JOURNAL OF INTERDISCIPLINARY HISTOPATHOLOGY, 2018
VOL 6, NO. 2, PAGE 61–66
10.5455/jihp.20181004012403

ORIGINAL RESEARCH

Open Access

Immunohistochemical identification of salivary pleomorphic adenoma subtypes using p63 and alpha smooth muscle actin myoepithelial markers

Adeola M. Ladeji[1], Kehinde E. Adebiyi[2], Akinwumi Komolafe[3], Olujide O. Soyele[1], Mofoluwaso Olajide[2], Henry A. Adeola[4,5]

[1]Department of Oral Maxillofacial Surgery and Oral Pathology, Faculty of Dentistry, Obafemi Awolowo University, Ile-Ife, Nigeria

[2]Department of Oral Pathology and Oral Medicine, Faculty of Dentistry, Lagos State University College of Medicine, Ikeja, Lagos, Nigeria

[3]Department of Morbid Anatomy and Forensic Medicine, Faculty of Basic Medical Sciences, Obafemi Awolowo University, Ile-Ife, Nigeria

[4]Department of Oral and Maxillofacial Pathology, Faculty of Dentistry, University of the Western Cape and Tygerberg Hospital, Cape Town, South Africa

[5]Division of Dermatology, Department of Medicine, Faculty of Health Sciences and Groote Schuur Hospital, University of Cape Town, Cape Town, South Africa

ABSTRACT

Background: Pleomorphic adenoma (PA) histopathologically represents a heterogeneous lesion with varying proportion of mesenchymal and epithelial tissue. Due to its heterogeneity, identifying various subtypes often presents a diagnostic dilemma. Hence, the aim of this study is to determine and compare the activities of salivary myoepithelial (myo) and basal reserve cells (BRC) within the subtypes of salivary PA using p63 and alpha smooth muscle actin (α-SMA) immunohistochemical markers.

Method: In this retrospective study, 24 archival tissue blocks of PA (12 stroma-rich, 6 cell-rich, and 6 classic subtypes) retrieved and subjected to immunohistochemistry using monoclonal p63 and α-SMA antibodies. Fraction of positive cells expressed as the percentage of total cells within each sample, labeling indices (LI) was computed.

Result: PA samples subjected to immunohistochemistry showed 100% p63 and 95.9% α-SMA immunoreactivity. Statistically significant difference ($p = 0.01$) was observed with p63 mean LI across PA subtypes. Overall, p63 LI (37.1 ± 13.5) was higher than α-SMA LI (26.5 ± 9.5). Staining pattern for both markers was similar within epithelial and stroma elements.

Conclusion: Disparity in Mean LI of the markers with higher index observed for p63, suggests additional activity of BRC, whose variable expression is associated with the differential ratio of epithelial to stroma components within PA subtypes.

ARTICLE HISTORY

Received October 04, 2018
Accepted November 06, 2018
Published November 12, 2018

KEYWORDS

Pleomorphic adenoma; p63; alpha smooth muscle actin; labeling index

Introduction

Pleomorphic adenoma (PA) of the salivary glands is an epithelial tumor which histologically presents a variable proportion of epithelial to mesenchymal components [1]. Seifert et al. [2] classified PA into four histological subtypes based on the proportion of the stroma-to-cellular components. Recently, this has been modified into three subtypes *viz*: stroma-rich, classic, and cell-rich subtypes [1]. The complex nature of PA has, however, been attributed to an interplay between the cells involved in the tumor and the proteins which they express [3].

Myoepithelial cells are modified epithelial cells which are normal cellular components of the major and minor salivary glands. They are, however, considered as key participants in the morphogenetic processes, responsible for the variable histopathologic appearances of many salivary gland tumors [4].

Basal cells are a subset of reserve cells which differentiate into the ductal components of the salivary glands and also function in the repair of these ductal cells. Studies have shown that the tumor formation can occur at any stage of morphogenesis

Contact Henry A. Adeola ✉ henry.adeola@uct.ac.za Division of Dermatology, Department of Medicine, Faculty of Health Sciences and Groote Schuur Hospital, University of Cape Town, Cape Town, South Africa.

toward full differentiation of these normal salivary glandular cells [5]. Pammer et al. [6], in their study, observed activity of these reserve cells in the epithelial and stroma components of PA.

