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ORIGINAL RESEARCH

Role of P63 Immunostain in the Histomorphological Analysis of Salivary Gland Tumors- In Tertiary Care Hospital

C. Shalini[1], Jostna Devi Akarapu[2], M. Mamatha[3], Swathi Cheruku[4]

[1]Assistant Professor, Department of Pathology, Mallareddy Institute of Medical Sciences, Suraram, Hyderabad, India
[2]Assistant Professor, Department of Pathology, Malla Reddy Medical College for Women, Hyderabad, Telangana State, India
[3]Assistant Professor, Department of Pathology, Malla Reddy Medical College for Women, Hyderabad, Telangana State, India
[4]Assistant Professor, Department of Pathology, Mallareddy Institute of Medical Sciences, Suraram, Hyderabad, Telangana State, India

ABSTRACT

Background: Salivary gland tumors are rare head-and-neck neoplasms. They demonstrate dual cell differentiation and morphologic overlap. Dual cell differentiation requires histomorphological study and immunohistochemistry to diagnose. p63 is a selective immunohistochemical marker expressed in nuclei of myoepithelial cells and basal duct cells in normal salivary glands and aid in the diagnosis of of salivary gland tumors by highlightining the biphasic nature of the tumors. Extent of p63 positivity can be a useful predictor of clinical outcome and could help in the aggressive mode of treatment in certain types of salivary glands tumors.To study the role of p63 in the diagnosis of salivary gland tumors.

Material and Methods: The present study was conducted at Malla Reddy Institute of Medical Sciences in the Department of Pathology on the surgically resected salivary gland specimens received for routine histopathological evaluation. A total of 60 cases of salivary gland tumors were included in the study. It is a retrospective study carried out from January 2018 to December 2021, on surgically resected salivary gland tumor specimens and Immunohistochemical analysis with p63.

Results: A Total of 60 Salivary gland tumors were studied with different cytomorphology and mixed architectural patterns. In the present study, most frequent age groups were between 40 and 60 years.. All tumors except Warthin's were female-predominant. The parotid gland was the most common site, with adenoid cystic carcinoma mostly seen in smaller salivary glands. Pleomorphic adenoma and Mucoepidermoid carcinoma were frequent tumors seen. Standard techniques of IHC with p63 antibody were carried out on all the 60 Salivary gland tumors. All the benign tumors were positive for p63. Malignant tumors with basal cell involvement, such as Adenoid cystic carcinoma 6/7 cases showed p63 positivity with varying intensities. Clear, intermediate and squamous cells of all Mucoepidermoid carcinoma were p63 positive. p63 was negative in Acinic cell carcinoma. Differential localization of p63 in various neoplasms was observed which has given information on myoepithelial cells.

Conclusion: P63 is an important Immunohistochemical marker which aids in diagnosis of salivary gland tumors and also helps in localization of myoepithelial cells by differential expression in various salivary gland tumors.

Keywords: Histopathology, Salivary gland tumors, dual cell differentiation, immunohistochemistry, p63.

Corresponding Author: DrSwathiCheruku, Assistant Professor, Department of Pathology, Mallareddy Institute of Medical Sciences, Suraram, Hyderabad, Telangana State, India. Email id:-swathimdpath@gmail.com

INTRODUCTION

Salivary gland tumors constitute 3 to 5 % of all head and neck tumors. There is a diverse range of morphologic spectrum and overlap in various salivary gland tumors. Both luminal cells (acinar and ductal cells) and abluminal cells(myoepithelial and basal cells) can be found in the salivary glands.[1]The majority of salivary gland neoplams originate from or differentiate into the same cell lines, namely epithelial (acinar and ductal), myoepithelial, and basal cells. As a consequence, there is a significant amount of morphologic overlap. Another factor that contributes to the difficulty in identification is the metaplasticalterations such as oncocytic, sebaceous, squamous, clear, and chondroid in certain tumors.[2,3]

The biological activity of these tumors, as well as their prognoses, is distinct from one another. A rigorous search and extensive morphologic examination, which is occasionally assisted by the judicious application of immunohistochemistry, are required to identify dual cell differentiation in these tumors. Recent research suggests that p63, a p53 homolog is a selective immunohistochemical marker expressed normally in nuclei of myoepithelial cells but absent in luminal cells of Salivary gland tumors, can assist in the diagnosis of salivary gland tumors by drawing attention to the biphasic nature of the tumor. The extent of p63 positivity can also be a good predictor of clinical outcome and may call for a more aggressive mode of therapy in certain types of salivary gland tumors.

MATERIALS & METHODS

Study Period: It is a retrospective and further prospective study carried out from January 2018 to December 2021, at Malla Reddy Institute of Medical Sciences, in the Department of Pathology on surgically resected salivary gland tumor specimens received for routine histopathological evaluation.

Data Collection Method: For prospective cases, we studied the salivary gland tumor specimens received in the pathology department in 10% formalin. In every case, the standard protocol for surgical grossing of the specimens was followed. After conventional processing, paraffin sections of 5 μm. Thickness was stained with hematoxylin and eosin (H & E) for histopathological study. In addition, 4 μm. Sections were cut from a paraffin block of tumor tissue and taken on four glass slides coated with an adhesive (silane) for immunohistochemistry (IHC) to detect p63 expression. For the retrospective cases, the histopathology reports, slides, and paraffin blocks were retrieved from the archives. Additional sections were made from the retrieved paraffin blocks.

P63 Immunohistochemistry: The technique for IHC was antigen retrieval in tris buffer in a microwave oven, blocking agent with endogenous peroxidase with 3% hydrogen peroxide, incubated with primary mouse monoclonal antibody (Novocastra, UK), linking with rabbit anti-mouse secondary antibody (Novocastra, UK), enzyme labelling with streptavidin-horseradish peroxidase (Novocastra, UK), developing chromogen with diaminobenzidine (DAB) and counterstaining with haematoxylin. Positive and negative controls were run with each batch of slides.[1,2]

Interpretation of P63 Staining: Sections were evaluated microscopically. The extent of p63 immunostaining was graded and scored as follows.

