PA (7), PLGAs (3), ACC (4), BAC (1), SDC (1), Clear cell salivary gland tumors (2) and unusual adenocarcinomas (2)] were selected from the record for the study. Formalin-fixed and paraffin embedded tissue sections of the tumors were immunohistochemically analyzed for the presence of SMA, CK, GFAP, c-kit, vimentin and S-100 protein (only in few cases). A standard streptovidin peroxidase procedure was used after antigen retrieval. To assure proper staining, salivary gland fragments, blood vessels and connective tissue fibers present in the sections adjacent to the tumor and within the tumor were used as internal positive controls.

Results:

PAs exhibited positivity to CK in 100% of cases, vimentin in 71% of cases, SMA in 57% of cases and c-kit in 71% of cases. PLGAs were positive to CK, vimentin and c-kit in 100% of cases and to SMA in 50% of cases. ACCs showed

positivity to CK in 50% of cases, vimentin in 25% of cases, SMA in 75% of cases and c-kit in 100% of

cases. GFAP staining was negative in all ACCs, PLGAs and 70% of PAs. BAC exhibited reactivity to all markers except GFAP, whereas SDC was negative to all markers (CK, c-Kit, GFAP, SMA & Vimentin). CCC demonstrated positivity in 100% of cases to CK and SMA (stroma only) and in 50% of cases to vimentin, while c-kit and S-100 were negative. Unusual adenocarcinomas were positive to CK and negative to GFAP in 100% of cases, whereas 50% of cases were positive in vimentin, c-kit and SMA. Out of the two unusual adenocarcinomas S-100 was used in only one case, where it was positive.

Conclusion:

This study suggests that the use of IHC as a supplemental diagnostic tool in border line or difficult salivary gland tumors may well augment the routine microscopic differential, especially when the pattern of reaction is taken into consideration.

Key words:

Immunohistochemistry; IHC; Salivary gland tumors; Pleomorphic adenoma; Adenoid cystic carcinoma; Polymorphous low-grade adenocarcinoma; Basal cell adenocarcinoma; Salivary duct carcinoma; Clear cell carcinoma; Canalicular adenoma; Basal cell adenoma; SMA, vimentin; S-100; GFAP; CK; c-kit.; Tamil Nadu Government Dental College; Chennai.

CONTENTS

LIST OF ABBREVATIONS

IHC	Immunohistochemistry
PLGA	Polymorphous low grade adenocarcinoma
PA	Pleomorphic adenoma
ACC	Adenoid cystic carcinoma
CA	Canalicular adenoma
MA	Monomorphic adenoma
BA	Basal cell adenoma
BAC	Basal cell adenocarcinoma
SDC	Salivary duct carcinoma
EMC	Epithelial myoepithelial carcinoma
MEC	Mucoepidermoid carcinoma
CCC	Clear cell carcinoma
CCA	Clear cell adenocarcinoma
CCASG	Clear cell adenocarcinoma of salivary gland
CCMEC	Clear cell myoepithelial carcinoma
RCC	Renal cell carcinoma
DPX	Dibutyl phthalate Xylol
No.	Number
WHO	World health organization
CK	Cytokeratin
GFAP	Glial fibrillary acidic protein
SMA	Smooth muscle actin
MSA	Muscle specific actin
CEA	Carcinoembryonic antigen

EMA	Epithelial membrane antigen
HMFG	Human milk fat globulin
LMK	Low molecular weight keratin
HMK	High molecular weight keratin
H & E	Hematoxylin & Eosin
NOS	Not otherwise specified
pCEA	Polyclonal carcinoembryonic antigen
GCDFP	Gross cystic disease fluid protein
SMM	Smooth muscle myosin
SMMHC	Smooth muscle myosin heavy chain
Rb	Retinoblastoma
LCA	Leukocyte common antigen
PCNA	Proliferating cell nuclear antigen
AR	Androgen receptor
BRST	Breast
EGFR	Epidermal growth factor receptor
Ly	Lysozyme
LF	Lactoferrin
Alpha 1-Ach	Alpha 1-antichymotrypsin
PK	Polyclonal keratin
NSE	Neuron-specific enolase
SC	Secretory component
PTAH	Phosphotungstic acid hematoxylin
PAS-D	Periodic acid schiff-diastase.
PAS	Periodic acid schiff

INTRODUCTION

Salivary gland tumors are a heterogenous group of neoplasms that may manifest different cellular and growth phenotypes. The latest WHO classification lists 40 named salivary gland tumors.[1] These tumors range from the more frequent benign salivary tumor, like PA, to more aggressive SDC or carcinoma ex PA. Therefore, distinction is an essential part of any diagnostic setup, which to the untrained or even for the experienced may pose considerable diagnostic challenge, especially with rare entities. However, most of the salivary gland tumor entities can reliably be distinguished by recognition of cell types and specific growth patterns. Nonetheless, some salivary gland tumors manifest overlapping morphological and cellular phenotypes often making diagnosis difficult.

The overlapping histological features are commonly encountered in tumors like PA, BA or PLGA, ACC and SDC. Thus, when a tumor does not fit within the established criteria for PLGA, ACC or MA (carcinoma) and mixed tumors with the use of routine stains, confirmation with IHC studies may be necessary or desirable. Although IHC have been regarded as confirmatory or supportive for the diagnosis of salivary gland tumors, no single marker would indicate a specific tumor type. Thus, IHC may often require a panel of immuno markers that are costly to employ for routine diagnostic use. However, the use of CK, GFAP, c-kit and selective muscle markers such as SMA and vimentin can often be found helpful in delineating PA, CA, BAC, PLGA, ACC and SDC. For example, a weak reaction with PLGA but a strong reaction with ACC by SMA may help in the distinction of these two entities. Similarly, a negative or weak reaction with GFAP can help to differentiate PLGA and ACC from CA or BAC and PA.

Most pathologists often employ commercially available IHC markers to

discriminate potential salivary gland tumors but are not clear whether they provide unequivocal diagnostic clues in routine practice. The purpose of the present study is to determine whether the use of selective commercially available immunomarkers will provide diagnostic distinctions in borderline or difficult cases of salivary gland tumors.

AIMS AND OBJECTIVES

1. The aim of this study is to identify difficult or borderline salivary gland tumors from a review of 225 salivary gland tumors.
2. To determine whether selective commercially available immunohistochemical markers may aid in the differential diagnosis of morphologically difficult salivary gland tumors.

REVIEW OF LITERATURE

Regezi et al in 1985[2] studied 17 PAs, 5 ACCs and a CA using the immuno markers GFAP and S-100. The authors have found that 16 PAs were positive to GFAP whereas CA and 5 ACCs were negative to GFAP. In PAs, cells in myxoid and cartilage areas and plasmacytoid cells showed more intense reaction with GFAP than other areas.

Morinaga et al in 1987[3] stated that the normal myoepithelial cells were positive for actin but negative for vimentin. Outer tubular cells of organoid double-layered tubular growth pattern which were seen in PA, MA and ACC, the 'cyst' -lining cells and the outermost cells of cribriform growth pattern in ACC were occasionally positive for actin. These outer tubular cells, 'cyst'-lining cells, and outermost cells were considered to be neoplastic myoepithelial cells. However, their staining reaction was much lower than that of normal myoepithelial cells. On the other hand, these neoplastic myoepithelial cells were always positive for vimentin. The 'mesenchymal'cells and hyaline cells of PA (modified myoepithelial cells) and indifferent cells of ACC were negative for actin, but positive for vimentin and occasionally positive for keratin.

Yamada et al in 1988[4] studied the expression of vimentin and keratin (KL1 7 PKK1) in 80 cases of PA. The authors have found that 50 (63%) cases were positive with vimentin (20 strongly positive and 30 moderately positive), 5 cases were very slightly positive (6%) or gave trace staining and 25 cases were negative (31%) to vimentin. Large foci with squamous metaplasia showed negativity to vimentin. Vimentin was expressed with variable intensities in fibrous stromal elements and peripheral/basal side of outer tumor cells (including some spindle cells) in the tubulo-

ductal structures and with strong staining in modified or neoplastic myoepithelial cells and osteochondroidal cells. The authors also showed that the keratin staining was observed in the luminal tumor cells.

Stead et al in 1988[5] performed an IHC study of 34 PAs of the major salivary glands using GFAP, keratin, S-100 protein, CEA, EMA and SC. The authors have showed that PAs exhibited keratin staining in luminal cells of the ducts and intense GFAP staining in periductal and stromal cells differentiating epithelial and myoepithelial cells. The authors have suggested that the presence of GFAP-like immunoreactivity in normal myoepithelial cells strongly supports the extensive involvement of these cells in PAs and the combination of keratin, S-100 and GFAP immunostaining is particularly useful in identifying the component cells in PAs of the salivary glands.

Mori et al in 1989[6] studied 41 cases of PA using the immuno markers keratin, vimentin and S-100. The authors have found that in PAs, great heterogeneity in the staining as well as multiple and co-expressions of these proteins were found in the outer tumor cells of tubulo-ductal structures and modified myoepithelial cells but not in the luminal tumor cells. Among the outer tumor cells, 85% were stained for vimentin and 97% for K8.12 keratin. The authors also showed that the modified and neoplastic myoepithelial cells showed similar expressions to those of outer tumor cells.

Simpson et al in 1990[7] in their study CCC with AE1/AE3, S-100, GFAP, vimentin, CAM 5.2, HMFG 1&2, CEA, NSE, thyroglobulin, desmin, actin and myosin showed that staining was strongly positive with AE1, equivocal with AE3 and negative with S-100, GFAP and vimentin.

Simpson et al in 1991[8] studied the immunoreactivity of 6 cases of PLGA and

6 cases of ACC with immuno markers AE1/AE3, vimentin, keratin, S-100, CAM 5.2, HMFG 1, HMFG 2, and CEA. The authors have shown that all PLGAs showed some cytoplasmic staining for CK AE1 and to a lesser extent for vimentin whereas ACCs displayed diffuse cytoplasmic staining with CKs and negativity with vimentin. The authors have suggested that the microscopic differences were mainly cytological and to a lesser extent morphological and the IHC reactions of two tumors were not sufficiently dissimilar to be of practical value.

Regezi et al in 1991[9] studied the histopathological features and IHC staining of 16 cases of PLGA, 17 cases of ACC and 21 other histologically similar minor salivary gland neoplasms with GFAP, vimentin, keratin (LMK & HMK), S-100 and MSA. The authors stated that the syncytium of tumor cells, often cribriform growth pattern was the H/P characteristic of PLGA. Small (single layered) ducts and fusiform spindle cells characterizing the peripheral aspects of the lesion and solid lobules with peripheral palisading enclosing central pseudoglandular structures were observed. In their study, mucous cells were found in 3 cases, clear cells in one case and the stroma ranged from densely collagenous to hyalinized to mucoid.

In these, authors showed that the cribriform growth pattern in ACC appears as discrete islands (in contrast to the cellular syncytium seen in PLGAs) and are surrounded by retraction spaces or clefts. In their study, cribriform growth pattern was present in all ACCs and was the dominant histologic feature in 14/17 cases, whereas tubular or trabecular pattern was found in only 3 cases. They also found that PLGA showed a single layer in the ducts compared to characteristic bilayered ducts in ACC. The authors have shown that among a total of 16 PLGAs and 17 ACCs, 1 PLGA and 2 ACCs showed focal or weak reaction with GFAP while vimentin and keratin expression were found in all tumors studied. In contrast, the MA in their series exhibited moderate immunostaining for GFAP (4 CAs & 3 BAs) and strong staining for keratin, whereas vimentin expression was found in the membranous variant.

In this study, the authors have found that the IHC staining helped in the interpretation of 11 tumors that were equivocal with H&E sections. It was shown that 3 PAs with PLGA features exhibited

moderate to strong staining for S-100, GFAP, vimentin and keratin. Of the 8 other equivocal tumors, staining patterns were similar to PLGA in 2 cases (strong S-100 and weak actin staining), ACC in 3 cases (weak S-100 and moderate actin staining) and no distinctive pattern in 3 cases which were labeled as adenocarcinoma (NOS).

Nishimura et al in 1991[10] examined immunoreactivity of 60 cases of PA, 5 cases of BA and 10 cases of ACC to keratin, vimentin, GFAP, actin, desmin, S-100 and SC. The authors showed that in cases of PA, vimentin immunoreactivity was present in almost all tumor cells of epithelial or mesenchymal differentiation, except for inner tubular cells and squamous epithelial cells. GFAP also showed the similar immunoreactivity but reactivity was significantly reduced in the mature chondroid cells with lacunar formation. They also showed that in both ACC and BA, vimentin was observed in some tumor cells but GFAP was consistently negative. The authors believe that GFAP may be useful in discrimination among the tumors they studied.

Norberg et al in 1991[11] in their study of IHC of 3 cases of PLGA with CKs AE1/AE3, vimentin, EMA, CEA, MSA and S-100 found that all cases were positive for vimentin and staining was expressed in discrete region of basal cytoplasm in some tumor cells except one case in which staining was infrequent. The authors also showed that all 3 cases were positive to AE1/AE3 in luminal tumor cells forming duct like structures in the solid growth pattern in 2 cases and no specific pattern of tumor cell staining in one case.

Ogawa et al in 1991[12] examined 3 cases of CCC with the immuno markers vimentin, S-100, GFAP, actin, keratin and EMA. The authors have found that case 1 showed extensive positivity for vimentin and S-100 protein with focal expression of GFAP, strongly suggested that the clear cells were myoepithelial in nature. In contrast, the clear cells from case 2 and 3 expressed keratin.

Jones et al in 1992[13] compared the immunoreactivity of α-SMA and S-100 protein in a wide range of salivary gland tumors. The authors selected 6 PLGAs, 3

ACCs, 10 PAs and 4 SDCs for their study and have found that among 6 PLGAs, 5 displayed some staining with α-SMA, but this was much less pronounced than the S-100 in 3 cases consisting of just isolated cells and 2 tumors expressed α-SMA in outer cell layer. Two out of three ACCs showed focal weak α-SMA immunoreactivity. They also found that in 10 PAs, the inner duct lining cells were negative for α-SMA, but focal weak positivity was noted in some tubules in an apparent myoepithelial cell layer and only occasional cells in the matrix expressed α-SMA. All SDCs were negative to α-SMA.