P63 is a selective histochemical marker for basal/stem cells of stratified epithelium and myoepithelial cells. It is a p53 homolog which plays an essential role in both morphogenesis of the epidermis and limb development [7]. Structurally, p63 gene is located on chromosome 3q27-29 where it encodes three isoforms; p63α, p63β, and p63γ. A direct role of p63 in tumor formation has not been demonstrated, however, amplification of the 3q27 region has been established in a number of tumors, which is suggestive of its role as an oncogene rather than as a tumor suppressor gene [7–9]. There are a few in-depth studies on the expression of p63 in PA of the salivary glands. Edward et al. [10], in their study of p63 expression in selected SGNs, suggested that p63 expression may represent myoepithelial cells or a population of p63 positive epithelial stem or reserve cells.

In addition, p63 has also been shown to be a sensitive marker for lung squamous cell carcinomas with reported sensitivity of between 80% and 100% and specificity of 70%–90% [11]. Furthermore, the identification of p63 in the myoepithelial cells of the normal ducts of the breast tissue and its utility for assessment of breast lesions due to its differential expression in the luminal, basal, and myoepithelial cells of the breast has been reported [12].

Alpha smooth muscle actin is a component of the microfilaments of the myoepithelial cells of the salivary glands. Various actin isoforms expressed in various epithelial and non-epithelial cell types vary in their amino acid sequence, however, in the salivary glands, antibody against α-SMA specifically recognizes myoepithelial cells [13,14]. Scarpellini et al. [14], in a study of 23 benign and malignant salivary gland neoplasms, also found that calponin and alpha smooth muscle actin were the markers, most frequently expressed by the myoepithelial cells and positivity was mostly located in the myoepithelial cells that constituted the external layer of the glandular or tubular neoplastic structures.

p63 and α-SMA immunohistochemistry will help to better define the relative roles played by myoepithelial and basal reserve cells and their influence on the different subtypes of PA. Proper understanding of these factors will help to better characterize the tumor, arrive at accurate diagnosis, and subsequently help in proper treatment planning for a better outcome.

Materials and Methods

Ethical consideration

This is a retrospective study where archival tissues were used in all cases and there was no contact between the patients and the investigator at any time. All information retrieved from surgical day book were only accessible to the investigator, they were given code numbers and no names were recorded. All data were transferred to a password protected personal computer. Ethical approval was granted by the research ethics committee of the Obafemi Awolowo University, Ile-Ife under the ethics and protocol numbers IRB/IEC/0004553 and ERC/2013/07/18, respectively.

Case selection and tissue preparation

Formalin fixed paraffin embedded tissue blocks of 24 PAs were selected from the departments of Oral pathology and Morbid Anatomy and Forensic medicine of the Obafemi Awolowo University Teaching Hospitals Complex. These included 12 stroma rich, 6 cell rich, and 6 classic subtypes. Demographic information regarding age, gender, and site of lesions were retrieved for each subtype. H&E staining of re-cut tissue blocks was done to reconfirm diagnosis (Figure 1a).

Immunohistochemistry

Nearly, 4.5 μm tissue sections cut from the selected blocks were mounted on positively charged slides, deparaffinized, rehydrated, and subjected to immunohistochemistry. P63 and α-SMA antigens were retrieved in citrate buffer put in an incubator (microwave) at 90° C for 20–25 minutes as appropriate. Positive and negative controls for the antibodies were processed along the other slides for the same antibody to ensure validity (prostatic tissue for p63 and endothelial lined vascular channel for α-SMA) (Figure 1e–h). Endogenous peroxidase was blocked using 3% hydrogen peroxide for 10 minutes. Immunoperoxidase staining was done using mouse monoclonal p63 antibody (Product code-PM 163 AA) clone 4A4 and mouse monoclonal α-SMA antibody (Product code-NCL-SMA) clone αsm-1 at dilution fractions of 1:100 and 1:50, respectively. Two2-step horseradish peroxidase (HRP) technique was used, specimens were incubated for an hour with 40–130 μl (depending on the surface area of the tissue) of an appropriately diluted NCL mouse monoclonal primary antibody for α-SMA and PM monoclonal antibody for p63. Incubation