Proportion of p63 staining in tumor cells	Positivity
Less than 50%	1+
50-75%	2+
More than 75%	3+

Inclusion Criteria:
All epithelial origin tumors, major and minor salivary gland tumors.

Exclusion Criteria:
1. All inflammatory and cystic lesions of salivary glands.
2. All mesenchymal derived salivary gland tumors.
3. Metastatic tumors in salivary glands.

Sample size- 60: Immunohistochemical staining was done on 60cases as per standard protocol and manufacturer‘s instructions with positive and negative controls using antihuman4A monoclonal p63 antibody.
Study design: An observational clinical study.

RESULTS

Table No 1: Age distribution in present study

Age group (years)	No. of cases	% of cases
<20	4	6.66
20-30	7	11.67
31-40	12	20
41-50	16	26.66
51-60	12	20
61-70	8	13.33
>70	1	1
Total	60	100.0

In the present study, maximum number of patients was between 41-50 years of age.

Table No 2: Age distribution of patients studied according to tumor type

Age in years	Age group(%) in Individual Tumors				
	PA	WT	MEC	AdCC	AciCC
<20	8.1%	0%	8.4%	0%	0%
20-30	15.8%	0%	19.1%	09.3%	0%
31-40	27.8%	0%	23.2%	38.9%	0%
41-50	30.5%	09.0%	27.5%	5.0%	100%
51-60	10.6%	46.5%	9.3%	46.9%	0%
61-70	7.1%	31.3%	12.8%	0%	0%
>70	00%	13.1%	0%	0%	0%

Most of the salivary gland tumors were common between the age group of 41-50 years. Mucoepidermoid carcinoma showed a wide age range.

Table No 3: Gender distribution according to tumor type

Gender	Tumor				
	PA	**WT**	**MEC**	**AdCC**	**AciCC**
Male	43.7%	78.5%	35.2%	38.2 %	0%
Female	55.3%	15.9%	65.3%	55.41%	100%

In the present study all salivary gland tumors were common in women except warthin tumor which was common in males.

Table No 4: Distribution of Salivary gland tumors

Salivary gland Tumor	No.of Cases	% of cases
Pleomorphic Adenoma	25	41.66
Warthin's Tumor	9	15
Mucoepidermoidcarcinoma	17	28.34
Adenoid cystic carcinoma	7	11.66
Acinic cell carcinoma	2	3.33
Total	60	100.0

The most common benign Salivary gland tumor was Pleomorphic Adenoma and the most common malignant Salivary gland tumor was Mucoepidermoid Carcinoma in the present study.

Table No 5: Location of tumor

Location	Tumor					Total
	PA	**WT**	**MEC**	**AdCC**	**AciCC**	
Parotid gland	71.9%	100%	72.8%	11.8%	100%	72.7%
Submandibular gland	7.2%	0%	0%	0%	0%	3%
Minor Salivaryglands	21.8%	0%	27.2%	85.7%	0%	24.2%

In the present study most common location of salivary gland tumor was Parotid except in case of AdCC which was more common in minor salivary gland.

Table No 6: Cytomorphology of Myoepithelial cells in benign salivary gland neoplasms

Benign Salivary gland Tumors	Morphology of Myoepithelial cells						
	Basaloid	Epitheloid	Clear	Spindle	Plasmacytoid	Mixed	Oncocytic
PA	00	70.6%	25.9%	72.8%	40%	78.2%	3.6%
WT	NA	NA	NA	NA	NA	NA	NA

PA had myoepithelial cells in mixed morphology with the commonest patterns being epithelial and spindle-shaped. WT had bilayered epithelium, an outer basaloid, and a luminal oncocytic epithelial layer.

Table No 7: Cytomorphology of Myoepithelial cells in malignant salivary gland neoplasms

Malignant Salivary Gland Tumor	Morphology of Myoepithelial cells					
	Basaloid	Epitheloid	Clear	Spindle	Plasmacytoid	Mixed
MEC	0	100%	65%	00	66.2%	66.2%
AdCC	100%	0	0	0	12.8%	14.8 %
AciCC	NA	NA	NA	NA	NA	NA

Malignant Salivary gland tumors also showed Myoepithelial cells with mixed morphology. In AciCCmyoepithelial /basal cells were not appreciated.

Table 8: Architectural patterns in benign salivary gland neoplasms

Benign tumor	Myxoid	Solid	Reticular	Cribriform	Microcystic	Mixed
PA	75.4%	35.8%	93.4%	7.2%	6.4%	70.8%

In PA main architectural pattern was mixed with the most common pattern being reticular.

Table No 9: Architectural patterns in malignant salivary gland neoplasms

Malignant salivary gland tumor	Myxoid	Solid	Reticular	Cribriform	Microcystic	Mixed
MEC	0	54.9%	5.3%	15.3%	68.4%	82.9%
AdCC	0	72 %	14 %	82.7%	0	69.4%
AciCC	0	100%	0	0	33%	100%

Most salivary gland tumors showed mixed architectural pattern. In MEC microcystic and solid patterns were predominant, whereas in AdCC and AciCC solid pattern was predominant

Table No 10: P63 Staining in different tumor types

P63Staining	Tumor				
	PA	WT	MEC	AdCC	AciCC
Absent	0%	0%	0%	12.8%	100%
Present	100%	100%	100%	85.6%	0%

All cases of PA, WT, and MEC showed p63 positivity. 6 out of 7 AdCC carcinomas showed p63positivity. All AciCC were negative for p63 immunostain.

Table No 11: Intensity of p63 staining in different tumors

Proportion of P63 Staining in tumor Cells	Tumor				
	PA	WT	MEC	AdCC	AciCC
Nil	0%	0%	0%	14.3%	100%
Upto 50% cells are positive for p63	54.9%	100%	26%.5	14.2%	0%

50-75% cells are positive	40.4%	0%	60.2%	70.6%	0%
More than 75%	3.6%	0%	10.2%	0%	0%

Salivary gland tumors which were positive for p63 immunostain showed variable intensity of staining. P63 staining of more than 75 %(3+) was found in PA and MEC.