Delgado et al in 1993[14] studied 14 cases of SDC using the immuno markers SMA, CEA, BRST, CKs (HMK & LMK), S-100, MSA and LeuM-1. The authors indicated that SDC was composed entirely of ductal cells without the participation of myoepithelial cells as the battery of myoepithelial markers (SMA & MSA) failed to stain neoplastic cells.

Williams et al in 1993[15] analysed 23 cases of BAC and 11 cases of BA using the immuno markers CK, GFAP, SMA, vimentin, S-100, CEA, EMA, B72.3, Ber-EP4 and HMFG. The authors have found that no distinct reactivity was present for any of the growth pattern within either BA or BAC. 100% cases of both BAC and BA showed intense reactivity with CK in cells bordering lumina, pseudolumina and occasionally in peripheral cells. Vimentin showed reactivity in 78% BACs and 73% BAs, whereas SMA showed positivity in 83% BACs and 91% BAs. Reactivity with vimentin and SMA were prominent within the stromal elements and many peripheral tumor cells in which vimentin demonstrated basilar dot like reactivity in peripheral cells and showed cytoplasmic reactivity within many remaining tumor cells whereas GFAP exhibited only faint staining in a few scattered tumor cells in 18% cases of BA and 13% cases of BAC. The authors stated that though IHC markers were not useful

in differentiating BAC from BA due to their similar IHC differentiation, this immunoprofile prove to be useful in distinguishing BAC from other malignant salivary and non salivary neoplasms.

de Araujo et al in 1994[16] compared the staining patterns of 7 cases of PA, 7 cases of ACC, 2 cases of MA and 6 cases of PLGA to immuno markers vimentin and actin. The authors have found that in PAs, vimentin was detected in almost all the nonluminal cells (polygonal, spindle shaped and hyaline or plasmacytoid cells) in which the staining was weak in the clear polygonal cells and strong in the plasmacytoid cells whereas actin stained only the outer cells of the ducts and faintly stained the polygonal cells. In cases of ACC, tubular growth pattern showed both vimentin and actin staining in the narrow band of cytoplasm of the outer tubular cells. The cribriform growth pattern showed that the cells lining pseudocysts and peripheral cells of cylinders were stained positively for both vimentin and actin, whereas in solid growth pattern with central necrosis, neither vimentin nor actin was detected in tumor cells.

The growth patterns in PLGA (lobular, trabecular and papillary) were positive for vimentin whereas out of 6 cases studied, 2 cases showed staining (weaker than vimentin) for actin and 4 cases were negative for actin. The authors also showed that BAs expressed vimentin in nonluminal cells including duct like structures and luminal cells in some cases, whereas actin reactivity was negative. Finally, the authors suggested that vimentin could be useful to investigate myoepithelial participation and distribution in salivary gland tumors.

Ferreiro et al in 1994[17]investigated 6 CAs, 5 PLGAs and 6 ACCs with a panel of immuno markers including AE1/AE3, vimentin, GFAP, S-100, EMA, CEA and MSA. The authors have found that all CAs showed strong cytoplasmic positivity

to AE1/AE3 and strong reactivity to vimentin in abluminal cells of tubules or canals and stroma whereas only one had focal cytoplasmic GFAP staining. All PLGAs and ACCs showed positive staining with AE1/AE3 and vimentin but negative staining with GFAP. Finally, the authors summarized that IHC may be useful as an aid in the differential diagnosis of CA.

Shrestha et al in 1994[18] evaluated the IHC of 14 cases of CCC using CK (K1.1 and K8.12), vimentin, S-100 alpha and beta subunits, NSE, GFAP, MAM-3, MAM-6 and PCNA, Ly, LF and alpha 1-Ach. The authors have found that H/P of the carcinoma was characterized by round or polygonal tumor cells growing in solid sheets, small nests or cords with collagenous stroma. The clear cells had nuclei with little pleomorphism and few or no mitotic figures. The authors showed that and CK was present in few tumor cells with almost negligible to strong reaction in all cases, vimentin in 6 cases, GFAP in 5 cases with multiple-expression of cytokeratin K8.12, vimentin and GFAP in 5 cases. S-100 protein immunoreactivity was the most prominent feature with more intense reaction.

Takai et al in 1995[19] selected 14 cases of PA for IHC evaluation of vimentin, GFAP, CK14 and MSA expression. The authors showed that 100% cases were extensively positive for vimentin with staining expressed in nonluminal tumor cells of all morphological types including plasmacytoid cells. In plasmacytoid cells, either entire cytoplasm was intensely stained or the cytoplasm contained pale round zone enclosed by a rim of strong positive cytoplasm. The degree of cytoplasmic staining for vimentin was strong in majority of nonluminal cells than luminal cells and out of 14 cases, only 4 cases failed to express vimentin in luminal cells. With GFAP, 94% of PA had a significant population of positive nonluminal cells (plasmacytoid cells, stellate to spindle shaped cells in myxoid material). The cytoplasmic distribution of GFAP in plasmacytoid cells in PA was either diffused or surrounded by weakly

stained central region as noted with vimentin and 6/14 (43%) cases showed positivity in luminal cells.

Mccluggage et al in 1995[20] studied a case of BAC using the immune markers SMA, CAM 5.2, S-100 and CEA. The authors have found that the tumor cells (smaller cell type) showed focal positivity for SMA mainly around the periphery of the cell groups, although small numbers of cells within the centers of the groups also showed positive staining. The authors stated that this staining pattern supported the concept that the cells showing myoepithelial differentiation were present in the malignant counterpart of BAC.

Seifert et al in 1996[21] studied halinizing CCC using the immune markers CK, S-100, actin, EMA and CEA. The authors have found that the tumor was histopathologically characterized by solid or trabecular formations of polygonal clear cells which are surrounded by a broad hyalinized desmoplastic connective tissue stroma. The clear cells expressed CK but not S-100 protein, actin or other markers of myoepithelial cells.

Savera et al in 1997[22] analysed immunoreactivity of 65 cases of PA to α-SMA, SMMH and calponin. The authors have classified different cell types within the PAs as inner tubular epithelial cells, myoepithelium-like cells (juxtatubular, cuboidal, and spindle), modified myoepithelium (myxoid, chondroid, hyaline), and transformed myoepithelium (solid epithelioid, squamous, basaloid-cribriform). The authors showed that 94% of their cases reacted with α-SMA and the staining was limited to the myoepithelial-like cells, whereas modified and transformed myoepithelial cells lacked these myofilaments and thus α-SMA is expressed only in the well differentiated neoplastic myoepithelium. None of the smooth muscle markers stained the inner-tubular epithelial cells. Hence, the authors found that the expression of α-

SMA in the neoplastic myoepithelium is to be associated with the state of morphologic differentiation and calphonin is the most sensitive marker of neoplastic myoepithelium.

Gnepp et al in 1997[23] evaluated the GFAP localization in 12 cases of PA and 12 cases of PLGA. The authors found that 2 cases of PLGA showed focal positivity only in the epithelial component and staining did not occur in the stroma. The authors also showed that in PA, all the 12 cases showed positive reactivity in stromal component in which only 5 cases showed positivity in epithelial component with weak and focal staining. In stromal component, majority of staining was seen in the periductal cells and in the plump to spindle mesenchymal like cells. Finally, the authors concluded that the positive staining in mesenchymal like cells equates the diagnosis of PA, whereas the epithelial but not the stromal component in PLGA may occasionally stain with GFAP, on the other hand the negative does not indicate a diagnosis of PLGA since occasional PA may have GFAP reactivity. In such situation, the authors suggested that other histopathologic criteria need to be used to establish the proper diagnosis.

Perez Ordonez et al in 1998[24] performed histopathlogical study as well as IHC study with vimentin, SMA, GFAP and few other immunostains in 16 cases of PLGA. The authors have found that the uniform cytology of PLGA indicated the low-grade malignant cytological features rather than different cell types forming these neoplasms. The neoplastic cells in PLGA composed of small cuboidal luminal cells and round, polygonal and spindle or oval nonluminal cells were usually observed in a combination of five basic architectural patterns including tubules and duct like structures, cords and trabeculae, solid nests, cribriform areas with pseudoluminal spaces and papillae.

The authors have found that vimentin was positive in all cases where all cell types showed intense reactivity except for single case. With SMA, staining was negative in 13 cases, weakly positive in 1 case and moderately positive in 2 cases with positive cells seen on the periphery of the nests and some solid cords whereas GFAP was focally present in only one case and thus the authors demonstrated a total absence of GFAP and actin-positive cells in most cases of PLGA. Finally the authors suggested that the luminal cells exhibits ductal type differentiation showing moderate or strong reactivity to LMK and vimentin and only occasional reactivity to HMK. In contrast, the nonluminal cells showed a phenotype consistent with basal cell differentiation and less commonly with myoepithelial cells demonstrating immunoreactivity to LMK, HMK, vimentin and SMA.

Ikeda et al in 1998[25] studied a case of BAC using the immuno markers CK, vimentin, SMA, EMA, CEA and S-100. The authors showed that all of the cells were reactive for CK and vimentin with focal reactivity for SMA.

Prasad et al in 1999[26] analysed 26 PLGAs, 13 ACCs, 17 CAs, 6 SDCs and 9 oncocytomas with the use of immuno markers SMA, SMMHC and calponin. The authors found that 100% cases of ACC showed reactivity with α-SMA. In the cribriform growth pattern, small basophilic neoplastic myoepithelial cells with angular nuclei lining the pseudocystic structures and forming contiguous solid growth pattern exhibited a narrow band of cytoplasmic positivity for α-SMA whereas in tubular growth pattern with double layers, the cuboidal inner epithelial cells were negative to α-SMA and the abluminal myoepithelial cells reacted intensively with α-SMA. Thus, there was unambiguous myoepithelial participation in all growth patterns of these tumors.

In contrast, none of the tumor cells of PLGA showed any reactivity with α-

SMA, because the cells lining the periductal and pseudoadenoid pattern in these tumors lack myofilament expression and were more closely related to basal ductal cells. However, the chances of false positivity may result due to the presence of myoepithelial cells of entrapped, non neoplastic salivary acini and peripherally located stromal fibroblasts. Thus, there was no evidence of myoepithelial differentiation in any histologic pattern in these tumors. All CAs were negative to α-SMA whereas in SDCs, some of the ductal structures (residual non-neoplastic myoepithelium of native glands) retained a peripheral rim of myoepithelium, tight cuff of myofibroblasts and dysplastic stromal cells which reacted with α-SMA and the tumour cells were nonreactive. All cases of oncocytoma were entirely negative for muscle markers. Finally, the authors concluded that the consistent difference of immunostaining patterns between PLGA and ACC have diagnostic significance.

Quddus et al, in 1999[27] studied 3 cases of BACs by means of IHC using AE1/ AE3, SMA, vimentin, EMA, CEA, p53, Ki-67, S-100, GFAP and c-erb-B2. The authors have found that all 3 tumors stained with SMA were strong positivity in peripheral tumor cells of the nests in the solid growth pattern whereas tumor cells towards the center showed weak positivity. The trabecular growth pattern revealed multifocal to diffuse staining confined to cells at the margins of the cords of tumor cells.

The authors also found that the immunoreactivity of vimentin was similar in all the 3 tumors with diffuse intense staining of the tumor cells, CK (AE1/AE3) also stained all the 3 tumors more peripherally in the solid growth pattern and usually centrally in the trabecular growth pattern. inally, the authors have concluded that the degree and type of differentiation, proportion, and arrangement of tumor cells were responsible for the variability of the staining in the different histologic patterns.

Michal et al in 1999[28] evaluated 8 cases of adenocarcinoma of the tongue using the immuno markers CK (AE1/AE3, CAM 5.2), SMA, S-100, MSA and thyroglobulin. The authors have found that on H/P study, solid, microcystic and tubular growth patterns were seen. Solid areas contained tumor cells with hyperchromatic peripheral layer which displayed peripheral palisading and also retraction clefts around the islands. Microcystic growth pattern was composed of lobules of neoplastic cells with cribriform or single layered tubular structures. Cytologically, tumor cells were characterized by overlapping, single pale vesicular nuclei with ground glass appearance and clear to oxophilic cytoplasm. The authors also showed that IHC revealed strong reactivity of tumor cells to CK AE1/AE3 and strongest positivity to SMA in cribriform areas with spindle shaped cells.

Synder and Paulino in 1999[29] studied a case of hybrid carcinoma composed of 80% SDC and 20% ACC using the immuno markers CK, vimentin, SMA, ER, PR, BRST-2 and S-100. The authors have found that on H/P study, the classiccribriform pattern of ACC containing basaloid tumor cells with angular nuclei, small nucleoli, scant eosinophilic cytoplasm and indistinct cells borders was present at the periphery. The cyst like spaces contained pale basophilic material as well as eosinophilic basal lamina material. There were solid nests of pleomorphic cells with central comedonecrosis and occasional dystrophic calcification. Mitotic figures were readily identified.

The authors also showed that SDC component was positive for CK but negative for SMA whereas in ACC, the inner layer was positive for only CK in contrast to the outer layer which was negative for CK but positive for SMA and vimentin. The authors concluded that when some histological features of different salivary gland tumors overlap, IHC was used as a valuable tool to delineate the

components of a hybrid tumor.

Berho et al in 1999[30] described the IHC pattern of two cases of primary intraosseous hyalinizing CCCs of the jaws to CKs AE1/AE3, SMA, vimentin, S-100, CEA, CAM 5.2 and EMA. The authors have found that the lesions were composed of sharply demarcated solid islands, trabeculae, and cords growing with infiltrative borders and characteristic heavily hyalinized fibrous stroma. At the periphery of the lesions, cords and small nests of neoplastic cells were seen between the remaining bony trabeculae. Two cellular phenotypes were noted where majority of the cells were polyhedral or round with cytoplasmic clearing containing small centrally located nuclei and the other cell type was smaller cells having an eosinophilic and granular cytoplasm. The authors showed that in both cases the majority of the cells showed strong cytoplasmic staining with AE1/AE3. In both cases vimentin stain was positive in the stromal cells and negative in the neoplastic cells whereas S-100 and SMA were uniformly negative.