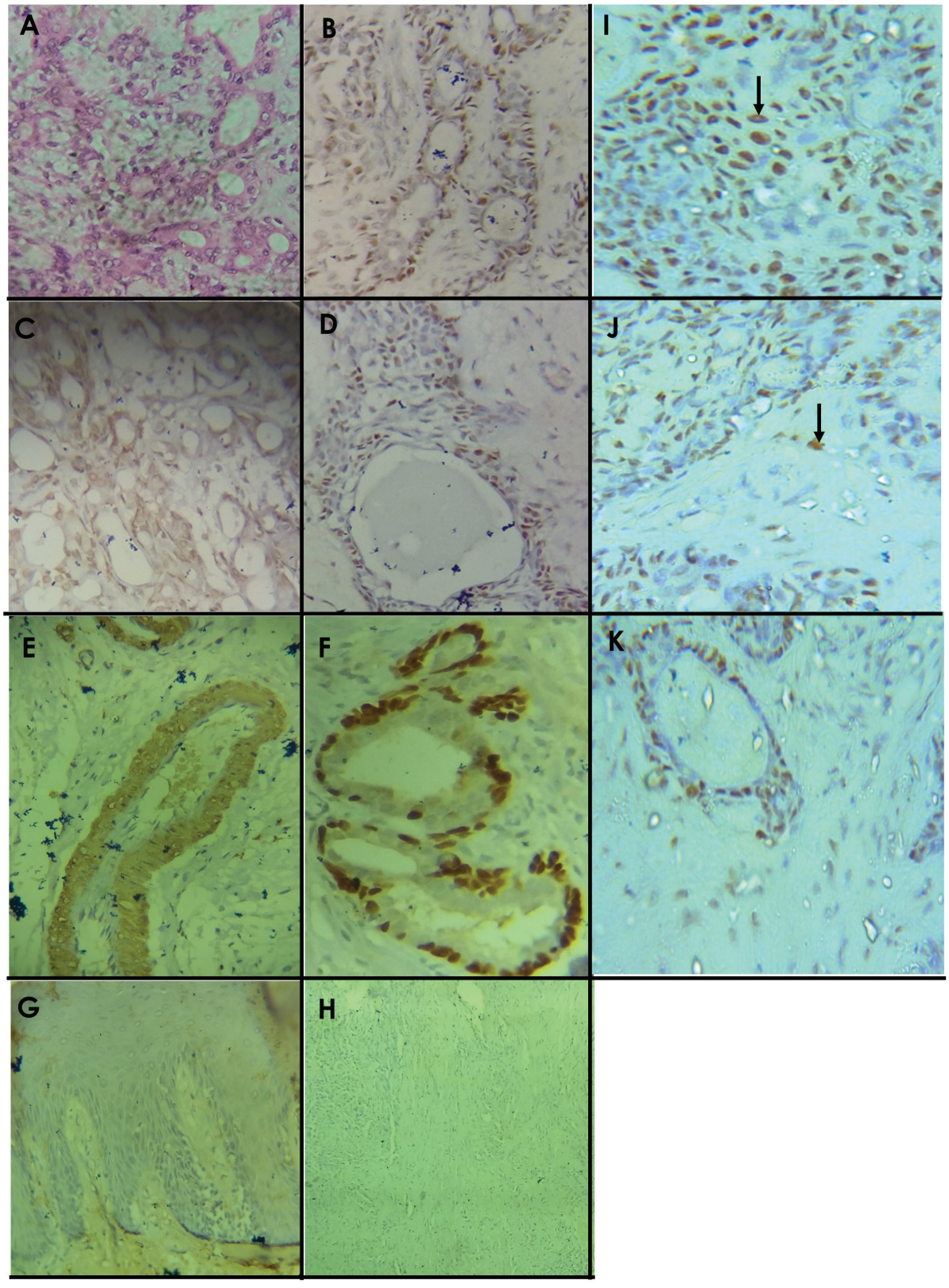

Figure 1. (a) Photomicrographs showing the immunohistochemical profile of a pleomorphic adenoma preceded by haematoxylin and eosin (H&E) staining. (b and d) p63 is strongly positive, with strong staining pattern observed in the nuclei of the abluminal (myoepithelial-like) cells and a few of the stroma cells (×100 and ×400 magnification, respectively). (c) Moderate cytoplasmic staining of the abluminal cells for α-SMA (×100 magnification). (e and g) Positive and negative controls for α-SMA, respectively; (f and h) positive and negative controls for p63, respectively. (i, j, and k) Degree of p63 immunopositivity for cell rich, classic (mixed) and stromal rich pleomorphic adenomas, respectively. Immunopositive cells are indicated with arrows.