Table No 12: Summary of the observations on IHC

Salivary gland tumors	**Total**	**p63positivity**	**Observation on IHC**
PA	25	25	Variable intensity which was not proportional to cellularity
WT	9	9	Basal cells were stained
MEC	17	17	Staining intensity was not proportional to grade
AdCC	7	6	Peripheral pattern
AciCC	2	0	Negative

Table No 13:Comparision of p63 staining in benignvsmalignant tumors.

p63 Interpretation	**Malignant salivary gland tumors**	**Benign salivary gland tumors**	**Chi square test**	**p value**
Positive	23	34	48.773	0.00(<0.05)
Negative	3	0		
Total no. of cases	26	34		

Present study showed p63 was positive in all of the benign salivary gland tumors whereas in malignant tumors it was 23/26 cases showed positivity with a significant P value of 0.00(<0.05)

Sensitivity and specificity-

Sensitivity of p63 was found to be 88.5% and specificity was 100% in the present study.

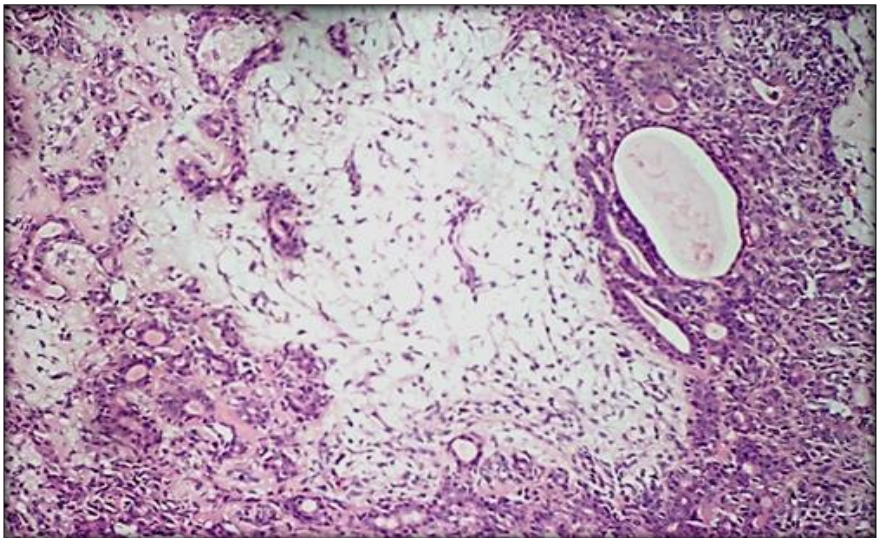

Figure 1(a): Pleomorphic Adenoma – Biphasic population of epithelial and myoepithelial cells in myxoidstroma(40 X)

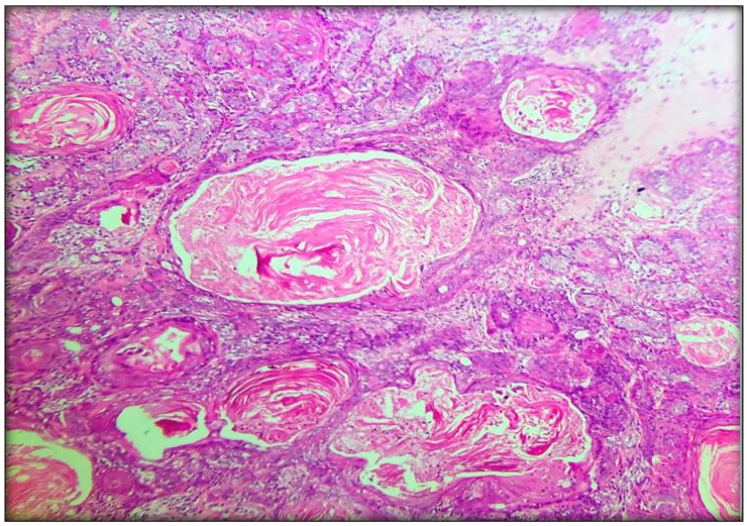

Figure 1(b): Pleomorphic Adenoma with squamous metaplasia (40X)

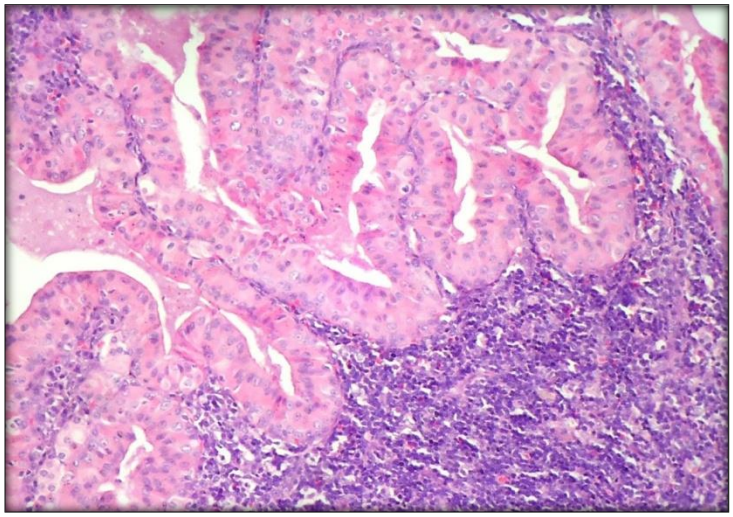

Figure 2:Warthin's Tumor – Papillary pattern with bilayered epithelium showing oncocytic change in a lymphoid stroma (40X)

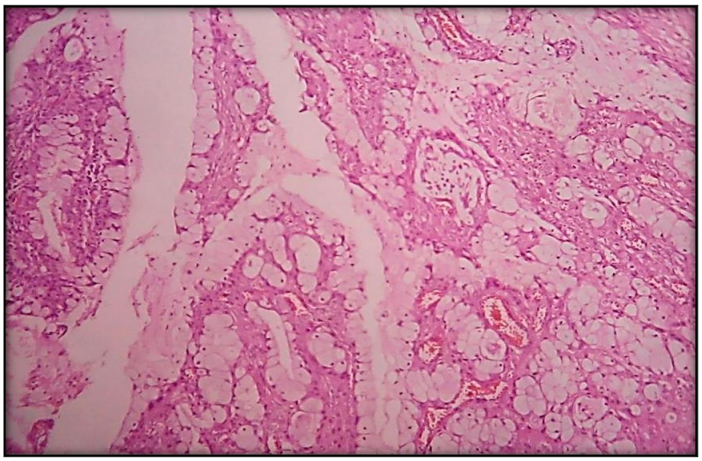

Figure 3: Mucoepidermoid Carcinoma, low grade - The cyst is lined by mucinous cells supported by an underlying layer of intermediate cells. The nuclei are typically bland looking (40X)