Rezende et al in 1999[31] analysed the IHC staining of 3 cases of CCASG using CKs, S-100, SMA, vimentin, GFAP, CEA and EMA. The authors showed that all the 3 cases were negative to S-100, SMA, vimentin and GFAP.

Foschini et al in 2000[32] stated that myoepithelial cells are contractile elements showing a combined epithelial and smooth muscle phenotype. They found that among the IHC markers, SMA, calphonin, SMM and heavy caldesmin employed to detect myoepithelialcells, SMA is the most widely used marker. The reactivity of smooth muscle markers is variable in the major and minor salivary glands. In minor salivary glands, all the four markers were equally strongly expressed. Although SMA is the most widely used in identifying the myoepithelial cells, it also stains stromal myofibroblasts, sometimes hampering the identification of myoepithelial cells.

Zarbo et al in 2000[33] studied 11 cases of CA and 14 cases of BA with use of α-SMA, SMMH, and calponin. The authors have showed that BAs were sub classified as members of the variants, trabecular-tubular, trabecular, tubular, membranous and solid. Except one trabecular-tubular variant, all other BAs contained cells highlighted by α-SMA and of the tubular variant, positively stained cells were seen in tumors composed of single layered tubules and bilayered tubules. In the trabecular and trabecular-tubular adenomas, the tumor cells at the peripheral stromal interface that formed trabeculae showed myoepithelial differentiation contrast to the central basaloid cells within the trabeculae. In addition, the stromal like spindled myoepithelial cells in between trabeculae also stained positive in the trabecular-tubular type. In the solid type, peripheral, palisading dark cuboidal cells stained positively whereas in membranous type, in addition to this staining pattern, the cells surrounding the hyaline cylinders within solid tumor islands also stained positively for α-SMA. The authors also showed that CA was negative for α-SMA and thus, with the exception of CA, all exhibit some degree of myoepithelial cell participation.

de Araujo et al in 2000[34]applied a panel of antibodies composed of CKs, vimentin, and actin to 114 minor salivary gland tumors. The authors have revealed that luminal cells of intercalated duct like structures seen in PA, BA, ACC and EMC expressed CKs 7,8,14 & 19 whereas the outer cells of the duct like structures in PA, BA and ACC exhibited vimentin. Finally the authors suggested that the panel of antibodies employed is effective in distinguishing among salivary gland tumors.

Kaneko et al in 2000[35] studied IHC of EMC using PK, S-100, SMA, GFAP, vimentin, EMA, NSE, amylase, LF and SC. The authors have found that the clear cells in both solid and tubular growth patterns reacted with S-100, GFAP, and vimentin and had diastase-soluble PAS granules in their cytoplasm.

Scarpellini et al in 2001[36] have used IHC to study a series of 23 benign and malignant tumors (PA, BA, ACC, EMC and myoepithelioma) using the markers SMA, calponin, caldesmin and SMMHC. The authors have found that all their tumors showed positivity to atleast one of the myoepithelial marker and of these markers, SMA was expressed most frequently. Staining was found in the myoepithelial cells that formed the outer cell layers of the glandular or tubular growth. Finally, the authors concluded that this panel of myoepithelial markers helps to disclose myoepithelial cell differentiation and can be a useful tool for the correct histopathological diagnosis of salivary gland tumors and among the four markers studied, calponin and SMA were the most useful to identify myoepithelial cell differentiation.

Lopes et al in 2001[37] described an intraoral SDC with H/P features of large duct like structures lined by pleomorphic cells with central comedonecrosis. The cells were cuboidal with round, clear nuclei, and prominent nucleoli and showed numerous atypical mitotic figures. The growth patterns observed were intraductal, solid and cribriform. The authors performed IHC study in this case with the markers CK, EMA, PCNA, Ki-67, collagen IV and laminin and showed that SDC was positive to CK.

Curran et al in 2001[38] studied 36 cases of PA and 42 cases of PLGA (minor salivary gland tumors) with GFAP. All PLGAs demonstrated tumor mass that was unencapsulated, partially circumscribed, and peripherally infiltrative arranged most commonly in solid and tubular growth patterns alone or in combination, whereas 3 cases contained a few areas with a papillary configuration and only 13 cases showed cribriform growth pattern. The stromal component was most commonly a densely fibromyxoid type. All PAs were completely or partially encapsulated containing both epithelial and myxomatous areas showing cuboidal to polygonal cells and spindled or

stellate cells respectively whereas 12 cases had prominent or scattered chondroid areas. The authors have shown that over 50% of cells showed positive reaction to GFAP in 66% of PA, and the remaining 33% of the cases exhibited less than 50% of positive cells. In PLGA, though 73% of cases failed to react with GFAP, remaining 23% cases had faint and spotty staining that was mainly confined to the less numerous medium-sized polygonal cells rather than the ovoid cells that comprise the bulk of the lesion. The authors stated that minor gland lesions differ from major gland lesions in that they are more often cellular, lacking a prominent fibrous capsule, and containing scant or absent myxoid and chondroid material and hence, the use of GFAP should be considered for differentiation of PLGA versus PA when there was overlapping architectural, background, and cellular features in the neoplasm of minor salivary glands.

Machado de Sousa et al in **2001**[39] analysed 3 cases of BA and 3 cases of CA with CKs 7, 8, 13,14, and 19, vimentin and MSA. The authors have found that in cases of BA, outer cells in duct like structures were stained with vimentin in contrast to the solid areas, which showed negative reactivity. None of the CA was reactive with vimentin.

Penner et al, in 2002[40] analysed c-kit expression in 9 cases of ACC and 14 cases of PLGA. The authors have found that c-kit was expressed by 100% of ACC compared to 57% of PLGA cases. The cribriform and tubular growth patterns in ACC showed positivity in inner cell layers (luminal cell layer) of the tumor, while the solid growth pattern expressed c-kit in all cells, most of which were thought to be of modified myoepithelial cells. The authors also showed that the expression of c-kit in PLGA was very focal and greatly decreased when compared with ACC. Finally, the authors consider that the use of c-kit may be helpful in the differential.

Chhieng et al in 2002[41]studied about BA, BAC, cellular PA and ACC using the immunostains CK, GFAP, chromogranin, LCA, CD 99 and desmin. The authors have found that the stromal cells in PA and myoepithelial cells in ACC were positive for GFAP, whereas PLGA, BA and BAC were negative to GFAP. Finally they suggested that consideration of cytological features along with the architectural pattern aided by IHC was essential to arrive at an accurate diagnosis.

Nagao et al in 2002[42] studied 9 cases of hybrid carcinoma which also includes BAC, SDC and ACC using the immuno markers AE1/AE3, SMA, vimentin, CAM 5.2, S-100, CEA, p53, EGFR, c-erbB-2, GCDFP-15 and MIB-1. Their BAC showed diffuse positivity to AE1/AE3 compared to SMA and vimentin which were restricted to the periphery in the solid growth pattern. SDC was frequently immunoreactive for AE1/AE3 but negative for SMA and vimentin whereas in ACC, vimentin and SMA were positive.

Wang et al in 2002[43] studied 20 cases of salivary primary clear cell tumors including 12 CCCs, 7 EMCs and 1 CCMEC CK, S-100, calponin and MSA. The authors have found that CCC appeared as islands and sheets of tumor cells with clear cytoplasm admixed with some pink cytoplasmic smaller cells. Tumor cells were relatively pleomorphic with high nuclear-cytoplasmic ratios and dark, condensed, eccentric nuclei. Collagen deposition is variable and no hyaline cells were seen. The authors showed that S-100 expression was strong in 1 case of CCC and weak to moderate in 2/8 cases of CCC.

Mino et al in 2003[44]evaluated KIT (antibodies-H300 and A4502) IHC reactivity in head and neck neoplasms including 53 cases of ACC, 16 cases of PA, 5 cases of AC, 4 cases of SDC and 8 cases of PLGA. The authors found that 94% of ACC were positive for atleast one of the two antibodies and 77% of cases were

positive for both antibodies. Tubular, cribriform and mixed subtypes of ACC showed a distinct IHC pattern (more pronounced in tubular subtype) of luminal epithelial cell positivity with peripheral myoepithelial cell negativity and solid subtype demonstrated a diffuse staining pattern. KIT expression was lowest among the cribriform subtype and highest among the tubular and solid subtypes of ACC.

The authors also showed that the PA, BA and PLGA showed little expression of KIT and BAC showed slightly increased KIT expression with strong diffuse staining.BAC had a heterogenous pattern reminiscent of the tubular and mixed subtypes of ACC. The authors suggested that the expression of KIT was potentially useful in distinguishing ACC from PA, BA, PLGA, BAC and basal cell carcinoma.

Edwards et al in 2003[45] studied 17 cases of PLGA, 17 cases of MA and 15 cases of ACC with c-kit. The reactivity was uniformly positive in the cytoplasm of luminal neoplastic cells in all cases of ACC with more prominent expression in solid variant and the intensity of staining was strongest in ACC of minor salivary gland origin. The authors also have found that regarding PLGA, 16 (94%) cases were reactive for c-kit with atleast 25% of the tumor cells being positive (faint focal to strong reactivity) and the overall intensity of staining was weaker in PLGA than in ACC. MAs also showed reactivity similar to PLGA with positivity in 16 (94%) cases and expression was observed in luminal cells similar to ACC. Finally the authors consider that the utility of c-kit may not be helpful in distinguishing ACC from PLGA.

Hemachandran et al in 2003[46] studied a case of BA with pancytokeratin, SMA, vimentin, S-100 and CEA. The authors found that there was diffuse strong positivity for pancytokeratin shown by the tumor cell within the islands whereas SMA positivity was noted in the palisading basaloid cells and vimentin positivity was

observed in the stromal cells. The authors stated that strong positivity for cytokeratin, SMA, and S-100 protein was noted in the epithelial cell component, indicating the myoepithelial nature of this tumor cells.

Furuse et al in 2003[47] analyzed 5 CAs and 10 PLGAs with the use of SMA, vimentin, CK7, CK8, CK13 and CK14 immuno markers. The authors have found that PLGAs located in the upper lip were composed of a single layer of columnar cells arranged in channels resembling the H/P feature of CA. They found negative reactivity in CA with SMA and vimentin. In cases of PLGA, vimentin stained all columnar cells and SMA was negative. Finally, the authors concluded that the positivity of PLGA to vimentin was the best means to differentiate the two entities.

Nagao et al in 2003[48]studied IHC staining of 6 cases of dedifferentiated ACC using CK AE1/AE3, α-SMA, GFAP, S-100, EMA, MSA, CEA, HER-2/neu, p53, pRb, cyclin D1 and Ki-67. The authors stated that the dedifferentiated ACC was a rare variant of ACC which was characterized histologically by two components, conventional low-grade ACC and high-grade 'dedifferentiated' carcinoma. The conventional low-grade ACC consisted of a mixture of cribriform and tubular patterns with scant solid areas whereas the high-grade dedifferentiated carcinoma was either a poorly differentiated adenocarcinoma or undifferentiated carcinoma.

The authors have found that low-grade ACC component showed positivity to AE1/AE3 in the cells located mainly on the inner (luminal) aspect of cells nests and to α-SMA in the abluminal tumor cells adjacent to connective tissue. In contrast, dedifferentiated carcinoma expressed diffuse immunoreactivity to AE1/AE3 and was negative to α-SMA staining. GFAP was negative in all cases.

Matsuzaka et al in 2004[49] reported a case of CA and its immunoreactivity with AE1/AE3, GFAP, SMA, vimentin, S-100, CK 7, 8, 13, 14 & 19 and PCNA. The

authors showed that the tumor cells were positive for AE1/AE3, partially positive for GFAP and negative for SMA and vimentin.

Nagao et al in 2004[50] analysed the IHC of 3 cases of PLGA using the markers CKs AE1/AE3, vimentin, α-SMA, S-100, CKs 7, 20 & 34bE12, EMA, CEA, E-cadherin, HER2/neu, MSA, Ki67 and p53. They found that all the tumors were positive for AE1 ⁄ AE3 and negative for α-SMA and GFAP. They also showed that tumor cells in all cases had a diffuse immunoreactivity for vimentin.

Sicurella et al in 2004[51] analysed the IHC of a case of CCC using CK AE1/AE3, S-100, SMA and vimentin. The authors have found that the neoplastic cells were intensely positive for AE1/AE3 and negative for S-100, SMA and vimentin.

Sun et al in 2005[52] investigated a case of hyalinizing CCC using the immuno markers CK AE1/AE3, S-100 and α-SMA. The authors showed that on H/P, the tumor cells were arranged in solid cell nests, trabeculae, islands or cords surrounded by a prominent hyaline stroma and occasional foci of myxoid stroma. Among the two types of cell populations found, the vast majority of the tumor cells were round to polygonal with clear periodic acid-Schiff (PAS) positive cytoplasm and the other population was smaller and consisted of plump nuclei and granular eosinophilic cytoplasm. Both populations of tumor cells lacked nuclear pleomorphism, and mitoses were very scarce. The authors also found that all the tumors were positive for CK AE1/AE3 and negative for S-100 and α-SMA.

Freier et al in 2005[53] studied KIT expression in 55 cases of ACC. The authors showed that the prevalence of positive KIT immunostaining in ACC was 89%. Of the different growth patterns, the tubular growth pattern showed 100% positivity in contrast to 92% positivity in cribriform growth pattern and 75% positivity in solid growth pattern. The staining intensity of reaction was strong in cribriform and tubular

subtypes and weak in solid subtype. The authors stated that KIT immunostaining was more frequently found in well-differentiated tumors and might get lost during dedifferentiation of ACC, indicating that different molecular pathways could be involved in the formation of histological ACC subtypes.

