Role of p63 immunohistochemistry in diagnosing Salivary gland neoplasms

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Abstract
Introduction: Salivary gland tumors are uncommon neoplasms encountered in the head and neck region. Salivary gland neoplasms have varied histopathological findings with dual cell differentiation with some of the tumors showing morphologic overlap. Detailed histomorphologic analysis along with judicious application of immunohistochemistry are required to identify dual cell differentiation and to make a proper diagnosis. p63 is a selective immunohistochemical marker of basal stem cells of stratified epithelium and of myoepithelial cells which aid in the diagnosis of salivary gland tumors.
Objective: To study the role of p63 in the diagnosis of salivary gland tumours.
Materials and Methods: The present study was conducted in the Pathology department of M.S Ramaiah Hospital on the surgically resected salivary gland tumor specimens received for routine histopathological evaluation, from January 2010 to December 2014. A total of 66 cases of salivary gland neoplasms were included in the study.
Results: Most tumors studied had myoepithelial cells with mixed cytomorphology and had mixed architectural pattern. 66 salivary gland tumors were immunostained for p63 antibody using standard procedures. Pleomorphic adenoma, Adenoid Cystic Carcinoma and Warthin tumor were positive for p63 immunostain with variable intensities. Mucoepidermoid Carcinoma showed positivity in clear, intermediate and squamous cells. Acinic Cell Carcinoma was negative for p63.
Conclusion: Proper diagnosis of salivary gland tumor is very important in the treatment and management of the patient. p63 is an important immunohistochemical marker which aid in the diagnosis of salivary gland tumors. The differential localization of p63 in these neoplasms has shed light on the myoepithelial cellular localizations in the salivary gland tumors.

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

Introduction
Salivary gland neoplasms account for 3-5.5% of all head and neck tumors.[1,2] There is a wide variety of benign and malignant salivary gland tumors. Salivary gland shows both luminal (acinar and ductal cells) and abluminal cells (myoepithelial and basal cells).[3] Most of salivary gland tumors arise from or differentiate towards the same cell lines i.e. epithelial (acinar and ductal), myoepithelial, and basal. This results in considerable morphologic overlap. Also some tumors undergo a variety of metaplastic changes (i.e. oncocytic, sebaceous, squamous, clear, chondroid) which makes the diagnosis difficult.[4] These tumors show differences in biological behavior and also in prognosis. In some tumours, careful search and detailed morphologic analysis, sometimes aided by judicious application of immunohistochemistry are required to identify dual cell differentiation.[5,6]

Recent studies show that p63, a p53 homologue which is a selective immunohistochemical marker of basal stem cells of stratified epithelium and of myoepithelial cells, aid in the diagnosis of salivary gland tumours by highlighting the biphasic nature of the tumour.[6]

Extent of p63 positivity can be a useful predictor of clinical outcome and could warrant a more aggressive mode of therapy in certain types of salivary gland tumours.

Objective
To study the role of p63 in the diagnosis of salivary gland tumours.

Materials and Methods
Study was conducted in the Pathology department of M.S Ramaiah Hospital on the surgically resected salivary gland tumour specimens received for routine histopathological evaluation, from January 2010 to December 2014. Ethical clearance was obtained for the study.

A total of 66 cases were studied. For prospective cases, the standard protocol for surgical grossing of the specimens was followed.

After conventional processing, paraffin sections of 5µm thickness was stained by haematoxylin and eosin (H & E) for histopathological study. In addition, 4µm sections was cut from paraffin block of tumour tissue and taken on 4 glass slides coated with 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 endogenous peroxidase with 3% hydrogen peroxide, incubating 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 deaminobenzidine (DAB) and counterstaining with

haematoxylin. Positive and negative controls were run with each batch of slides.

Immunohistochemical staining was done on all 66 cases as per standard protocols and manufacturer's instructions with positive and negative controls using antihuman 4A4 monoclonal p63 antibody.

Results

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

Staining of the tumour cells	Positivity
Less than 50%	1
50-75%	2
More than 75%	3

Inclusion Criteria

All epithelial origin, major and minor salivary gland tumors

Exclusion Criteria

1. All inflammatory and cystic lesions of salivary glands.
2. All mesenchymal origin salivary gland tumors.
3. Metastasis in salivary glands.