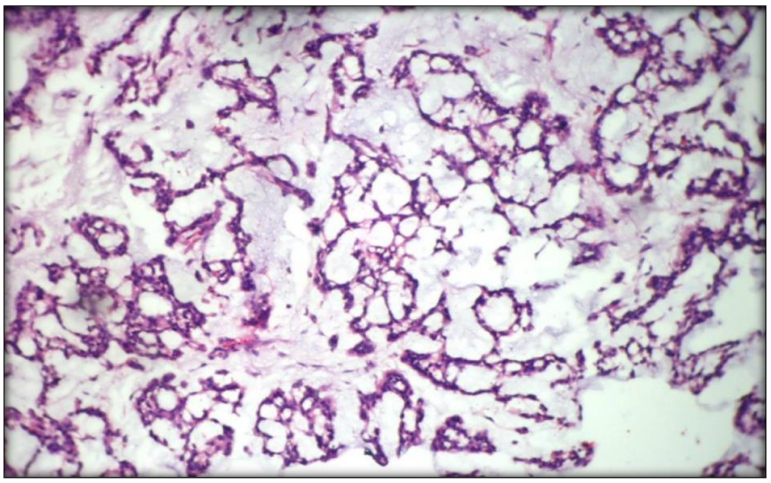

Figure 4: Adenoid Cystic Carcinoma- Tumor showing cribriform pattern with myxohyalinestroma(40X)

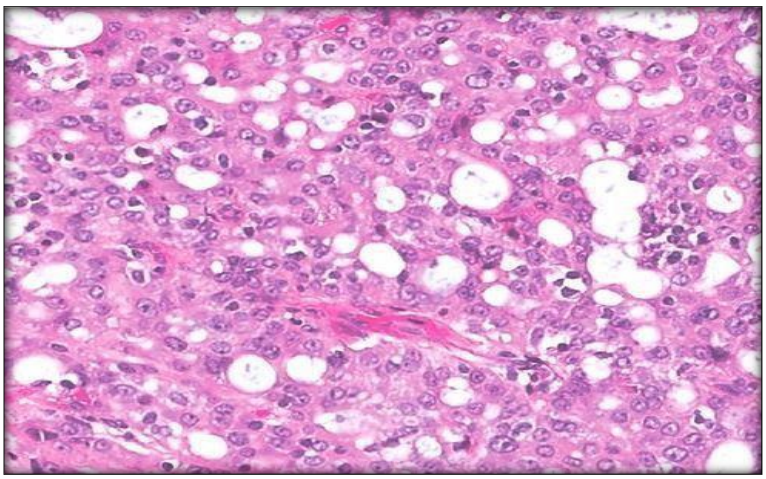

Figure 5: Acinic Cell Carcinoma- showing microcysticpattern (40X)

P63 Staining Pattern in Salivary Gland Tumors

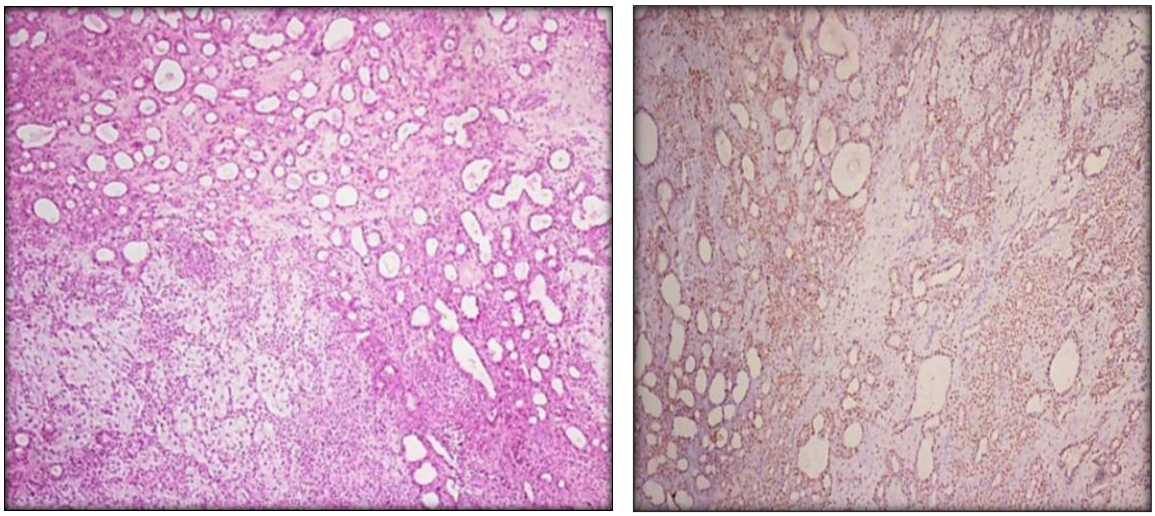

Figure 6(a) - Pleomorphic Adenoma Figure 6(b): P63 positivity (3+) (10X) H&E (10X)positive expression 3+ (10X)