Margaritescu et al in 2005[54] have done IHC evaluation of 4 salivary gland BA using AE1/AE3, vimentin, SMA, CK19, CEA, S-100 and PCNA. The authors showed that the positive reaction to vimentin was present in all the investigated cases with variable intensity. Both the epithelial and stromal components showed positivity and the reactivity of epithelial component was limited only to some of the basaloid cells from the periphery of epithelial neoplastic proliferations that corresponded to the dark basaloid cells observed in routine light microscopy. In epithelial component, less than 20% cells were positive with weak to moderate intensity whereas stromal component showed reactivity in interstitial fibroblasts and stellate shaped cells in myxoid areas.

With SMA, staining pattern was similar to vimentin in epithelial component but the light basaloid cells and luminal ductal cells were negative. In stromal areas with myxoid differentiation, fusiform cells with myoepithelial-like morphology were observed with weak intensity. The reactivity to AE1/AE3 was confined to tumoral epithelial component with intense reactivity in luminal ductal cells (tubular growth pattern) and positivity was observed in 75% of cells. The authors also noticed that 2 cases of solid growth pattern showed positive reaction to AE1/AE3 with weak intensity in dark basaloid cells. All the 3 markers exhibited homogenous cytoplasmic staining pattern.

Furuse et al in 2005[55] compared the immunoexpression of 5 myoepithelial cell markers, α-SMA, vimentin, calponin, h-caldesmon and S-100-protein in 16 PAs,

15 ACCs and 3 EMCs of salivary glands. The authors have found that in PAs, α-SMA was observed mostly in nonluminal cells of the duct-like structures, spindle shaped cells and some cells of the chondroid areas whereas polygonal, plasmacytoid and star-shaped cells of the myxoid stroma were negative to SMA. In contrast, vimentin was expressed by most of the myoepithelial cells, regardless of their phenotype.

The authors also showed that in ACC, tubular type exhibited reactivity to SMA and vimentin in outer tubular cells whereas in cribriform type, cells lining the cyst-like spaces and peripheral cells of the arrangements stained positively for α-SMA and vimentin. Solid type of ACC showed negativity to SMA and slight positivity to vimentin, being strong only in few cells. The authors have concluded that α-SMA was useful for identification of myoepithelial cells, especially in cribriform and tubular ACC while polygonal and plasmacytoid cells of PA and solid ACC showed negativity with α-SMA.

Andreadis et al in 2006[56] in their IHC analysis of 20 PAs, 14 ACCs, 14 PLGAs, 1 oncocytoma, 3 SDCs and 1 BA using a polyclonal c-kit antibody observed that in normal salivary glandular tissues, c-kit showed weak expression in intercalated and striated ducts of major and minor salivary glands whereas mucous; myoepithelial and oncocytic cells were negative. The authors have found that 80% cases of ACC were positive for c-kit with strong expression in almost all neoplastic cells of the solid growth pattern, the luminal cells of tubular structures and the inner layer of cells lining pseudocystic spaces of cribriform growth pattern. PLGA showed expression more frequently in the luminal and less often in the nonluminal cells. In ACC, more than 50% of neoplastic cells were positive in contrast to PLGA in which less than 50% of neoplastic cells were positive for c-kit. The authors also found that in BA, 10-25% of cells of all structures were membrane stained for c-kit in a non-specific

manner and all cases of PA showed moderate (25-50%) mainly, membrane positivity exclusively of the inner luminal neoplastic cells of the duct-like structures whereas the tumor cells in solid, trabecular or reticular areas were negative. Oncocytic adenomas and SDCs were negative for c-kit whereas neoplastic cells in the center of the solid islands of BA were positive for c-kit. Finally, the authors concluded that c-kit expression was used in differential diagnosis of ACC and other subtypes of salivary gland neoplasms.

Beltran et al in 2006[57] studied 10 cases of PLGA and 12 cases of ACC with the immuno markers c-kit, SMA, Ki-67 and MSA. The authors showed that c-kit staining in PLGA was negative in 8 cases, weak in 1 case and strong in 1 case whereas ACC showed staining in luminal cells in all 12 cases with weak staining in 5 cases and strong in 7 cases. The authors also showed that SMA expression in PLGA samples were negative in 5 cases, weak in 2 cases and strong in 3 cases and ACC samples showed negativity for SMA in 2 cases of solid histologic variant and strong staining in abluminal cells (myoepithelial cells) in 10 cases. The authors concluded that c-kit and SMA were potential adjunctive diagnostic tools in differentiating PLGA from ACC in histologic sections.

Albores-Saavedra et al in 2006[58] studied the immunoreactivity of 2 cases of sclerosing variants of ACC with AE1/AE3, SMA, vimentin, S-100 and collagen IV. The authors found that the predominant myoepithelial cells showed immunoreactivity for SMA and vimentin and negativity to AE1/AE3. In contrast, the epithelial cells that lined ductal structures were negative for SMA and vimentin and positive to AE1/AE3.

Epivatianos et al in 2007[59] analysed IHC expression of vimentin, SMA and c-kit in 12 cases of ACC and 12 cases of PLGA. The authors stated that ACC and PLGA are distinct types of salivary gland adenocarcinomas with different prognosis

but due to overlapping histological features (tubular/ductular and cribriform growth patterns) may occasionally result in a diagnostic pitfall complicating the therapeutic decision making. In these cases, the authors employed IHC to support the histological diagnosis with the use of a panel of markers (c-kit, α-SMA and vimentin). The authors have found the expression of c-kit in both ACC (83%) and PLGA (41%) with more than 50% of c-kit positive cells in ACC and less than 50% of c-kit positive cells in over 80% and 40% of ACC and PLGA respectively. They also found different IHC reactivity pattern in ACC and PLGA. In ACC, staining was limited to the luminal cells of the ductular pattern and staining was uniform in all cells of solid growth pattern. On the other hand, PLGA showed reactivity in both luminal and non-luminal cells of ductular, trabecular and lobular growth patterns. The authors have also found that 100% of both ACC and PLGA showed positivity with vimentin. In ACC, the expression was limited to the nonluminal cells of tubular and ductular growth patterns, and in the tumor cells lining the pseudocysts and proper tumor cells of cribriform structures whereas expression of vimentin in PLGA was observed in the nonluminal cells of lobular, ductular and trabecular growth patterns, tumor cells lining the pseudocysts, dispersed proper tumor cells in cribriform structures. In the other growth patterns of PLGA, staining was variable. The expression of α-SMA in ACCs (100%) were similar to vimentin with moderate to strong intensity, while in PLGA, 25% of cases were positive with reactivity limited to the luminal and nonluminal cells of ductular and tubular growth patterns, central and peripheral cells of the solid and fascicular growth patterns and single layer cells of ductular and tubular structures. Finally, the authors concluded that use of α-SMA and c-kit may offer an adjunctive aid in differential diagnosis of ACC and PLGA.

Cavalcante et al in 2007[60] analysed immunoreactivity of 12 cases of PA, 8

cases of ACC and 4 cases of PLGA to vimentin, calponin and HHF-35. The authors have found that vimentin was expressed in all cases of PA and PLGA and 62.5% cases of ACC and its expression was diffuse in all tumours. In PA, the vimentin immunostaining was concentrated in almost all nonluminal cells , including polygonal, spindle-shaped, hyaline and plasmacytoid cells, located at the periphery of the sheets, nests, cords, tubules or ducts. Chondroid matrix cells were intensely stained for vimentin and no staining of luminal cells was observed. In PLGA, focal staining was observed mainly in nests and cords and also in trabeculae and cells lining the pseudocysts of cribriform growth pattern. In ACCs, vimentin immunoreactivity was mainly observed in cells delimiting the pseudocystic structures and occasionally in nonluminal cells of tubules and ducts.

Curran et al, in 2007[61] *examined 27 CAs, 21 PAs, 30 PLGAs and 3 BAs to determine their immunoreactivity to GFAP. The authors showed that 26 CAs and 3 BAs demonstrated either strong or weak intracytoplasmic reactivity that was confined to a row of cells at the tumour/ connective tissue interface whereas one case of CA demonstrated weak diffuse positivity of tumor cells but also demonstrated a row of strong immunoreactive cells at the tumor/connective tissue interface and one CA was not reactive. The authors also found that all PLGAs showed little or no immunoreactivity to GFAP with the exception of occasional faint positivity in luminal cells. Contrast to PLGA, all PAs demonstrated either strong or weak cytoplasmic positivity within the tumor cells. Additionally, 2 cases of PA demonstrated a weakly linear row of immunoreactive cells at the tumor/connective tissue interface. The authors stated that quantification of GFAP positive tumor cells was useful in differentiating PA from PLGA but not when differentiating PLGA or PA from*

CA. The authors suggest that the pattern of single row of distinct GFAP immunoreactive cells at the tumor/connective tissue interface can be used as a reliable diagnostic criterion for differentiating among CA, PLGA and PA.

Parashar et al in 2007[62] reported immunoreactivity of 2 cases of BAC with SMA, GFAP, vimentin, S-100, Bcl-2, CKs 7 & 20, EMA, CEA and p53. The authors showed that fewer than 20% of cells were weakly positive for vimentin in only 1 tumor and both tumors were negative for GFAP and SMA.

Kikuchi et al in 2007[63] studied a case of SDC using the immune markers CK AE1/AE3, SMA, vimentin, CAM 5.2, AR, BRST-2, S-100 and GCDFP-15. On IHC analyses, they showed that neoplastic cells were positive for AE1/AE3 and negative for SMA and vimentin.

Meer and Altini in 2007[64] studied 24 PAs, 22 ACCs, 21 PLGAs and 3 SDCs using the immuno markers CK 7, CK 20 and CK AE1/AE3. The marker AE1/AE3 was used as a guideline. They showed that 100% cases of PA, ACC, PLGA and SDC were positive for AE1/AE3.

Di Palma et al in 2007[65] analysed a case of PA with multiple foci of oncocytic metaplasia using the immuno markers CKs 5, 6, 8, 18 & 14, vimentin, α-SMA, p63, S-100, β-catenin, EGFR, HER2, Ki-67 and p53. The authors have found that α-SMA was positive in the spindle cells and to a lesser extent in the epithelioid components of PA. PA was also positive to vimentin. In contrast, α-SMA and vimentin were negative in oncocytic cells. Both PA and oncocytic cells were positive to CK 8/18 whereas absence of myoepithelial markers in oncocytic foci indicated lack of myoepithelial differentiation in oncocytic cells.

Pereira et al in 2007[66] reported immunoreactivity of a case of CA using CKs AE1/AE3, S-100, EMA, CEA, calphonin and p63. The authors have found that

most of the cells were positive for AE1/AE3 CKs within the cytoplasm and for S-100 protein in the outer portions of tubules or canals. Finally they summarized that the strong and frequent staining for S100 protein represents the main characteristic of CA.

Suzuki et al in 2007[67] presented the IHC reactivity of a case of CCC to pancytokeratin (AE1+AE3), S-100, vimentin, GFAP, MSA and SMMH. The authors showed that all the tumor cells displayed positive result only for pancytokeratin and negative results for S-100, vimentin and GFAP.

Angiero et al in 2007[68] reported the IHC of a case of hyalinizing CCC to the markers pancytokeratin, SMA, S-100, vimentin, GFAP, EMA, HMB45, CD68 and CEA. The authors have found that the neoplastic cells were immunoreactive to pancytokeratin, but negative for SMA, vimentin, S-100 protein and GFAP.

Pujary et al in 2008[69] investigated a case of hyalinizing CCC using the immuno markers CK AE1/AE3, S-100 and vimentin. The authors have found that H/P, the tumour was partly encapsulated with infiltrative margins, and was composed of large polygonal cells with clear cytoplasm and distinct cell borders admixed with smaller cells having amphophilic cytoplasm and the nuclei were large, oval to slightly irregular with coarse chromatin. The neoplastic cells were arranged in nests and trabeculae, surrounded by fibrous stroma showing hyalinization. PAS stain with and without diastase showed intracytoplasmic glycogen in some of the tumour cells. In their study, IHC showed the tumour cells to be positive for CK AE1/AE3 whereas S-100 and vimentin were negative.

Deihimy et al in 2008[70] in their study of 25 cases of PA with the immuno markers GFAP, vimentin, MSA and S-100 found that all nonluminal cells and chondromyxoid areas in all PAs were positive (more than 50% tumor cells) for GFAP and vimentin.

MATERIALS AND METHODS

MATERIALS

The archival paraffin blocks of 20 salivary tumors from a review of 225 reported salivary gland entities were retrieved from the Department of Oral Pathology & Microbiology, Tamil Nadu Government Dental College & Hospital, Chennai 600 003. Of these 20 cases, both classic and difficult to diagnose cases with routine and special sections included PA (3), PA with overlapping features (4), ACC (2), ACC with overlapping feature (2), ACC/SDC (1), PLGA (3), BAC (1), tumors with clear cells (2) and unclassifiable (2). These 20 cases were subsequently studied with selective immunohistochemical markers to support or confirm the original diagnosis in the classic cases and the provisional microscopic diagnosis in the difficult cases. Streptovidin-peroxidase technique was employed to these cases immunohistochemically. To assure proper staining, salivary gland fragments, blood vessels and connective tissue fibers present in the sections adjacent to the tumor and within the tumor were used as internal positive controls. The antibodies used in the study as well as specific protocol information are listed in the Table 1.

METHODS

HEMATOXYLIN AND EOSIN STAINING PROCEDURE:[71]

1. Sections were deparaffinised with xylene.
2. Hydrated with descending grades of alcohol.
3. The sections were drained and transferred to hematoxylin, where they were left for 10 minutes.
4. The slides were then drained and washed in running water until the sections were

blue.