with an undiluted labeled polymer HRP conjugated anti-mouse secondary antibody (8 µg/ml) for 30 minutes followed. The slides were rinsed with phosphate buffered saline (PBS) and areas surrounding the tissue were wiped dry with dry gauze and 1 ml of substrate-chromogen (i.e. diaminobenzidene) solution was added to cover the specimen. The slides were incubated in humidity chamber for 15 minutes. Thereafter, they were immersed in a bath of aqueous haematoxylin. Assessment of stains was done by three certified pathologists in an objective manner using a grid. Positive result was evidenced by the brownish coloration of the cellular nucleus for p63 and cytoplasm for α-SMA. From each quadrant, five points were identified from a circular view observed under the 10× objective lens. The identified points were labeled as north, south, west, east, and centre. In each of the identified points, 20 consecutive cells were evaluated for the presence and intensity of staining in the cells using the quantitative scoring system for immunohistochemical staining [15]. The total number of cells assessed per quadrant was 100 cells. Intensity of stain recorded was an average score per 25 cells. The exercise was repeated in the remaining three quadrants and the summation of this was recorded as the intensity of staining per 100 cells.

Grading of lesions was conducted using the quantitative scoring system for immunohistochemical staining (Histo-Score) [15] are as shown in Table 1. Labeling Index (LI) was defined as the fraction of the positive cells expressed as the percentage of positive cells within each tumor type and their variants; while mean Labeling index (MLI) was defined as the mean of the positive cells seen with each of the markers within the different neoplasms and their variants.

Statistical analysis

Data were analyzed using Statistical Package for Social Sciences version 12 (SPSS v.12.0; SPSS, Chicago, IL, USA). Descriptive statistics were carried out for socio-demographic variables such as age, sex, site of tumor, and histopathological characteristics. Quantitative variable (age) was expressed using parameters such as mean, median, minimum, maximum, and measures of variability. Qualitative descriptive variables such as sex, site of tumor, histopathological subtypes of PA were expressed as frequencies and percentages. P63 and α-SMA expression by the tumors were categorized using a quantitative scoring system. Comparison of the immunoperoxidase staining pattern within PA and its subtypes was analyzed using the likelihood ratio χ^2-square. Kruskal Wallis One-way analysis of variance for more than two variables was further used to compare the mean of the fraction of positive cells (MLI) within PA and its variants. Statistical significance was inferred at $p < 0.05$.

Results

All cases of PA were found to occur between 16 and 70 years of age, with mean and median ages of 35.8 and 33 years, respectively. Female predominance was observed, with a female:male ratio of 1.4:1. Parotid gland was the commonest site (n = 11, 45.8%), followed by submandibular gland (n = 6, 25.0%), palate (n = 4, 16.7%), labial (n = 2, 8.3%), and buccal mucosa (n = 1, 4.2%).

Nearly, 100% immunoreactivity to p63 was observed, while α-SMA showed 95.9% immunoreactivity. The range of staining for p63 was between weak (45.8%) and strong (4.1%) staining. α-SMA showed a range between negative (4.2%) and moderate (29.2%) staining, most tumors were weakly positive for αSMA (66.7%) (Table 2 and Figure 1c).

Three histopathologic subtypes were identified, cell rich (n = 6, 25%), classic (n = 6, 25%), and stroma rich (n = 12, 50%) as shown by immunopositivity to p63 (Figure 1i–k). Within the histopathologic subtypes of PA, highest mean LI for p63 was recorded with cell rich PA and immunoexpression of p63 within the variants was statistically significant at $p < 0.001$, αSMA showed no significant difference within the histologic variants (Table 3). P63 showed an overall index of 37.1 ± 13.1 which is higher than α-SMA which was 26.5 ± 9.5 (Table 4 and Figure 1b, d).

Discussion

P63 is a marker of the basal cells of stratified squamous epithelium and myoepithelial cells [7]. Similar to a previous study by Di Como et al. [16], all the 24 samples of PA used in this study were positive for p63 antibody. p63 staining was observed in the myoepithelial-like (abluminal) cells where they are seen to express a range of weak (45.8%) to moderate (50%) positivity. These positive abluminal cells were seen to be merging with the peripheral stroma, which is similar to reports by Edward et al. [10] A number of the spindle cells and chondrocytes within the stroma were also observed to express positivity supporting the reports of Nagao et al. [3], Alves et al. [17], and Bilal et al. [18]. The mean LI amongst PA was highest for cell rich followed by

Table 1. Parameters for the labeling index.