Results

Distribution of Benign tumor in Patients

Out of 66 cases studied, 38 cases (57%) were benign tumors. Most common benign salivary gland tumor was pleomorphic adenoma 29 cases (44%) (Fig. 1), Warthin tumor (Fig. 2) constituted 9 cases (13%).

Distribution of Malignant tumor in Patients

Of the total 66 cases, 28 cases (43%) were malignant salivary gland tumors. Most common malignant tumor was mucoepidermoid carcinoma (Fig. 3), 19 cases (29%) followed by Adenoid cystic carcinoma 7 cases (11%) and Acinic cell carcinoma 2 cases (3%).

Age distribution in Patients Studied

Most of the patients with salivary gland tumors were between 41-60 years.

Gender Distribution According to Tumor Type

All salivary gland tumors were common in women except warthin tumour which was common in males.

Location of the Tumor

Parotid gland (72.7%) was the most common site for the tumors studied except in case of Adenoid Cystic Carcinoma which was more common in minor salivary glands (Table 1)

p63 Staining in Different Tumors types

All cases of Pleomorphic Adenoma, Warthin Tumor, Mucoepidermoid carcinoma showed p63 positivity. 6 out of 7 Adenoid Cystic Carcinomas showed p63 positivity. All Acinic Cell Carcinomas were negative for p63 immunostain (Table 2).

Intensity of p63 Staining in Different Tumours

Salivay gland tumours which were positive for p63 immunostain showed variable intensity of staining (Table 3).

Pleomorphic adenoma showed p63 positivity in all the 29 cases studied with variable intensities. Basal cells were stained in Warthin tumor. All 19 cases of mucoepidermoid carcinoma showed p63 positivity. 6 cases of Adenoid cystic carcinoma showed peripheral staining pattern. Acinic cell carcinoma showed p63 negativity. (Table 4).

Discussion

Pleomorphic Adenoma

Pleomorphic Adenoma was the most common salivary gland tumor and representing 76.3% of benign and 43.9% of total salivary gland neoplasms.

Depending on the proportion of both epithelial and mesenchymal-like tissues Pleomorphic Adenoma were sub-classified into stroma-rich in 14 cases (48.2%), cell-rich in 05(17.2%) and classic in 10 cases (34.4%) of Pleomorphic Adenoma.

Correlation between cell type and p63 expression in Pleomorphic Adenomas is seen in those cells with differentiation along myoepithelial lineages which are p63-reactive and those with luminal lineages are p63 unreactive.

29 of 29 pleomorphic adenomas demonstrated p63 nuclear staining in the modified myoepithelial cells (Fig. 1). p63 reactivity did not correlate with tumor type (whether or not the tumor was predominantly myxoid or cellular) which was similar to the findings of Bilal etal.[7] Immunoreactivity was diffuse in only one case, although there was great variability in the approximate proportions of reactive tumor cells (between 10% and 90% of tumor cells). All cell types were reactive, including stellate, plasmacytoid, spindle, clear cells, and cells appearing to be in transition from one type to another. The modified myoepithelial cells surrounding duct-like structures were often the most obviously reactive. In contrast, p63 reactivity was always absent in cuboidal or columnar epithelial cells lining duct lumens (Table 5).

Warthin Tumour

Nine cases of Warthin Tumor were encountered with all of them presenting in parotid glands of elderly males.

The analysis of the immunostain for the p63 has indicated nuclear positivity in all the investigated cases (Table 6), with variable intensity but limits at the level of the basal cell level which is in line with the results of the above mentioned studies. The palisading nuclei of luminal columnar cells were unreactive. The immunohistochemical study of the bilayer epithelial component of Warthin tumor showed different immunstaining of the two types of epithelia, the oncocytic columnar and the basal layer, similar to those found in the salivary gland ducts (Fig. 2).

Adenoid Cystic Carcinoma

Adenoid Cystic Carcinoma constituted 10.6% of all tumors and 25% of malignant ones.

6 out of 7 Adenoid Cystic Carcinoma studied showed selective staining of the peripheral myoepithelial cell layer. One case of Adenoid Cystic Carcinoma was negative for p63 staining (Fig. 3). These results were in similar to results of a series by Edwards et al[12] in which only 5 of 8 solid variants showed strong positivity whereas 2 were negative and 1 was weakly positive (Fig. 4). (Table 7).