5. The sections were dipped in acid alcohol where they were agitated for a few seconds and again washed in running water until blue again.
6. The sections were counterstained with Eosin for 30 seconds.
7. The sections were washed in running water for 3-4 minutes, to differentiate the Eosin.
8. After draining, the sections were dehydrated in ascending grades of alcohol.
9. The sections were cleared with xylol, where they were given two changes for 30 seconds each..
10. The sections being clear, the slides were dried and mounted with DPX (Distrene 80 Dibutyl phthalate Xylol) under a cover slip.

Results

1. Nuclei: Blue.

2. Cytoplasm: Varying shades of pink.

Special stains like mucicaramine, PAS, PAS-D, PTAH were employed for selected cases.

MATERIALS USED FOR IMMUNOHISTOCHEMISTRY STAINING PROCEDURE:

1. Primary antibodies:

i) Anti actin antibody (1A4)

ii) Anti vimentin antibody (V9)

iii) GFAP antibody (GA-5)

iv) Anti CD 117 (C-KIT) antibody

v) Anti cytokeratin antiboby. (AE1/AE3)

vi) S100 (few cases)

2. Secondary antibody kit: (Super Sensitive™ Polymer-HRP IHC Detection System)

i) BioGenex peroxidase block

ii) BioGenex super enhancer

iii) BioGenex polymer-HRP complex

iv) BioGenex power block

v) BioGenex DAB chromogen

vi) BioGenex DAB substrate buffer

3. Counterstaining reagent:

i) Harri's hematoxylin

4. Dehydrating agent:

Graded alcohol (100%, 95%, 90%, 70% and 50%)

5. Wash buffers:

i) Tris buffered saline (TBS) (pH-7.6) – 0.30 gms of Tris buffer mixed with 4 gms of Nacl.

ii) Distilled water

6. Xylene

7. Antigen retrieval solution

Citrate buffer (pH-6) – 500 ml of distilled water mixed with 1.05gms of citric acid salt.

8. Pap pen

9. Mixing vial

10. Pressure cooker

11. PH analyse meter.

MATERIALS USED FOR HEMATOXYLIN AND EOSIN STAINING PROCEDURE:

1. **Clearing agent:**

   Xylene

2. **Dehydrating agent:**

   Graded alcohol (100%, 90%, 70% and 30%)

3. Hematoxylin
4. Eosin
5. **Mounting agent:**

   DPX (Distrene 80 Dibutyl phthalate Xylene)

6. Cover slip.

IMMUNOHISTOCHEMISTRY STAINING PROCEDURE[72]

1. SECTIONING:

Tissue sections of 3 micron thickness were made in leica semi-automated (Rm 2245) microtome and taken onto the gelatin coated slides.

2. DEPARAFFINISATION:

The sections were deparaffinized by immersing the slides in xylene for 2 changes.

3. REHYDRATION:

The sections were rehydrated by taking them through 2 changes of alcohol. Then the slides were kept immersed in distilled water for 30 seconds.

4. ANTIGEN RETRIEVAL:

Antigen retrieval was done by using standard pressure cooker method containing boiling citrate buffer solution for 5 minutes.

5. IHC STAINING PROCEDURE:

All the reagents stored in refrigerator were brought into room temperature (24-28 degree Celsius) prior to immunostaining. At no time, the tissue sections were allowed to dry during the staining procedure.

Step 1: Blocking of peroxidase activity

After tapping off the excess buffer from the slides, the sections were covered with 3% hydrogen peroxide block for 15 minutes following which slides were gently washed with Tris buffer (TBS) and kept for 5 minutes in the same solution.

Step 2: Power block

Sections were treated with power block for 10 minutes. After the power block, slides were not treated with Tris buffer.

Step 3: Primary antibody application

The excess buffer was tapped off and the sections were covered with optimally diluted primary antibody. The slides were incubated for 1 hour with CK, SMA and GFAP and 1½ hour with vimentin and half-an hour for S100. Then slides were washed gently with TBS and kept in TBS bath for 5 minutes.

Step 4: Link (secondary antibody application)

After tapping off excess buffer, the sections were incubated with link antibody for 45 minutes and then rinsed gently with TBS and kept in TBS bath for 5 minutes.

Step 5: Streptavidin peroxidase application

The excess buffer was tapped off and the sections were incubated in streptavidin peroxidase for 30 minutes. Then slides were washed gently and kept in TBS bath for 5 minutes.

Step 6: Substrate chromogen application

The tissue sections were completely coated with freshly prepared substrate

chromogen solution using pasteur pipette for 5-10 minutes (or) until acceptable colour intensity has reached. The slides were then washed with TBS and counterstained.

Step 7: Counterstaining

The sections were immersed in Harri's hematoxylin for 10 minutes and then washed gently under running water and allowed to dry.

Step 8: Mounting

The sections were dehydrated, cleaned in xylene and then mounted using DPX.

OBSERVATIONS & RESULTS

PATHOLOGIC FINDINGS

PLEOMORPHIC ADENOMA

A total of 7 PAs was included in this study, out of which, 3 were classic cases and 3 had resemblance to ACC and the other resembled PLGA. Histopathological features of theses cases were as follows:

Classic case 1, showed clear oncocytic cells with oval nuclei as well as few cells with eosinophilic granular cytoplasm and also displayed focal areas of stellate shaped cells in a myxoid stroma. There was no capsule and the tumor mass was under the cover of overlying epithelium.

Classic case 2, showed a well encapsulated tumour with foci of chondroid areas, double layered ductal structures with clear cells in the outer layer, adipocytes and squamous metaplasia with keratin pearl were also noted. Uninvolved normal minor salivary tissue was found at the edge of the lesion.

Classic case 3, showed sheets of plamacytoid cells, glandular epithelial cells and hyaline globules. The lesion was well circumscribed by fibrous capsule.

PA that resembled PLGA, showed partially circumscribed lesion consisting of isomorphic cells with washed out nuclei arranged in sheets and ductular configuration. Papillary projections into the cystically dilated ducts were also found. Admixture of adipose tissue was also evident.

PA that resembled ACC, showed cribriform areas and tubular growth patterns with dark staining angular cells and the stroma ranged from myxoid to hyaline.

PLGA

PLGA comprised 3 cases in this study. In general, all the PLGAs exhibited diverse morphologic growth patterns. Two PLGA showed solid lobular or organoid

growth pattern, cystic and fascicular growth patterns. The peripheral cells in solid lobular growth pattern showed palisading. The cells were either isomorphic, pale oval shaped nuclei with washed out nuclei or large, eosinophilic cells with granular cytoplasm. Areas of clear, squamous and mucous cells were also seen strikingly. Spindle cells comprised the fascicular growth pattern. The solid lobular growth pattern was surrounded by PAS positive hyaline material. The stroma of these tumors varied from fibrous to focal myxoid areas or fibrous to hyalinized areas. Both were partially encapsulated. The third PLGA showed tumors cells arranged as solid islands with cribriform spaces, and solid nests arranged in an organoid growth pattern. The individual cells were isomorphic and showed pale staining nuclei.

ACC/SDC

ACC comprised of a total of 4 cases. The first case of ACC showed two component: an ACC component and BA component. The ACC component showed hyperchromatic cells arranged in a cribriform growth pattern. In the BA component, solid, trabecular and tubulo-ductular growth pattern with interconnecting strands were noted. The individual cells appeared basaloid and columnar in shape. The solid island showed squamous eddies. The stroma in this field was more vascular. Both foci merged with each other imperceptibly.

The second case of ACC showed an ACC component and CA component; the ACC foci showed cribriform and tubular growth pattern. The tubules were lined by an inner layer of darker staining cells and outer clear cells. In the centre of the field, the CA foci showed interconnecting strands of double layer of epithelial cells supported by vascular stroma. The lesion was encapsulated but showed capsular infiltration by the ACC component.

Third case of ACC was entirely comprised of solid islands with small,

hyperchromatic, angular cells with inconspicuous cytoplasm.The tumor islands were surrounded by retraction spaces in some islands. No other growth pattern was evident in the section.

The fourth case of ACC exhibited nodules of tumor cells arranged in cribriform and tubular growth patterns. The individual tumor islands were hypocellular and contained abundant hyalinized tissue organized into small globules or spherules. The hyaline material was surrounded by hyperchromatic, angular to ovoid cells. Basophilic mucin predominated in cribriform and ductular spaces. The cribriform nests contained cells with round to oval hyperchromatic nuclei with scant cytoplasm and in some cribriform islands, cells with pale nuclei surrounding vascular spaces were noted. At the periphery of the tumor, perineural and perivascular invasion as well as infiltration into the minor salivary gland was noted.

SDC

Histologically, the tumor consists of solid and pseudo cribriform nests. Many of the nests showed central comedonecrosis and tumour islands were surrounded by dense fibrous tissue. Neoplastic cells were cuboidal to polygonal with moderate amount of cytoplasm and showed higher grade of nuclear cytology**.**

BAC

It was composed of uniform, densely packed small basaloid cells and large polygonal cells forming solid lobules, sheets, islands or nests. The periphery of the tumor nests was lined by a row of palisading hyperchromatic cells. Some nests showed foci of hyalinized material within the tumor islands. In most islands a PAS positive hyaline material was found. The overlying epithelium was ulcerated and the tumor cells showed infiltration into the minor salivary glands.

UNUSUAL ADENOCARCINOMA:

Two cases of adenocarcinoma defied categorization. On of the case showed multiple growth patterns that include solid islands, cribriform, tubular, cystic, trabecular and trabeculo-tubular growth patterns. The cribriform and ductular spaces contained basophilic to eosinophilic material. In some areas, the tumor cells were arranged into interconnecting strands with luminal columnar and basal cells. In general, the cells were isomorphic but the tumor islands with or without cribriform spaces showed high grade nuclear cytology, especially at the periphery. The stroma ranged from fibrous to myxoid in nature. Perineural invasion was also observed.

The other adenocarcinoma showed features consistent with CA and PLGA/ACC. CA foci displayed double layered columnar to cuboidal cells arranged in canalicular pattern. The stroma was fibrillar to vascular. Solid interconnecting islands with lumina were also evident. In other fields, solid islands and trabecular-tubular pattern reminiscent of BA was also noted. Foci of cribriform and tubular growth pattern resembled PLGA or ACC. Infiltration into bone was also noted.

CLEAR CELL SALIVARY GLAND TUMORS

Two cases of clear cell salivary gland tumors comprised in this study showed clear cells arranged in an organoid nest and surrounded by fibrous to hyaline stroma. In one case small eosinophilic cells were also noted. One was unencapsulated and the other showed partial encapsulation. PAS was reactive in one case but was diastase labile. Both cases did not react with either mucicarmine or PTAH stain.

IMMUNOHISTOCHEMICAL FINDINGS – TABLE 1.

PA

Classic (case 1) showed intense expression of c-kit as well as CK in the clear (oncocytic) cells and in the inner cells of ducts. SMA was expressed by myoepithelial cells and vimentin was observed in the stromal vessels. GFAP was negative.

Classic (case 2) showed SMA reactivity limited to stromal vessels whereas c-kit, GFAP and vimentin were negative. In contrast CK was weakly expressed by luminal cells of bilayered and single layered ducts.

Classic (case 3) exhibited intense positive reactivity to vimentin and CK in plasmacytoid cells. GFAP showed focal positivity of plasmacytoid cells. In contrast c-kit and SMA were negative**.**

In the PA resembling PLGA, c-kit was strongly expressed in the luminal cells of ductal stuctures. Vimentin showed intense reactivity restricted to abluminal cells of ductular structures and showed diffuse reactivity in the stromal component. CK was expressed strongly in luminal cells of ductular structures, whereas SMA showed focal reactivity limited to abluminal cells of ductular structures. GFAP was found to be negative.

The other 3 cases of PA resembling ACC, showed positivity to c-kit exclusively of the inner luminal cells of the duct-like structures and cribriform areas, whereas SMA positivity was observed only in the abluminal cells of ductular structures. Out of these 3 cases, 2 cases were positive for vimentin, mainly in the abluminal cells of ductular structures and in epithelial component, while only one case showed focal reactivity to GFAP.

PLGA

Of the 3 cases of PLGA, two PLGA with predominant oncocytic cells showed intense reaction to c-kit, which showed diffuse cytoplasmic reactivity in the oncocytic cells. SMA staining was limited to the stromal vessels, while vimentin was found in the basal part of cell membrane in one and in few scattered cells in both PLGA. CK was reactive in both PLGA. In contrast, in the other PLGA without oncocytic or mucous cells, both SMA and vimentin showed similar staining pattern in the peripheral cells in the organoid nests, and in the peripheral cells, lining cells and rest of the tumor cells in the solid cribriform growth. However, staining with SMA was more intense than vimentin. CK showed luminal cell reaction in the respective growth patterns. No reaction was found with GFAP in all three PLGA.

BAC

Vimentin, CK and c-kit showed diffuse reaction in the solid islands and sheets. SMA expression was limited to the vessels in the stroma. GFAP was negative.

ACC

In ACC with BA component, c-kit and CK showed similar expression pattern in the luminal cells and squamous eddies of the solid islands in the BA foci. In the ACC foci the expression was noted intensely in the cribriform growth pattern. SMA expression was expressed found in the abluminal cells in the BA foci and in the cribriform growth of the ACC foci, while vimentin and GFAP was negative in both foci.

In the ACC with CA foci, c-kit, vimentin and SMA staining were negative in the CA foci, whereas in the cribriform growth pattern of ACC foci showed expression with SMA and c-kit but not to vimentin. GFAP and CK were negative in both foci.

In the ACC with extensive sclerosis, SMA reactivity was observed in the cribriform and tubular growth patterns. Vimentin staining was confined to the

capsule. In the larger islands with and without cribriform spaces or sclerotic areas were either negative or weakly reactive to SMA. CK expression was noted around the blood vessels within the cribriform growth pattern. Similar staining pattern was also noted for c-Kit. No staining reaction was obtained with GFAP staining.

In the solid ACC, tumour cells were generally unreactive to SMA and vimentin but few cells showed reaction with vimentin. Instense staining with these markers were observed in the stromal connective tissue fibres. c-Kit showed diffuse reaction in the tumor cells of the solid islands.