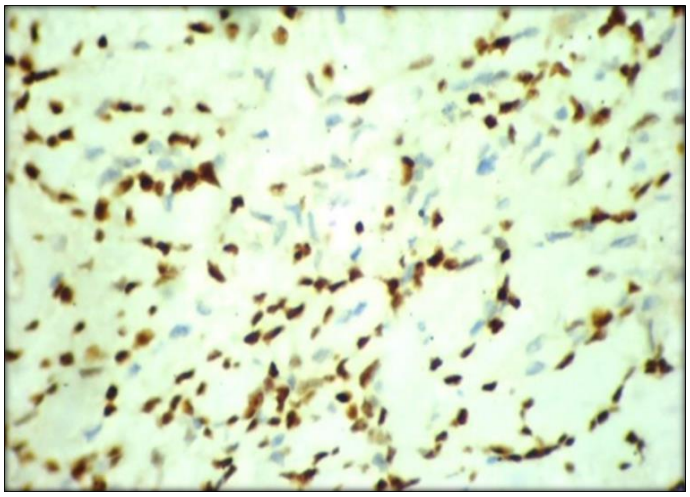

Figure 6(c): Pleomorphic adenoma, p63 positive expression in myoepithelial cells

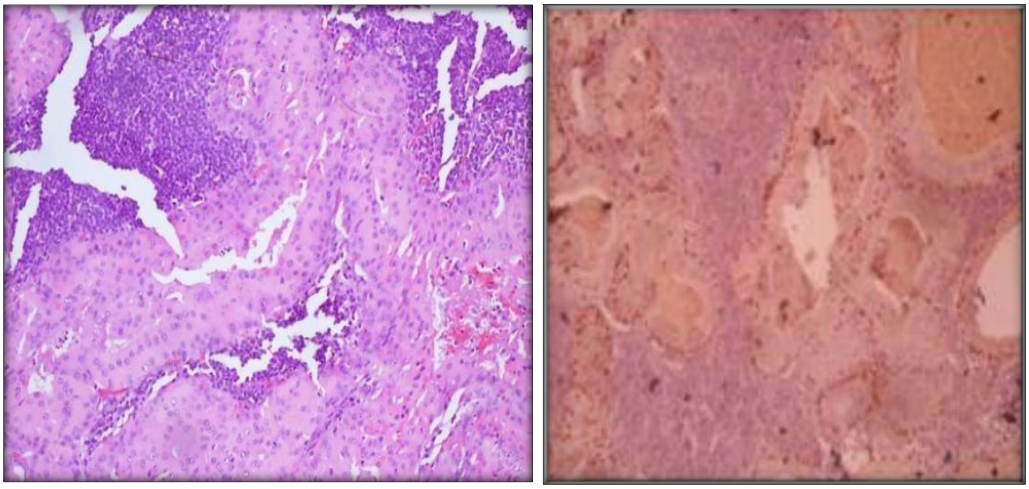

Figure 7(a): Warthin'sTumor- P63 Positive expression in Basal cells (40X)

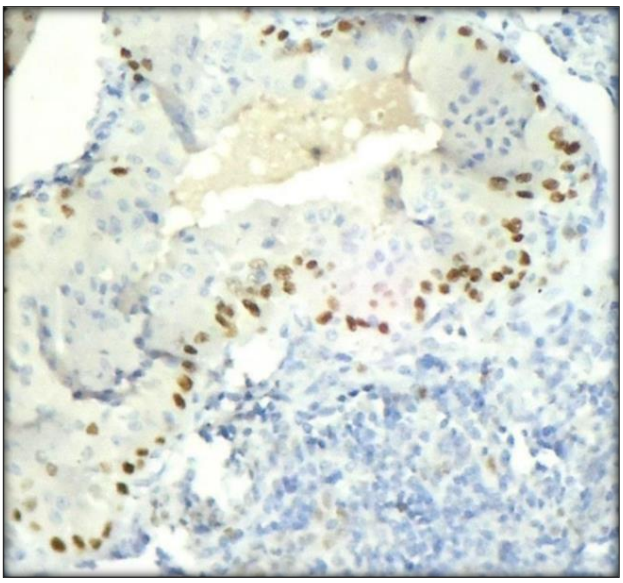

Figure 7(b): Warthin's Tumor- p63 positive basal cells and p63 negative luminal cells (100X)

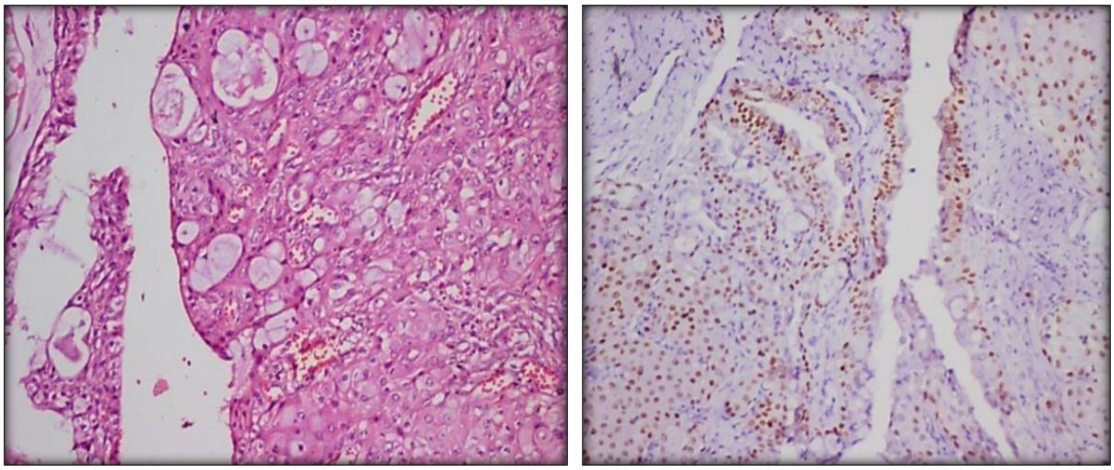

Figure 8(a): Mucoepidermoid carcinoma bpositive for p63 immunostain(40X)