Morphologic distinction of high-grade adenoid cystic carcinoma from basaloid squamous cell carcinoma can be difficult. Equivocal diagnoses can mislead treatment. Basaloid squamous cell carcinomas consistently displayed diffuse p63 positivity, with staining of nearly 100% of tumor cells. In contrast, adenoid cystic carcinoma displays a selective staining of a single peripheral layer of p63-positive cells surrounding centrally located tumor cells that were p63-negative. p63 immunostaining constitutes a specific and accurate means of distinguishing adenoid cystic carcinoma from basaloid squamous cell carcinoma. p63 positivity in adenoid cystic carcinoma appears to be homologous to that seen in the basal and/or myoepithelial compartments of salivary gland and other epithelia, and may signify a stem-cell-like role for these peripheral cells. Diffuse p63 positivity in basaloid squamous cell carcinoma suggests dysregulation of p63-positive stem cells in poorly differentiated squamous carcinoma. p63 positivity in Adenoid Cystic Carcinoma presumably represents a neoplastic recapitulation of the p63-positive basal/myoepithelial cell phenotype present in normal salivarygland.[11]

The extent of p63 positivity in Adenoid Cystic Carcinoma may be a useful predictor of clinical outcome. Although staging continues to play a pivotal role in the management of patients with Adenoid Cystic Carcinoma, high p63 expression could warrant a more aggressive mode of therapy. Conversely, low p63 expression might identify a patient population more likely to survive both short-term and long-term.

Acinic Cell Carcinoma

Differentiation of salivary gland Acinic Cell Carcinoma from Mucoepidermoid carcinoma can be diagnostically challenging as both may have prominent mucin production. p63 is an immunohistochemical stain that can potentially aid in differentiating unusual Acinic Cell Carcinoma with prominent mucin production from Mucoepidermoid carcinoma of the salivary gland.[13] In our study, Acinic cell carcinoma was always negative for p63 immunoreactivity (Fig. 5). while Mucoepidermoid carcinoma was always positive (Fig. 6).

p63 staining was negative in acinic cell carcinomas (Table 8). In contrast to classic Acinic Cell Carcinoma and Acinic Cell Carcinoma-High Grade Type, more than half of the tumors historically categorized as zymogen granule poor Acinic Cell Carcinoma actually represent Mammary Analogue Secretory Carcinoma (MASC). There are subtle morphologic and immunophenotypic differences between true zymogen granule poor Acinic Cell Carcinoma and MASC, ETV6 testing will separate these two groups more reliably.[14]

Mucoepidermoid Carcinoma

In our study we had all grades of Mucoepidermoid carcinoma. Out of 19, 8 mucoepidermoid carcinomas were predominantly cystic and solid in four cases. Using the AFIP criteria (Ellis and Auclair 1996),[15] tumors could be classified into low (eight cases), intermediate (seven cases), and high (four cases) grade. Strong and diffuse nuclear staining was noted in each case. There was no correlation between p63 immunoreactivity and microscopic grade. In cystic tumors, reactive nuclei were found in foci of intermediate or squamous cells and in basal cells at the periphery of mucous cells that lined cystic spaces. In solid tumors, sheets or islands of intermediate, squamous, and clear cells demonstrated p63 nuclear reaction. This findings were similar to the study conducted by Bilal et al[7]

Differentiation of salivary gland acinic cell carcinoma from mucoepidermoid carcinoma can be diagnostically challenging as both may have prominent mucin production. p63 is an immunohistochemical stain that can potentially aid in differentiating 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 while mucoepidermoid carcinoma is always positive (Table 9).

Salivary gland tumours with dual luminal and bluminal differentiation

All cases of Pleomorphic Adenoma showed a dual luminal – abluminal differentiation. p63 was positive for the myoepithelial/basal (abluminal) derived stellate, plasmacytoid, spindle and clear cells but was clearly negative for the cuboidal or columnar epithelial cells (luminal) lining the duct lumens.

In warthin tumour the dual differentiation is highlighted by the p63 immunostain.In all cases the basal cells (abluminal) were positive for p63 were as the palisading luminal cells were negative.

In case of Adenoid Cystic Carcinoma p63 showed selective staining of basaloid cells (abluminal) with negative staining of the luminal cells.