No reaction was noted with GFAP.

SDC

A negative reaction was noted in the tumor cells of SDC for all markers. The expression of SMA was restricted to the blood vessels and connective tissue fibers at the periphery of the tumour islands. Vimentin showed diffuse expression in the stromal fibres.

UNUSUAL ADENOCARCINOMA

In the unusual adenocarcinoma, CK expression was found in the centre cells and vimentin at the periphery in the trabecular, trabecular-tubular growth pattern. In the solid growth pattern CK expression was diffuse, staining both the centre and peripheral cells. Vimentin and SMA expression in the solid islands was similar to the trabecular growth pattern. Both showed expression in the solid sheets with pseudolumina at the periphery and in scattered tumor cells. Expression of c-kit was diffuse without any recognizable pattern of expression. No reaction was obtained with GFAP.

In the other unusual adenocarcinoma with features of CA and ACC/PLGA, S100 showed strong positivity in the luminal cells (nuclear staining) that lined the

canals or lumen. In the solid islands with tubules the expression of S100 was found in a mosaic pattern. CK showed weak reactivity in the luminal cells and no reaction was noted in the ACC or PLGA component. Expression of SMA was noted in a dot-like pattern at the periphery of cords with lumina or canals. SMA did not stain the tumor cells in the ACC or PLGA component. GFAP, vimentin and c-kit were found to be negative.

CLEAR CELL SALIVARY GLAND TUMORS

Of the two CCC, CK, c-Kit and vimentin expression was noted in the tumor cells (clear and eosinophilic cells) of a CCC. No staining with S100 was found and SMA staining was limited to the blood vessels. In the other CCC, CK showed positive reaction but c-Kit, S100 and vimentin did not react with the tumor cells. However, SMA intense staining of the stromal fibres but remain unstained in the tumor cells.

DISCUSSION

Salivary gland tumors comprises a heterogenous group of neoplasms and the

recent WHO classification list some 40 named salivary gland tumors.[1] While some are relatively common tumors, others are quite uncommonly encountered in routine practice. Tumors like PA are considered to be the most common benign salivary gland tumor, and as such, most pathologists are well aware of the histomorphological spectrum of this entity. In comparison, tumors like CA are uncommon in the Indian context and certain malignant tumors, due to rarity and similarity, pose diagnostic difficulty in routine practice. This holds not only true for a subset of tumors (ACC/PLGA/BAC/SDC) but also to a wide variety of salivary gland tumors, as they manifest histological diversity and overlapping features, making pre-operative diagnosis in routine sections difficult.[9, 38, 55] The literature provides adequate information with regard to the use of IHC in salivary gland tumors.[2-70, 73-97] Most pathologists often employ commercially available IHC markers to discriminate potential salivary gland tumors but is not clear whether they provide unequivocal diagnostic clues in routine practice. Tumors like PA, PLGA, ACC, BAC, CCC and unusual tumors were assessed selectively with IHC markers to address the utility in the differential diagnosis.

PLEOMORPHIC ADENOMA

PA is one of the well recognized tumours of salivary glands.[73] It is characterized by its morphological diversity in that it exhibits both gland-like epithelial and mesenchyme-like features. The epithelial component may show ductal proliferation in the form of intercalated ducts with lumina that are lined by a single layer of ductal epithelium encircled by darker staining, clear, angular myoepithelial cells. The cellular proliferation may take nests, solid sheets, or anastomosing cords. The stroma ranges from myxoid and chondroid to adipose tissue. The relative proportions of the epithelial and mesenchymal components vary from more myxoid to

more cellular. The individual cellular phenotype includes plasmactyoid, polygonal or spindle or occasionally, oncocytic in appearance. In most cases diagnosis of PA can be apparent from routine sections but in certain situations it may closely resemble other salivary gland neoplasms that include BCA, PLGA and ACC.[9, 38, 55]

IHC with SMA in PA may show positive reaction in abluminal and spindle cells but not in plasmacytoid, stellate or polygonal cells. The chondroid foci may also be negative with SMA but show positive reaction with vimentin or GFAP.[4, 10, 36, 55, 70] On the other hand, others consider that expression of vimentin can be found, with exception in the luminal and squamous cells, in almost all cells of PA with both epithelial and myoepithelial differentiation.[10, 16, 55] Furuse et al,[55] regard combination of SMA and vimentin to be more useful in identifying myoepithelial cells in PA.

In the present study three classic PA [each predominated by ductal and squamous pearl formation, clear cells (oncocytic) and plasmacytoid cells, respectively] were stained with CK, SMA, vimentin, GFAP and c-kit markers.

In the three classic PA, c-Kit expression was negative in two cases and expression was identified mainly in the clear cells (oncocytic cells) in the positive case, while GFAP was found to be positive in only one. In these classic PA, vimentin was found to be negative in the PA with luminal and squamous differentiation, while expression of SMA was focal and mostly restricted to the stromal vessels (Figure 15 E - H). In the PA with clear cells (oncocytic), SMA was positive in myoepithelial cells but vimentin was restricted to the stromal vessels (Figure 15 D). On the other hand, the clear cells (oncocytic) showed intense expression with CK and c-Kit (Figure A - C). The c-kit expression in clear (oncocytic) cells is interesting in view of its reported unreactivity in oncocytomas.[26, 56] The lack of expression of SMA and vimentin is attributed to lack of myoepithelial differentiation in oncocytic foci.[65]

Yamada et al[4] & Nishimura et al[10] considers that PA with luminal or squamous cell differentiation are less likely to react with vimentin. In the PA characterized by plasmacytoid cells, vimentin, GFAP and CK were positive but SMA and c-Kit was negative (Figure 16 A - D). The variable staining with the muscle markers indicate lack of differentiation towards myoepithelial phenotypes in two PA,[3, 74] as inbuilt controls within the sections showed positive reaction with these markers in the present study (Figure 15 F & G), while reaction with muscle markers in the more cellular PA correlates with the observation by others.[3, 10, 16, 55,60] Interestingly, CK expression was also found in the plasmacytoid cells, which are considered to be modified myoepithelial cells.[22] Staining of plasmacytoid cells by CK have also been reported by Ogawa et al,[75] According to him, plasmacytoid cells in PA are related to luminal cells and do not originate from myoepithelial cells, but myoepithelial markers were also found to be expressed in the plasmacytoid cells in the present study. This is not an unexpected finding as myoepithelial cells contain both epithelial and muscle filaments.[32, 76]

The variable IHC results with regard to GFAP in PA, where all but one was negative, is in contrast to the noted GFAP findings in PA.[5, 23, 38, 61] In the GFAP positive cases staining was limited to the spindle and plasmacytoid cells and in the stroma that conforms to the reported observation for GFAP staining in PA, where positive reaction can be found in the myoepithelial cells or its derived stroma.[5, 19, 38, 61, 70]

PLEOMORPHIC ADENOMA WITH OVERLAPPING FEATURES

Since PLGA may show encapsulation and ductal or tubular growth pattern

accompanied by myxoid or hyaline stroma, differentiation from PA is often difficult on routine sections. [9, 24, 73] On the other hand, cellular and cribriform areas in PA cause diagnostic difficulty with ACC.

In this study, four PA showed close morphological resemblance to PLGA and ACC in view of the cellular and ductal features in the former (one PA), and cellular and cribriform features with regard to ACC (three PA). In view of this potential pitfall, IHC markers like c-kit and GFAP in conjunction with SMA and vimentin was employed to narrow the differential.

According to Zarbo,[77] PLGA may also show focal reactivity with myoepithelial markers, but is generally said to be limited to the spindle cells and cribriform areas.[28] Outer tubular and ductular structures were shown to stain with vimentin and SMA in both PLGA and ACC.[9] In comparison, PA usually show intense reactivity for muscle markers.[77] In the present study, although SMA and vimentin showed reactivity in accordance with the noted observation,[10, 16, 55, 60] their expression was not consistently found in all this four PA. More over, expression of these markers remained equivocal in the area of interest, necessitating further confirmation with the use of c-Kit and GFAP to refine the IHC results. Expression of c-Kit was found in these four PA, whereas c-kit was positive in only one of the three classic PA in the present study.

Interpretation of c-Kit expression in these PA assumes significance, since it is also reported to be expressed in PLGA and ACC.[40, 44, 56, 78] In ACC expression of c-kit can be found in the luminal cells, in the lining cells of pseudocysts, at the periphery and in the proper tumor cells, [44, 56, 57, 59] while in PLGA c-kit can be seen in the luminal and non-luminal cells of ductular and tubular and solid lobular growth patterns but rarely in the lining cells of pseudocysts.[59]

Although c-Kit expression can be found in both ACC and PLGA,[44, 56] the luminal cell expression of c-Kit in these four PA were in accordance with the observation made by others[56](Figure 17 E, Figure 18 D & Figure 19 D). However, in view of similar expression pattern in CA and BA, caution should be exercised when the differential involves these neoplasms rather than PLGA and ACC.[56]

GFAP was reactive in one of the PA that had resemblance to ACC but not in the other PA that had resemblance to PLGA (Figure 18 E). GFAP staining in PLGA may only be limited to the epithelial cells or negative,[23, 24, 38, 61] and those cases where they are positive are generally focal when compared to the focal to diffuse pattern of staining in PA.[23] On other hand, ACC lacks GFAP reactivity. Although staining with GFAP helped in the differential with ACC in one PA (Figure), the pattern of c-Kit expression was more helpful in the differential with other ACC and PLGA in our study. The utility of GFAP in the differential was found to be less useful in this study since it was expressed in only two of the PA.[9, 23]

BASAL CELL ADENOMA

BA was considered as distinct from PA because of lack of myxochondroid but with hyalinized matrix and isocellular features without myoepithelial cell participation.[33] However, periductal, epitheloid and spindled (stroma-like) myoepithelial cells may contribute to the neoplastic proliferation in BA, indicating its pleomorphic cell content with regard to staining with muscle markers.[33, 79] According to Zarbo et al,[33] all patterns of BA show myoepithelial differentiation but most pronounced in the trabecular-tubular variant of BA, and as such may show positive reaction with muscle markers. The pattern of reactivity also differs in different subtypes. In the tubular form the abluminal cells and in the trabecular or trabecular-tubular forms at the tumor-interface was found to be reactive with SMA, whereas the

central cells were unreactive. In the solid form, the peripheral, palisading dark cuboidal cells have been shown to stain positively,[15, 33,] and in the membranous type, peripheral cells and cells surrounding the hyaline droplets can be stained with SMA.[33]

CANALICULAR ADENOMA

It is a benign salivary gland tumor found often in the minor salivary glands of the upper lip. It is a well circumscribed lesion that manifests a characteristic growth pattern with isomorphic and cuboidal to columnar cells with eosinophilic cytoplasm. The nuclei are regular, oval and elongated, and occasionally, the nuclei impart a pseudostratified appearance. The canalicular growth pattern is considered to be characteristic yet not diagnostic.[47] It is composed of rows of columnar ductal-luminal cells rather than basal type cells. The cells form a double row of strands that may branch and then come together. Hence, the descriptive name canalicular adenoma.[73] The epithelial elements are supported by a stroma that is parvicellular, edematous and finely vascular. Canalicular adenoma may also show solid, trabecular and adenoid pattern (cribriform).[73, 77] These patterns may lead to confusion with BAC, ACC, and PLGA especially when there is no capsule. CA characteristically show intense S100 positivity but only focal GFAP reactivity or lack SMA and vimentin expression.[9, 26, 33, 77] Both CA and BA have also been shown to react with c-kit immunomarker.[56]

In the present study two cases of ACC had acceptable foci of CA and BA, respectively. The ACC with CA features showed double layered columnar strands with vascular stroma and pseudocystic (cribriform) spaces enclosing connective tissue. Areas of classic ACC with solid and tubular configurations were also identified to warrant a diagnosis of ACC on routine sections (Figure 8). The other showed features more of BA than CA in an otherwise acceptable ACC (Figure 9).

IHC results showed positive reaction with CK, SMA and c-Kit but negative

reaction was observed for vimentin and GFAP in the ACC with BA foci (Figure 20 A, B & C). Expression of CK was identified in the luminal cells and in squamous eddies but was negative in the ACC areas. SMA reacted with the abluminal cells in the BA foci and in the cribriform growth pattern in ACC areas (Figure 20 C & D). Expression of c-kit was identified similar to CK expression in the BA foci and was similar to SMA in the cribrifom growth pattern in ACC areas. Vimentin was negative in both foci, while CK was negative in the ACC foci. The IHC observation in the present study is consistent with SMA and CK reactivity in BA,[15, 46] where SMA in abluminal and CK in luminal cells react positively. The positive SMA reaction pattern and negative CK expression is also consistent with IHC findings in ACC.[55]

In the other ACC with CA foci, SMA, vimentin and c-Kit were negative in the CA foci, while SMA and c-Kit but not vimentin was positive in the ACC foci. GFAP stained none of the foci in both cases (Figure 21).

The c-Kit expression in both cases especially in the cribriform areas of ACC foci showed intense reaction (Figure 20 D & Figure 21 B), which is in contrast to Mino et al,[44] who observed that c-Kit expression will be least pronounced in the cribriform growth pattern of ACC. However, Freier et al,[53] have found more intense reaction with c-Kit in the cribriform growth pattern compared to the solid variant of ACC. They surmised that less intense or lack of expression in the solid variant could be due to loss of antigens in a more aggressive growth phenotype, and believe, that differences in clonal evolution of distinct growth patterns in ACC could possibly account for the varied reaction among the subtypes of ACC with c-Kit. In this study intense reaction was not only observed in the cribriform growth pattern in these two ACC but also in the solid and cribriform growth patterns in all the ACC examined as well.

The literature indicate that SMA and vimentin are generally unreactive in canalicular adenoma as it shows only luminal cell differentiation and lacks myoepithelial participation when stained with SMA compared to ACC, which normally show intense staining with SMA.[33, 39, 47] These results and the observation (variable staining in the respective foci) of the present study suggests that ACC could possibly originate from monomorphic adenomas as stated in the literature.[20, 80] Thus, IHC observation in the present study contributed to the diagnosis of ACC ex monomorphic adenoma in these two cases.