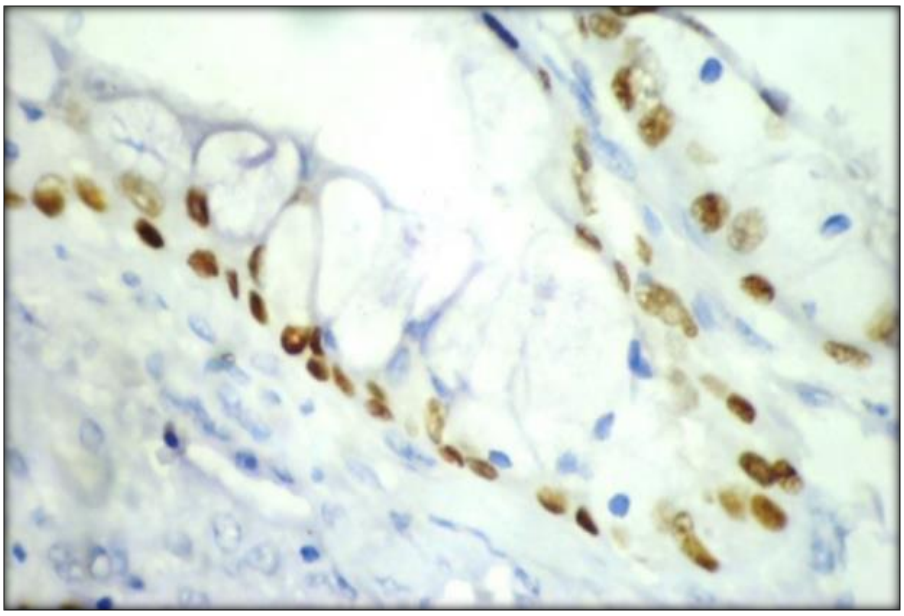

Figure 8(b): Mucoepidermoid Carcinoma - p63 positive basal nucleus of mucous cells lining the cystic spaces (100X)

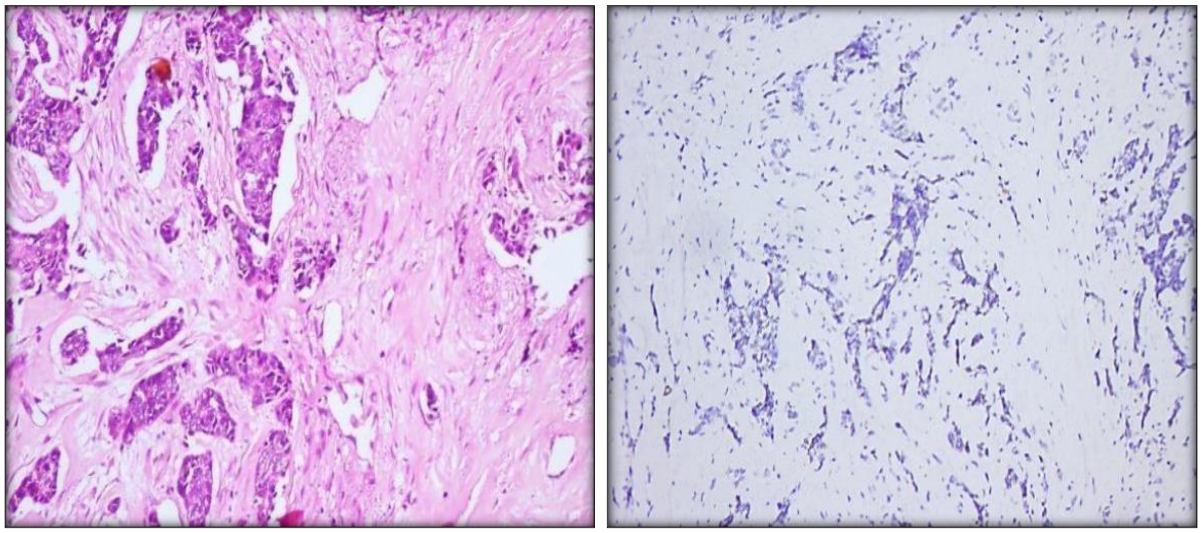

Figure 9(a): Adenoid cystic carcinoma negative for p63 (40X)

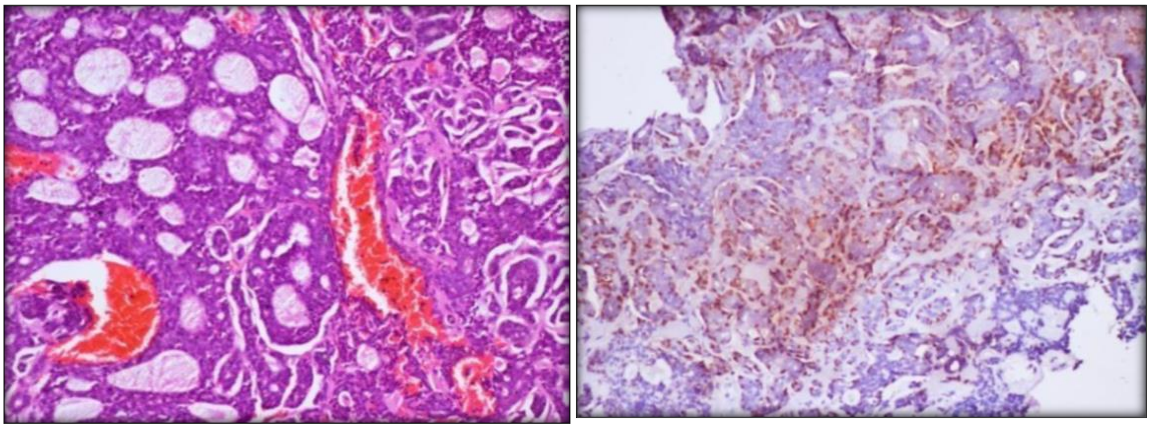

Figure 9(b): Adenoid cystic carcinoma positive for p63 in a peripheral pattern (40X)