Conclusion

Neoplastic myoepithelial cells are considered to be a key cellular participant in morphogenetic process responsible for various histological appearances of salivary gland tumors. Understanding the myoepithelial cells has thus 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. A better characterization of salivary gland myoepithelial cells with the help of proper IHC markers may provide valuable information regarding maintenance of this tissue, histogenesis and oncogenesis of salivary gland

tumors, and may have clinical utility for the diagnosis.

T able 1: Location of the tumor

Location	Pleomorphic adenoma	Warthin Tumor	Mucoepidermoid Carcinoma	Adenoid Cystic Carcinoma	Acinic Cell Carcinoma	Total
Parotid	72.4%	100%	78.9%	14.3%	100%	72.7%
Submandibular	6.9%	0%	0%	0%	0%	3%
Minor salivary Gland	20.7%	0%	21.1%	85.7%	0%	24.2%

Table 2: p63 Staining in different tumor types

p63 Staining	Pleomorphic Adenoma	Warthin Tumor	Mucoepidermoid carcinoma	Adenoid Cystic Carcinoma	Acinic Cell Carcinoma
Absent	0%	0%	0%	14.3%	100%
Present	100%	100%	100%	85.7%	0%

Table 3:Intensity of p63 staining in different tumours

P63 Staining of tumor Cells	Pleomorphic Adenoma	Warthin Tumor	Mucoepidermoid Carcinoma	Adenoid Cystic Carcinoma	Acinic Cell Carcinoma
Nil	0%	0%	0%	14.3%	100%
Upto 50% cells are positive for p63	55.2%	100%	26.3%	14.3%	0%
50-75% cells are positive	41.4%	0%	63.2%	71.4%	0%
More than 75%	3.4%	0%	10.5%	0%	0%

Table 4: Summary of the observations on IHC

H & E Diagnosis	Total	p63 positivity	Observations on IHC
Pleomorphic Adenoma	29	29	Variable intensity which was not proportional to cellularity
Warthin Tumor	9	9	Basal cells stained
Mucoepidermoid carcinoma	19	19	Staining intensity was not proportional to grade
Adenoid Cystic Carcinoma	7	6	Peripheral pattern
Acinic Cell Carcinoma	2	0	Negative

Table 5: Comparison of p63 immunoreactivity for Pleomorphic Adenoma

	Number of cases	Positive	Cell type
Bilal et al[7]	15	15	Myoepithelial
Weber et al[8]	42	42	Myoepithelial, Squamous, metaplastic
Present study	29	29	Myoepithelial

T**able 6:** Comparison of p63 immunoreactivity for Warthin Tumor

	No of cases	Positive cases	Cell type
Bilal et al[7]	4	4	Basal
Faur A et al[9]	42	42	Basal
Seethala[10]	21	21	Basal
Present study	9	9	Basal

Table 7: Comparison of p63 staining in Adenoid Cystic Carcinoma

	No. of Cases	Positive Cases	Cell Type
Bilal et al[7]	11	11	Myoepithelial
Seethala et al[10]	16	16	Myoepithelial
Emanuel et al[11]	14	14	Myoepithelial
Edwards et al[12]	15	13	Myoepithelial
Current	7	6	Myoepithelial

Table 8: Comparison of p63 staining in Acinic Cell Carcinoma

	No. of cases	Positive cases	Cell type
Bilal et al[7]	4	1	Intercalated duct cell
Seethala et al[10]	8	0	-
Current	2	0	-

Table 9: Comparison of p63 staining in Mucoepidermoid carcinoma

	No. of cases	Positivecases	Celltype
Bilal et al[7]	9	9	Clear, Intermediate, squamous
Maruya et al[16]	9	9	Intermediate, squamous
Seethala et al[10]	4	4	Squamous
Current	4	4	Clear, Intermediate, squamous