ADENOID CYSTIC CARCINOMA

ACC is a clinically and pathologically well described entity, and occurs both in major and minor salivary glands, especially the palate.[73] The growth patterns can be categorized into three types: (1) the cribriform pattern, (2) the tubular pattern and, (3) the solid pattern. A sclerosing variant of ACC has been emphasized in recent years and was considered to be histologically distinct from the three subtypes of ACC.[58] This variant typically elaborate excessive basement membrane-like material by the tumor cells as a measure of immuno-denfence by the tumor cells.[81]

In ACC, a mixture of growth patterns usually occurs within a single tumor but foci of the cribriform type can usually be found even when one of the others predominates.[73] The cells forming these patterns have been described as isomorphic, in that, they are uniform in size, shape and staining qualities,[73] and show both luminal and myoepithelial differentiation.[81]

A number of tumors share certain common histomorphological features with ACC. The tumors that may pose difficulty in separation from ACC include PA, BA, CA, BAC, PLGA and SDC of minor salivary gland origin, where small biopsies often contribute to diagnostic complexities. Nevertheless, certain characteristic histological

features aid in the separation of these lesions – see below.

PA can be distinguished when it presents its diverse mesenchymal features. Canalicular adenoma show encapsulation and characteristic bilayered columnar strands associated with prominent vascular stroma.[82] BA and BAC may pose considerable diagnostic difficulty in view of its multiple growth patterns, where solid islands of BAC more closely resemble ACC but show peripheral palisading. Although occasionally ACC show palisading, they often show cribriform structures which are uncommon in BA.[73, 83] PLGA and SDC may show solid, tubular and cribriform growth patterns similar to ACC.[73] However, the cribriform spaces in ACC are distinct from PLGA.[84] The cribriform spaces in ACC are composed of replicated extracellular matrix and dual population of cells, while the spaces may contain stromal elements and a single cell type in PLGA.[84] In SDC, the cribriform spaces are surrounded by cells with higher nuclear cytology and comedonecrosis in the solid islands.[14, 63, 84] Despite these subtle histological distinctions, accurate diagnostic interpretations arise when tissue specimens are inadequate, often necessitating the use of a panel of IHC markers to resolve the differential. Vimentin, SMA and c-Kit have been shown to be helpful in delineating ACC from other salivary gland tumors.[40, 44, 53, 56, 57, 59]

In this study two tumors comprised entirely of solid islands permitting no clear cut differentiation from ACC or SDC, although each appeared to be ACC or SDC on routine sections. The cells were more angular and hyperchromatic with high nuclear to cytoplasmic ratio and showed no comedonecrosis in ACC (Figure 10 A) and in SDC, it was characterized by a higher grade nuclear cytology, discernible eosinophilic cytoplasm and comedonecrosis (Figure 10 B & C).

IHC showed negative reaction for SMA and vimentin in the tumor cells but positive reaction in the stromal connective tissue - more intense with vimentin than

SMA, in both cases. Expression of c-kit was found in one of the two (Figure 22).

Since lack of staining with muscle markers characterizes both the solid variant of ACC and SDC,[14, 16, 26, 42, 57, 63] confirmation relied on the role of c-kit in the distinctions. A number of studies have shown more positive correlation with c-kit expression in ACC than in SDC.[40, 45, 56, 57, 59, 78] In contrast, only a single case of SDC has been shown to react with c-Kit in an analysis of 104 salivary gland tumors.[44] These observations indicate that cellular and growth features should be taken into consideration when interpreting IHC with c-Kit in a similar instance like the present study. There were subtle differences, as stated in the preceding, in the cellular and growth phenotypes of these two neoplasms that enabled to categorize them as ACC and SDC, respectively.

In the cribriform growth pattern expression of vimentin and SMA can be found in the cells lining the pseudocyst and proper tumor cells in both PLGA and ACC but the staining intensity or lack of staining in PLGA with SMA is considered to be more helpful in the differential, especially in this growth pattern.[26, 57, 59]

In the present study, an ACC was characterized by extensive sclerosis resembling collagenous spherulues (Figure 10 D – F) IHC revealed SMA reactivity in the cribriform and tubular growth patterns but vimentin staining was limited to the capsule (Figure 23). In the same section, the staining intensity with SMA was weak or absent in the larger islands that either showed cribriform spaces or lacked (Figure 23 E) CK stained mainly blood vessels within the cribriform islands, while c-Kit expression was similar to CK, but it also stained some luminal cells. The results indicate that SMA expression is variable and CK expression limited to the cells around blood vessels than to the expected luminal cell reaction in the cribriform growth pattern[48] – see below.

Sousa et al,[39] have stated that the cribriform spaces represent attempted luminization in a less differentiated tumor that may show positive reaction with CK, while inconsistent expression of CK and vimentin could possibly be linked to the undifferentiated nature of cells in ACC.

Chen et al,[86] proposed that the adenoid cystic carcinoma should be divided into two IHC groups: a group of neoplastic cells with prevalence of ductal formation positive to low and high molecular weight CK's, and the other group of neoplastic cells reactive with SMA and low molecular weight CK.

The lack of CK expression, vimentin and inconsistent SMA reaction in this study is consistent with the above observations in ACC.

BASAL CELL ADENOCARCINOMA/POLYMORPHOUS LOW-GRADE ADENOCARCINOMA

It resembles basal cell adenoma in its growth patterns and lack of cellular pleomorphism but is defined by infiltrative features that include invasion of nerves, vascular or lymphatic spaces, and occasionally, pleomorphism.[73] This tumor is characterized by growth patterns such as solid, membranous, trabecular, tubular and adenoid (cribriform).

The morphologic features of BAC are well described.[73] Solid growth pattern of BAC is characterized by uniform, densely packed basaloid cells forming variably sized nests. The cells in the centre of the clusters are larger and oval to elongated in shape with ill-defined cytoplasmic borders and contains pale nuclei, while the peripheral cells are small and have darkly stained basophilic nuclei with scant cytoplasm that often show peripheral palisading. Within the nest there may be squamous eddies or hyaline droplets.[41, 73] In the trabecular pattern, the tumor cells are arranged in branching, anastomosing cords that had occasional tubular structures with

pseudolumina and areas of microcyst formation.[15, 82] Nuclear palisading will be minimal in this pattern.

PLGA is a distinctive adenocarcinoma characterized by diverse morphological growth patterns thought to be found exclusively in the minor salivary glands.[73, 87] PLGA is often described in most standard text books as a low-grade tumor characterized by infiltrative growth, morphological diversity and cytological uniformity. The uniform cytology is often interpreted as related to the single cell type in PLGA. However, it refers to the low-grade malignant cytological features and not to the different cell types that can be found in PLGA.[24] The varied growth patterns that can be found in PLGA include solid, glandular, cribriform (pseudoadenoid), ductular, tubular (usually a single layer), trabecular, lobular, fascicular (streaming) and occasionally, cystic. The cell types found in PLGA are, generally, described as cuboidal to columnar shaped with a round to oval or spindle, vesicular to stippled or basophilic nuclei that may often overlap.[28, 73, 87] The nuclear to cytoplasmic ratio is less, the cytoplasm is amphophilic to eosinophilic and the nuclei in most instances appear washed out. The cell borders are not distinct.[24, 73, 87] The tumor cells may be associated with mucoid, hyaline or fibrovascular stroma.[73, 87] A slate gray-blue stroma is regarded as characteristic of PLGA.[87] PLGA may also show metaplastic changes, including squamous, sebaceous, and oncocytic features.[87] However, the latter feature have not been found to be prominent in most PLGA described so far.[28, 50, 87, 88]

The histological features of PLGA when present do not pose great diagnostic challenge, but in most instances, because of the diverse growth patterns and histological overlap of these growth patterns with other salivary gland tumors, PLGA may cause considerable dilemma even to an experienced specialist pathologist.[24]

While PLGA have been considered to occur exclusively in the minor salivary

glands, BA and BAC are generally thought to occur in major salivary glands.[15, 73] Since cases of BAC have also been reported to occur in minor salivary glands,[27, 62, 89] distinction of PLGA from BA and BAC can be quite difficult for two reasons; firstly, both may show solid lobular growth pattern with peripheral palisading, as well as intercellular hyaline droplets, and secondly, similar growth patterns [ductular (single layered), tubular and trabecular] can exist in these neoplasms.[15, 28, 41, 42, 73, 87, 90]

Although PLGA can be separated from BA because of lack of encapsulation, the same might make it difficult to separate it from BAC. According to the literature,[9, 15, 27, 56, 62] IHC with BAC have shown to react with CK, c-Kit, SMA and vimentin. On the other hand, PLGA also react with these markers in addition to GFAP reactivity.[16, 23, 24, 28, 38, 61, 77, 87] Since c-kit expression can also be found in BAC, the solid lobular pattern seen in two PLGA of the present study may pose problems with the solid lobular pattern in BA, where peripheral palisading and hyaline material surrounding the solid islands can be seen on routine sections – see above, as also found in two of the PLGA in our study (Figure 11 B & C) More over, oncocytic cells and mucous cells are not features of BAC,[15, 20, 27, 62, 73, 82, 89, 90] but found in these two PLGA.

The pattern of SMA and vimentin expression observed in the present study showed that expression of SMA was limited to the blood vessels in the stroma in these PLGA in agreement with others,[9, 24, 26, 88] while vimentin staining was limited to the peripheral basal part of the cell membrane in one of these two PLGA (Figure 24). In addition, few scattered cells within the solid lobular growth pattern also showed positive reaction with vimentin in both cases of PLGA. Araujo et al,[16, 88] considers that vimentin show reaction in PLGA, while others, [9, 26] is of the view that muscle markers are generally unreactive in PLGA. The observation in the present study is consistent with the latter proposition that myoepithelial cells are not the main

component of these two PLGA. However, the PLGA in the Araujo et al[16, 88] series showed reaction with vimentin in the tumor cells but not in the oncocytic cells, which showed intense reaction with CK 8 and 18. Staining with CK was intense and vimentin did not stain majority of cells (oncocytic and other cells) except for staining in the basal part of peripheral cells and in few scattered cells in the present study.

In contrast to these two PLGA, vimentin staining was found to be diffuse but more intense in the peripheral cells in the solid membranous variant of BAC in the present study similar to the reported IHC results in BAC [9, 15, 27, 62] (Figure 25 G – J). On the other hand, SMA staining was limited to the blood vessels in the stroma but c-kit and CK expression was diffuse and less intense compared to the intense staining in the two PLGA cases**.** The CK and SMA results of the present study are in agreement with the IHC observation in BAC by others.[62].[25] GFAP was found negative in both PLGA and BAC in our study.

Overall, the IHC results with regard to BAC in our study is consistent with the noted observations,[9, 15, 25, 27, 62, 56] while the intense c-kit expression noted for the PLGA in the solid lobular pattern (predominantly oncocytic and mucous cells) is an observation contrast to the negative c-Kit expression in tumors with oncocytic cells.[26, 56] In PLGA, variable (negative through moderate to positive) results were obtained with c-Kit by others.[40, 44, 45, 56, 78]

Another PLGA, in this study, with solid islands and small nests arranged in an organoid pattern but without peripheral palisading showed reaction with CK, c-Kit, vimentin and SMA but negative for GFAP.

The staining pattern with SMA and vimentin were more or less similar but the intensity of SMA and vimentin staining showed subtle differences. While both stained the larger solid islands characterized by cribriform spaces and pseudolumina in the

cells lining these structures, proper tumor cells and the cells at the periphery, the staining of vimentin was more on the basal part of the peripheral cells compared to the more intense membranous pattern of staining with SMA. The small nests arranged in an organoid pattern showed staining of the peripheral cells, while the central cells remained unreactive with SMA and vimentin. Off the two markers, SMA showed intense reaction (Figure 25 C & D) CK showed luminal staining in both the solid islands and organoid nests, while c-Kit showed a mosaic pattern of staining (Figure 25 F).

Although the solid islands with cribriform spaces and pseudolumina are not typical of BAC on morphological grounds, the pattern of expression of SMA and vimentin may be confused with BAC, if morphological features and CK expression are disregarded.

The literature indicates that staining with these markers have shown positive staining of the cells at the periphery more intensely than cells in the centre of the solid islands with both SMA and vimentin.[27] These IHC observations in BAC are very similar to the observation in PLGA, in the present study, with regard to SMA and vimentin staining pattern in the organoid nests but CK expression was noted only in the luminal cells (tubular and solid) and SMA plus vimentin stained the solid islands with or without cribriform spaces similar to the observation in PLGA (Figure 25 A – E).[11, 16, 24, 57, 59] The expression of c-Kit was found in a mosaic pattern in the solid islands with cribriform spaces or pseudolumina similar to the observation made by Epivatianos et al[59] (Figure 25 F), who found c-Kit staining in PLGA in the luminal and non-luminal cells of ductular and tubular and solid lobular growth patterns but rarely in the lining cells of pseudocysts. On the other hand, expression of c-Kit in BAC has been reported in a membranous pattern in all forms of BAC in less than 25%

of cases.[56] In the present study with regard to three PLGA and one BAC, morphological correlation with IHC confirmed the diagnosis made on routine sections in three PLGA and BAC in one.

UNUSUAL ADENOCARCINOMAS

Morphological diversity (growth and cellular features) is characteristic of most salivary gland tumors. Tumors manifesting different growth characteristics are often difficult to categorize into specific entity. Such tumors are at best be categorized as 'not otherwise specified'. At other instances, a well differentiated low-grade tumor may show a higher grade focus typical of other defined entities and these tumors have been described as de-differentiated.[42, 48, 91] The most noted finding is the presence of SDC focus in an ACC. On the other hand, hybrid tumors consisting of two defined entities in a single topographical region have been described in both major and minor salivary gland tumors.[42] The most common is PA and EMC or ACC and EMC; others include SDC and ACC, BAC and PLGA.