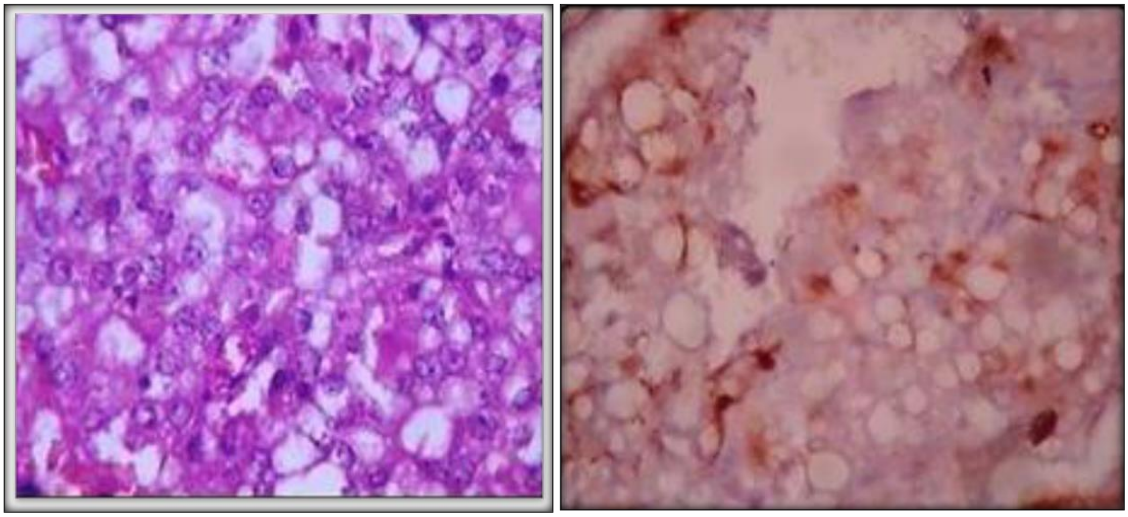

Figure 10: Acinic cell carcinoma negative for p63 staining(100X)

DISCUSSION

P63 is a marker that can be found in the basal duct cells as well as in nuclei of myoepithelial cells in normal salivary glands. In salivary gland tumors which differentiate towards luminal and myoepithelial cells like pleomorphic adenoma, basal cell adenoma, adenoid cystic carcinoma and epithelial- myoepithelial carcinomas- p63 was positive in myoepithelial cells and luminal cells were negative.(Bilal et al).

Pleomorphic Adenoma

In present study, PA was the most common salivary gland tumor with female preponderance and all 25cases (100%) showed positivity for p63. Based on the proportion of epithelial and mesenchymal like matrix PA was sub classified into stroma rich- 10cases (40%), cell rich 05cases (20%) and classic types 10cases (40%). It has been suggested that recurrence is more frequent for stroma-rich tumours, which have a higher chance of spillage of mucoidstroma during operation. On the other hand, highly cellular tumors may be more prone to malignant change.[12]

The prototypic histologic appearance of PA is partiallypseudocapsuled lesion with tumor islands appearing to melt into the sea of the chondromyxoidstroma.. In the present study, the predominance of the myxoid and chondroidstroma was clear. Hyalinization of the stroma was present in 3 cases (7.2%). Prominent zones of hyalinization have been related to aggressive behaviour or malignant transformation of PA. However, hyalinization as an isolated fact is not sufficient to predict this progression.[13]

Metaplasia within the stromal elements of benign mixed tumors is also a common finding. Stromal adipose was found in only 2 cases (4.8%). Extensive stromal lipoid metaplasia has also been described earlier in the literature, with fat comprising up to 90% of the tumor in some case reports. Cutaneous adnexal differentiation is well recognized in benign mixed tumours occurring in cutaneous sites. When cutaneous adnexal differentiation occurs in salivary gland pleomorphic adenomas, it can present a diagnostic pitfall that must not be misinterpreted as carcinoma at biopsy, fine needle aspiration, or frozen section. But in this study, we didn't find any PA with cutaneous adnexal differentiation.

Extensive squamous metaplasia and keratin pearl formation could be mistaken for malignant tumors such as mucoepidermoid carcinoma and squamous cell carcinoma. In present study extensive squamous metaplasia with keratin pearl formation was found in 1 out of 25 PA cases. Acellular keratin with basaloid and squamous epithelial cells without the characteristic matrix material that is normally diagnostic of a pleomorphic adenoma poses a diagnostic challenge.

In our study, epithelioid and spindle cells were the most frequent cell types. Because plasmacytoid cells are only found in pleomorphic adenoma and myoepithelioma, their identification has a high diagnostic value. In this study, we found 41.4% of PA with plasmacytoid morphology. Ellis et al. suggest that these cells appear to be in transition from one form to the other form. Interestingly, recent studies have shown that plasmacytoid cells in PA originate from luminal cells rather than myoepithelial cells.[15]

Correlation between cell type and p63 expression in PA- In present study 25 out of 25 cases showed showed p63 nuclear expression in myoepithelial cells. P63 reactivity did not correlate with tumor type (like myxoid or cellular rich PAs) which was similar to Bilal etal. Diffuse immunostaining(3+) was seen in only one case, although there was great variability in approximate proportion of reactive tumor cells(between 10% to 90%). All the cell types were reactive like stellate, plasmacytoid, spindle, and clear cells with modified myoepithelial cells surrounding duct like structures showing obvious positivity. In contrast, p63 reactivity was absent in cuboidal and columnar epithelial cells lining the duct lumens.

BasselTarakji et al found that p63 was significantly expressed in tumor duct cells in 6 out of 29 instances, while 16 (55.2 percent) cases showed moderate staining, and 7 (24.1 percent) cases expressed weak staining,[17] which was in contrast to present study.

Warthin's Tumour

In the present study, there were nine (15%) cases of WT and all the patients were elderly males who had symptoms related to their parotid glands.

The examination of the immunostaining for p63 revealed nuclear positivity in all of the cases that were investigated. The intensity of the nuclear positivity was variable, but it was limited to the level of the basal cells, which is consistent with the findings of the studies that were mentioned earlier. The nuclei of palisading cells in luminal columnar cells did not react to P63. The immunohistochemical analysis of the bilayer epithelial component of the Warthin tumors demonstrated different immunostains of the two forms of epithelia, the oncocytic columnar and the basal layer. These two types of epithelia are comparable to those that are seen in the salivary gland ducts.[2]

Adenoid Cystic Carcinoma

The present study found that 11.6%(7 cases) of all tumors and 26% of all malignant tumors were classified as AdCC. The tumors have an heterogeneous histology on microscopy, consisting of varying amounts of 3 distinct growth patterns. Cribriform, tubular, and solid growth patterns are the three patterns that have been described for AdCC. According to the present study, cribriform (85.6%) was the most common pattern seen.The grading of AdCC can be done on the basis of percentages of each pattern according to Szanto et al. Grade I

tumors have only tubular or cribriform growth patterns; Grade II tumors have cribriform or tubular growth with less than a 30% solid component; and Grade III tumors have a 30% solid component and more. Despite the fact that the prognostic importance of this grading system has been called into doubt, the existence of a solid component has been shown to be a reliable indicator of a poor outcome across multiple series.[21]

In the current study, out of 5 AdCC with a solid pattern, only 2 had a solid pattern that was greater than 30 percent. As a result, only 2 AdCC were of Grade III, while the remaining 3 cases were of Grade II.