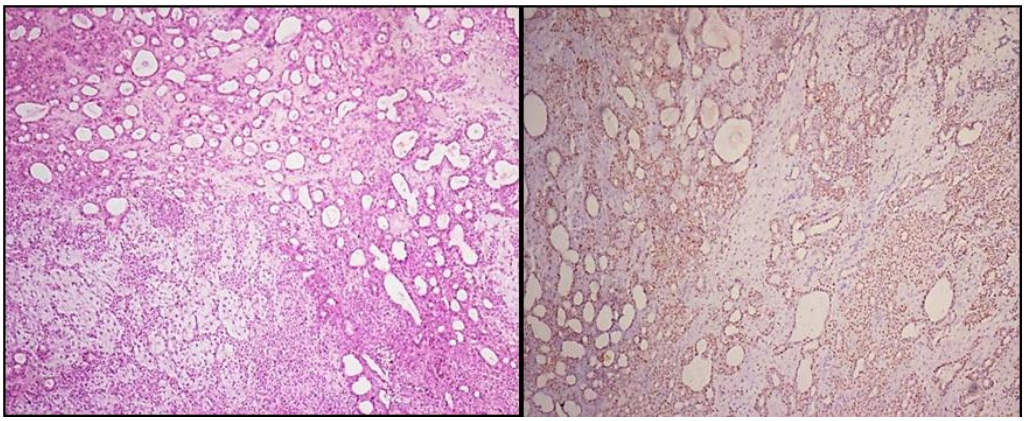

Fig. 1: Pleomorphic adenoma p63 positivity for modified myoepithelial cells.

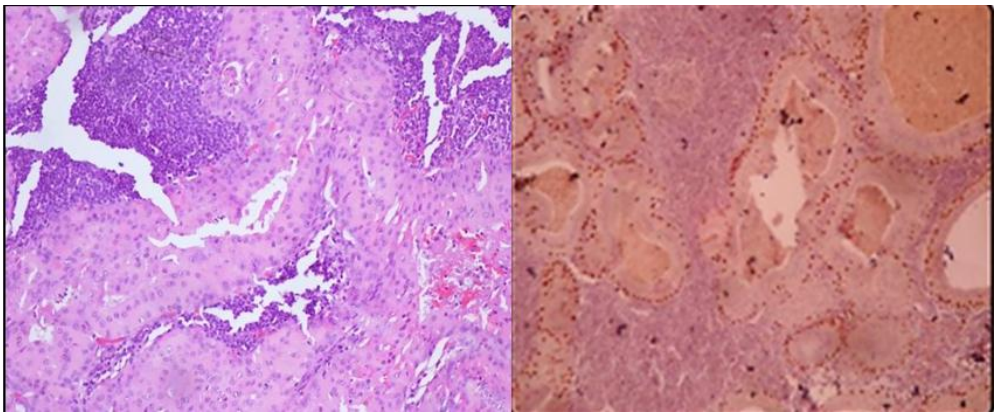

Fig. 2: Warthin tumour basal cells positive for p63 immunostain

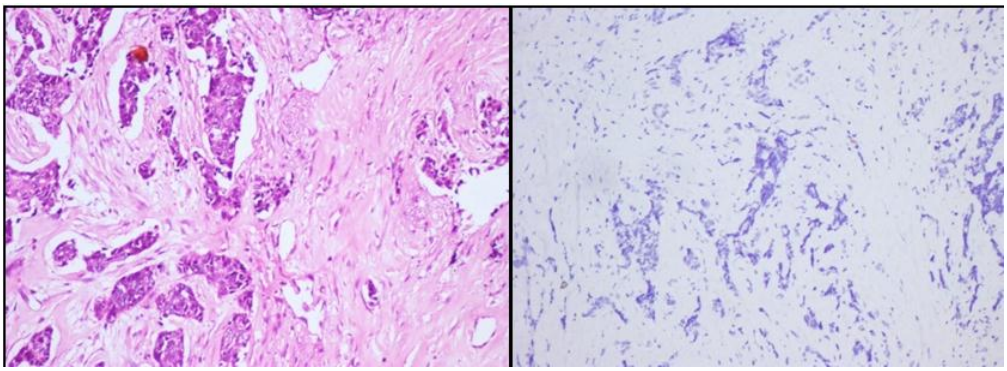

Fig. 3: Adenoid cystic carcinoma case which came out negative for p63.