In the present study two adenocarcinomas with overlapping features was also studied. One was characterized by multiple growth patterns (solid, cribriform, tubular, cystic, trabecular and trabecular-tubular forms) and the other primarily showed features consistent with CA but had focus that could be seen in either PLGA or ACC. Both were difficult to classify on routine sections. They were similarly stained with the IHC markers used in this study; in addition, S100 was also employed.

In the adenocarcinoma represented by multiple growth patterns, IHC showed CK expression in the luminal cells and vimentin in the abluminal cells in the trabecular growth pattern**,** while CK expression was diffuse in the solid islands and vimentin stained the peripheral cells (Figure 26). Similar IHC patterns have also been reported in the trabecular growth pattern in BAC.[15, 27] Further more, the peripheral cell

reaction with vimentin in the solid islands of BAC reported by Quddus et al,[27] is also similar to our staining reaction with this marker but the pattern of CK expression is different from their report, where it was unreactive in the central cells in the solid islands**.** In constrast, Nagao et al**,**[42] have observed diffuse CK reaction in the solid islands similar to the present study.

On the other hand, the pattern of vimentin expression in the solid sheets with pseudolumina would simulate the pattern of expression in PLGA,[59] where vimentin have been shown to react with cells at the periphery, proper tumor cells and around the cribriform spaces (Figure 27 B, D & E). Similarly, the pattern of CK expression in both luminal and abluminal cells (Figure 27 A)**,** as noted here, would lead to misinterpretation if care is not exercised to distinguish the cribriform spaces in PLGA from the pseudolumina commonly seen in BAC.[15, 20, 27, 82] While cribriform spaces are characteristically seen in PLGA or ACC, they are not typical of BAC.[77] The diffuse staining obtained with c-Kit is also consistent with BAC.[56]

Taken together, the observation of morphological growth patterns and cellular features in conjunction with the varied IHC results for different growth patterns is more consistent with BAC. It may be noted that although in some fields the growth or cellular features were more similar to PLGA or SDC (Figure 13 D), such areas could possibly represent NOS in an otherwise BAC or the phenomenon of de-differentiation.[48, 91] Some consider that overlapping features can occur among tumors that show myoepithelial differentiation and in such cases, they do not alter the basic diagnoses or prognostic outcomes**.**[92]

In the other adenocarcinoma with features of CA and ACC/PLGA, S100 marker was utilized in addition to other markers employed in this study. The lesion showed S100 reaction strongly in the luminal cells that lined the canals or lumen,

while it showed a mosaic pattern in the rest of the tumor cells. The intense reaction with CK and S100 is consistent with the observation found in the literature,[9, 17, 33, 77] where it is stated that CA characteristically show intense S100 positivity, but the reactivity was noted most intense in the luminal columnar cells (mainly in the nuclei) that lined the canals or tubules, while it showed a mosaic pattern in the solid areas in the present study (Figure 28 B & C). This observation is opposite to the staining of outer cells in CA with S100 noted by others.[17, 66]

Although CA have been noted to lack expression of SMA by most others,[17, 26, 33, 39, 47, 49, 77] the expression of vimentin in CA is conflicting. [17, 39, 47, 49] Ferreiro et al,[17] have found strong vimentin expression in the outer portions of the tubules or canals, while occasional tumor cells and stroma showed positive reaction.[17, 47]

In the current study, vimentin was negative whereas SMA was positive in the outer portions of tubules or canals and stromal fibres in regions that had typical features of CA. Contrary to the present observation, Zarbo et al,[33] believe that no such staining was possible even in the CA that had overlapping features with BCA. An observation they consider important in distinguishing CA with BCA features from BCA. It is beyond the scope of this study to explore further into the possibility of uni (luminal) or bicellular (luminal/myoepithelial) differentiation in CA. However, the distinction is not important with regard to the biology of these entities. On the other hand, both vimentin and SMA was negative in the transformed foci that showed tubular and cribriform growth patterns corresponding to PLGA or ACC foci. The latter observation is diametrically opposite to the expression pattern noted with these markers in PLGA or ACC by others,[9, 11, 13, 16, 26, 47, 55, 57, 59, 60] as well as in the present study – vide supra**.**

Since PLGA may also show bilayered cells characteristic of CA, Furuse et

al,[55] regard the use of vimentin in the differential between CA and PLGA, where vimentin strongly stains the PLGA but not CA. The lack of SMA or vimentin expression in the adenocarcinoma foci (ACC/PLGA) indicate that these antigens might have lost during transformation. However, this observation is also in contrast to the observation made in the present study with regard to the ACC with CA foci, as noted above.

Lack of encapsulation, as noted in the present study, was also noted by most others in CA,[93, 94] and as such would lead to a consideration of malignant transformation especially in view of the adenocarcinoma foci (PLGA/ACC) observed here.

As to whether this lesion is nothing but a multifocal CA [73, 82, 93] or a malignant transformation of CA, the literature emphasizes the importance of the former possibility than the later likelihood.[94] However, the clinical presentation and microscopic evidence of invasion of the adenocarcinoma component indicates a transformation process rather than multifocality in this study.

CLEAR CELL CARCINOMA

Clear cells can be found in many salivary gland tumors that mainly include oncocytoma, mucoepidermoid carcinoma, acinic cell carcinoma, myoepithelial carcinoma, and principally, in epithelial-myoepithelial carcinoma and clear cell carcinoma.[73, 95] Most or all of these neoplasms show reactivity with PAS but some are labile with PAS-D indicating the presence of glycogen, while acinic cell carcinoma show cytoplasmic granules when digested with diastase. On the other hand mucoepidermoid requires mucin stains to confirm the clear cell nature. Therefore, diagnosis of tumors with predominant clear cells always poses diagnostic challenge in routine sections.[43, 73, 95] Oncocytoma may show positive reaction with PTAH but in

some cases requires electron microscopic evaluation.[73, 95]

In the present study two salivary gland tumors comprised almost or entirely of clear cells arranged in an organoid pattern and solid nests surrounded by dense fibrous tissue. One of these showed cells with eosinophilic cytoplasm but no ductal/tubular differentiation, bimorphic cell layers or spindle and hyaline cells were identified in both lesions. One was unencapsulated and the other was partially encapsulated. Both were stained with the special stains mentioned – see above. Negative reaction with mucicarmine, PTAH and lack of secretory granules with PAS-D helped to exclude mucoepidermoid carcinoma, oncocytom and acinic cell carcinoma. Since epithelial-myoepithelial carcinoma may be composed of predominantly clear cells,[73] IHC with the markers used in this study plus S100 was employed in to refine the differential.

IHC showed CK expression in both tumors, while S100 was negative. IHC with SMA showed reaction in both tumors mainly in the stroma (Figure 29 C & Figure 30 A & B). On the other hand, vimentin showed intense reaction both in the stroma and tumor cells (clear and eosinophilic) in one of these two but was negative in the other (Figure 29 A).

The literature revealed that CK, S100 and muscle markers are generally positive in epithelial-myoepithelial carcinoma, indicating participation of ductal and myoepithelial cells in this tumor compared to luminal cell differentiation in clear cell carcinoma. [43, 73, 95] Therefore, this IHC pattern will be of use in the differential with clear cell carcinoma and others.[7, 26, 43, 96] However, in the present study S100 was unreactive in both tumors, which are generally intense and diffuse in epithelial-myoepithelial carcinoma predominated by clear cells.[35, 43, 73] On the other hand, muscle markers are reported to be negative in clear cell carcinoma,[7, 31, 43, 96,] but was found in this study.

Wang et al,[43] consider that clear cell carcinomas are devoid of myoepithelial markers. Nevertheless, some clear cell carcinomas may show positive reaction with muscle markers.[12, 18] Felix et al,[97] argues against the possibility of myoepithelial cell participation in CCC based on the lack of relation between the hyaline stroma seen in CCC and the basement membrane type material elaborated by tumors with myoepithelial cell participation. In contrast, the stroma was intensely positive to SMA in focal areas in the present study. Some consider that stromal reaction to SMA or other more specific myoepithelial markers might indicate the acquisition of smooth muscle phenotypes by stromal elements in salivary gland tumors.[33]

The positive reaction with muscle markers but negative reaction with S100 is more indicative of clear cell carcinoma and excludes the possibility of epithelial-myoepithelial carcinoma, clear cell myoepithelial carcinoma, mucoepidermoid carcinoma, acinic cell carcinoma and oncocytoma.[7, 21, 26, 67, 75, 90, 96] However, the immunoprofile of the present study should be carefully evaluated when the differential involves renal cell carcinoma in view of their positive reaction with vimentin.[31] Reaction with S100 and SMA was reported to be negative in renal cell carcinoma.[31] Additionally, one of the two clear cell carcinomas showed moderate (clear cells) to intense (eosinophilic cells) c-Kit reaction, a finding not been reported either for clear cell carcinoma or other primary salivary gland tumors with clear cell predominance.

Although the present immunoprofile in the two clear cell carcinomas was useful to exclude other clear cell salivary gland tumors, the appreciation of lack of pronounced atypia and dilated vessels, and clinical examination was more helpful in the differential with renal cell carcinoma than the reliance on the use of IHC in the differential. It is not clear whether c-Kit will be reactive in renal cell carcinoma, and

hence, their usefulness in the differential cannot be over emphasized.

APPLICATION OF IHC IN ROUTINE PRACTICE

The literature provides adequate information on the use of IHC in the assessment of salivary gland tumors, and as such, shall be considered to provide documentary evidence for comparison. However, such studies usually involve a large sample, yet restricted tumor types, and the results represent the overall positive or negative findings, implicitly conveying an impression of better correlation for diagnostic uses. But for routine diagnostic practice purpose the use of IHC may actually complicate diagnostic interpretations, especially when IHC sections are reviewed by another pathologist or pathologist having limited experience regarding the complexities of cellular and growth patterns that may occur in a variety of salivary gland tumors. For example, a focal area of positive reaction at the edge of the section may result from edge effect and when this is overlooked may lead to false positive interpretations. Similarly, when a given marker stains only the cells at the tumor/stroma interface and cells in the stroma rather than the majority of tumor cells would likely be construed as negative with that particular maker(s). This was evident in some of the PLGA in this study where the staining intensity with a certain maker was found to be variable, that ranged from less intense to focal or intense staining depending upon the cellular composition and growth patterns. When an unexpected result occurs, as found in the two PLGA with oncocytic cells and in the clear cell carcinoma, the final interpretations may be complicated when the growth patterns and cellular heterogeneity are not taken into consideration. The same inference can also be drawn for unusual tumors (in this study) if the pattern and intensity of IHC reactivity in the whole section are overlooked. Tumors showing features of two defined entities may lead to errors in interpretation when the secondary focus are not recognized as

minor component or given more importance. In such situations, it should be categorized based on the predominant morphogenetic growth features rather than based on the IHC outcome. On occasion the field of interest may not be found in the IHC sections either due to technical deficiency in the IHC procedure or due to loss of tissue material in the block. In such situations it is difficult or impossible to determine the specific diagnosis for what IHC was actually intended. Such difficulty encountered in the present study necessitated re-staining in few instances but this will add to the financial burden for the patients in routine practice. Nevertheless, IHC may serve as a useful tool if all these potential pitfalls are kept in mind and when the IHC interpretations are made in conjunction with a reference H & E section.

SUMMARY

About 20 salivary tumors were retrieved from a review of 225 reported salivary gland entities from the Department of Oral Pathology & Microbiology, Tamil Nadu Government Dental College & Hospital, Chennai 600 003. Of these 20 cases, both classic and difficult to diagnose cases with routine and special sections included PA (3), PA with overlapping features (4), ACC (2), ACC with overlapping feature (2), ACC/SDC (1), PLGA (3), BAC (1), tumors with clear cells (2) and unclassifiable (2). These 20 cases were subsequently studied with selective immunohistochemical markers to support or confirm the original diagnosis in the classic cases and the provisional microscopic diagnosis in the difficult cases. Streptovidin-peroxidase technique was employed to these cases immunohistochemically.

The results of the present study showed that PAs exhibited positivity to CK in 100% of cases, vimentin in 71% of cases, SMA in 57% of cases and c-kit in 71% of cases. PLGAs were positive to CK, vimentin and c-kit in 100% of cases and to SMA in 50% of cases. ACCs showed positivity to CK in 50% of cases, vimentin in 25% of cases, SMA in 75% of cases and c-kit in 100% of cases. GFAP staining was negative in all ACCs, PLGAs and 70% of PAs. BAC exhibited reactivity to all markers except GFAP, whereas SDC was negative to all markers (CK, c-Kit, GFAP, SMA & Vimentin). CCC demonstrated positivity in 100% of cases to CK and SMA (stroma only) and in 50% of cases to vimentin, while c-kit and S-100 was negative. Unusual adenocarcinomas were positive to CK and negative to GFAP in 100% of cases, whereas 50% of cases were positive in vimentin, c-kit and SMA. Out of the two unusual adenocarcinomas S-100 was used in only one case, where it was positive. Of the markers used in this study, SMA, vimentin and c-Kit have been found most useful in the differential of PA, PLGA, BAC, and ACC. Further, the pattern of reaction with

these markers both between different tumor entities and within an individual growth pattern of a tumor was found to be more useful than the outcome of quantitative or qualitative staining.

CONCLUSION

The use of IHC as a supplemental diagnostic tool in border line or difficult salivary gland tumors may well augment the routine microscopic differential as found in this study for most cases**.** However, caution should be exercised when interpreting the IHC results because of similar but not identical results observed for certain tumors and in certain fields of the same section in a given tumor for a given marker. The latter observation is important when interpreting IHC sections of a tumor with NOS or transformed foci. Therefore, it is observed that reliance on the positive or negative IHC results without actually observing the pattern of reactivity or lack of it may likely lead to inappropriate diagnosis, especially when growth patterns and cellular features are overlooked or not interpreted in conjunction with H & E slides. Additionally, the cost and time factor should be considered before advocating IHC for tumors that do not require different therapeutic measures.

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