Because the detection of perineural invasion has been demonstrated to be of greater prognostic value, careful documentation of perineural invasion should be done during staging. This is of particular importance. In the course of our research, we found evidence of perineural invasion in five out of seven AdCC. The majority of AdCC showed a desmoplasticstroma.

The study found that preferential staining of the peripheral myoepithelial cell layer was seen in six out of seven of the AdCC samples. There was one case of AdCC that did not show any evidence of p63 staining. These results were similar to what Edwards et al. found in a previous study, where only five of eight solid variations had a strong positive correlation, two had a negative correlation, and one had a weak positive correlation. It can be difficult to tell the difference between high-grade adenoid cystic carcinoma and basaloid squamous cell carcinoma based on their morphology alone. Equivocal diagnoses can mislead therapy.[22]

Acinic Cell Carcinoma

The distinction between AciCC and MEC, which can both have considerable mucin production, might be difficult. The immunohistochemicalstain p63 can help differentiating unusual AciCC with prominent mucin production from MEC. In present study, we found that AciCC consistently failed to exhibit p63 immunoreactivity, while MEC showed 100% positivity. This was in correlation with Kumar et al. where acinic cell carcinoma was never found to have p63 immunoreactivity.[2]

In contrast to classic Acinic Cell Carcinoma and Acinic Cell Carcinoma-High Grade Type, more than half of the tumors that has traditionally been classified as zymogen granule poor Acinic Cell Carcinoma actually represents Mammary Analogue Secretory Carcinoma. Other types of acinic cell carcinomas include classic acinic cell carcinoma and acinic cell carcinoma-high grade type. This is due to the presence of zymogen granules in traditional acinic cell carcinomas as well as acinic cell carcinomas of the high grade kind (MASC).

As opposed to classic AciCC and AciCC-HGT, more than half of the tumors that was traditionally classified as zymogen granule poor AciCC are in fact MASC. This is in contrast to both classic AciCC and AciCC-HGT. ETV6 testing is the method that will successfully differentiate between the real zymogen granules poor AciCC and the MASC. There are slight morphologic and immunophenotypic changes between the two groups. Even though they are different on a molecular level, it is not yet known whether or not this distinction has any important clinical repercussions.[23]

Mucoepidermoid Carcinoma

Kumar et al. revealed that distinguishing salivary gland acinic cell carcinoma from mucoepidermoid carcinoma can be difficult because both produce a lot of mucin. p63 is an immunohistochemical stain that may help distinguish unusual acinic cell carcinoma with prominent mucin production from mucoepidermoid carcinoma of the salivary gland. According to this study, acinic cell carcinoma is always negative for p63 immunoreactivity, whereas mucoepidermoid carcinoma is always positive.[2,3]

In the present study all grades of MEC were seen. Eight of 17 cases of mucoepidermoidcarcinomas were predominately cystic, whereas 9cases were solid.

According to the AFIP standards (Ellis and Auclair 1996), grading of MEC could be categorised as low (present study 8/17 cases), intermediate (present study 4/17 cases), or high (present study 5/17 cases), depending on the severity of the condition. In every instance, there was a discernible and widespread nuclear stain. There was no link found between p63 immunoreactivity and the microscopic grade of the tumor. In cystic tumors, reactive nuclei were seen in foci of intermediate or squamous cells as well as in basal cells at the periphery of mucous cells that lined cystic spaces Solid tumors, sheets or islands of intermediate, squamous, and clear cells, all of which have been shown to have a p63 nuclear response. The findings of this investigation were comparable to those found in Bilal et al.'s research.

Salivary gland tumors with abluminal differentiation-

There are cancers of the salivary glands that display both luminal and abluminal differentiation.[6] In every incidence of PA, there was a clear distinction between the luminal and abluminal layers. p63 was positive for the myoepithelial/basal (abluminal) derived stellate, plasmacytoid, spindle, and clear cells, but it was overwhelmingly negative for the cuboidal or columnar epithelial cells (luminal) that lined the duct lumens. The p63 immunostain draws attention to the dual differentiation that is present in warthin's tumor. In every instance, the palisading luminal cells were negative, whereas the basal cells, also known as abluminal cells, were positive for p63. In the instance of AdCC, p63 showed selective labelling of basaloid cells (abluminal), while the luminal cells displayed negative staining. It will be easier to narrow down the list of possible diagnoses and arrive at a conclusive diagnosis if you have a thorough understanding of the salivary gland tumors that exhibit dual luminal abluminal differentiation.[1,2]

CONCLUSION

Neoplastic myoepithelial cells are considered to be a key cellular participant in the morphogenetic process responsible for various histological appearances of salivary gland tumors. Nevertheless, controversy still exists concerning its participation in some salivary gland neoplasms. This has been due to the difficulty in fully characterising the wide spectrum of morphological and immunophenotypic expressions of neoplastic myoepithelium compared with its normal counterpart. Understanding the myoepithelial cells has important implications for clarifying diagnostic problems and improving the classification of salivary gland tumors. p63 is expressed in the nuclei of normal human salivary gland myoepithelial and basal duct cells as well as the modified myoepithelial and basal cells of human salivary gland tumors. With the help of the right IHC markers, a better understanding of salivary gland myoepithelial cells could give us important information about the maintenance of this tissue, the histogenesis and oncogenesis of salivary gland tumors, and could help with the diagnosis.

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