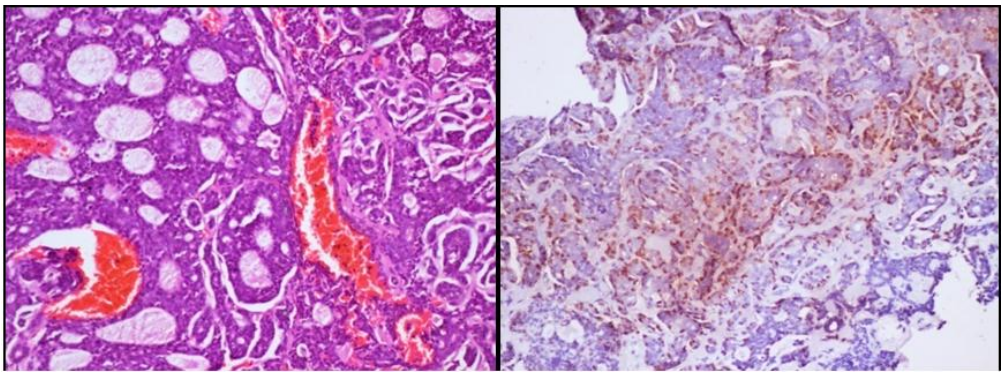

Fig. 4: Adenoid cystic carcinoma positive for p63 in a peripheral pattern

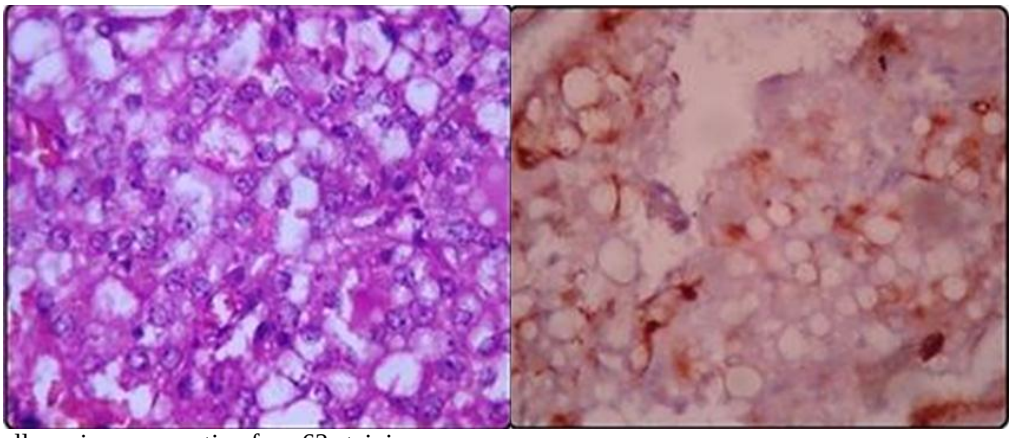

Fig. 5: Acinic cell carcinoma negative for p63 staining.

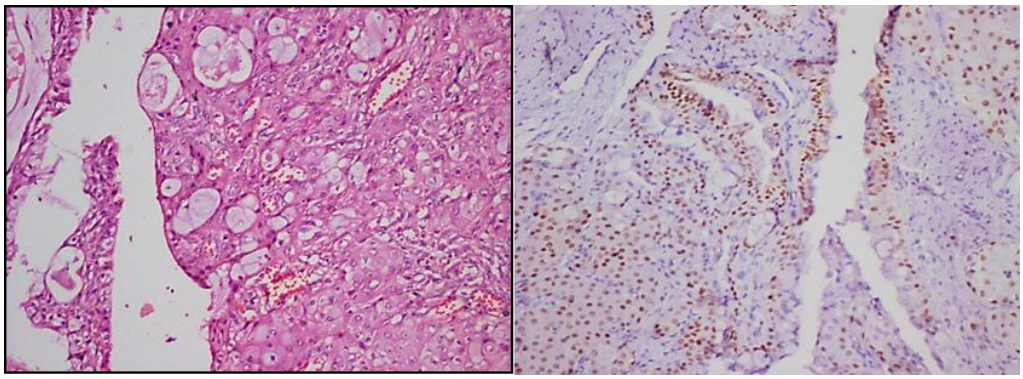

Fig. 6: Mucoepidermoid carcinoma positive for p63 immunostain.

Funding Source: None.

Conflict of Interest: None.

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How to cite this article: Kumar AA, Rakshitha HB, Alva NK, Reginald SR, Role of p63 immunohistochemistry in diagnosing Salivary gland neoplasms. *J Diagn Pathol Oncol* 2019;4(2):114-120.