Score	Positive cells
Negative reactivity (0)	<10%
Weakly positive reaction (1+)	10%–25%
Moderately positive reaction (2+)	26%–50%
Strongly positive reaction (3+)	51%–75%
Very strongly positive reaction (4+)	>75%

Table 2. Indirect immunoperoxidase staining for p63 and αSMA.

Positive cell	p63	αSMA
Negative staining	0 (0.0)	1 (4.2)
Weak staining	11 (45.8)	16 (66.7)
Moderate staining	12 (50.0)	7 (29.2)
Strong staining	1 (4.1)	0 (0.0)
Very strong staining	0 (0.0)	0 (0.0)

classic subtype. This difference was statistically significant at $p < 0.001$ and suggests more myoepithelial or epithelial stem/reserve cell activity in these two variants.

Alpha SMA is an immunomarker for myoepithelial cells. Reactivity within PA was 95.9% (23 cases), there was a case with no reactivity. Positivity was observed in the cytoplasms of the abluminal cells of ductal structures, which appeared to be merging with the surrounding stroma, with focal reactivity within the stroma cells as well, comparable to observations by Margaritescu et al. [19] and Nagao et al. [3]. Mean labeling index between the variant of PA pointed out higher indices in the cell rich PA (27.0 ± 4.0) and classic PA (28.3 ± 8.2).

Similar staining pattern observed for both markers reflects activity of myoepithelial cells within the epithelial and stroma elements of PA. Overall mean LI for p63 (37.1 ± 13.1) was observed to be higher than α-SMA (26.5 ± 9.5), furthermore, mean LI within the cell rich (50.0 ± 0.0) and classic (46.7 ± 10.8) subtypes were much higher than for α-SMA suggesting possible activity of basal/reserve cells in addition to the myoepithelial cells with p63, especially, within the cell rich and classic subtypes. The significant difference ($p < 0.001$) observed with p63 within the variants indicates that the activity of these basal reserve cells is associated with the variable cellularity seen in the subtypes of PA.

Neoplastic myoepithelium is considered a key cellular participant in the morphogenetic processes responsible for variable histologic appearances of PA, however, controversy still exists concerning the extent of its activities in salivary gland tumours. This study however, may have been able to establish relative roles played by the myoepithelial and basal reserve cells in PA of the salivary glands and its different subtypes.

Table 3. Comparison of mean labeling index within variants of pleomorphic adenoma.

Marker	Cell rich	Classic	Stroma rich	*p*-Value
p63	50.0 ± 0.0	46.7 ± 10.8	25.8 ± 7.0	<0.001
α-SMA	27.0 ± 4.0	28.3 ± 8.2	25.3 ± 12.0	0.619

Table 4. Mean labeling indices for p63 and αSMA.

Marker	Mean ± SD
p63	37.1 ± 13.5
α-SMA	26.5 ± 9.5

Conclusion

Myoepithelial cells play key roles in the histopathological variability of salivary pleomorphic adenoma. In addition to the influence of myoepithelial cells, activity of basal/reserve cells has also been found to be more prominent in the epithelial elements of the individual tumors. This may explain the observed relative cellularity within the tumor subtypes. This preliminary immunohistopathological study demonstrates that p63 and α-SMA are potential biomarkers for classification of PA of the salivary gland.

Acknowledgments

Adeola M. Ladeji wishes to thank Dr T.O Oyedele and Prof O. A Fatusi for the editorial help provided with the manuscript. Henry A. Adeola thanks the South African Medical Research Council (SAMRC) for a mid-career scientist research grant and the South African National Research Foundation (NRF) for incentive grant for rated researchers.

Conflict of interest

The Authors declare that they have no conflict of interest.

Authors' contributions

Adeola M. Ladeji conceptualized, designed, prepared, and critically revised the manuscript, tables, and figures. Kehinde E. Adebiyi, Akinwumi Komolafe, Olujide O. Soyele, and Mofoluwaso Olajide were involved in the design, data collection/analysis, and preparation of sections of the manuscripts. Henry A. Adeola was involved in the design, critical intellectual revision, and finalization of the

manuscript, figures and tables. All authors had final approval of the submitted version.

References

[1] Ito FA, Jorge J, Vargas PA, Lopes MA. Histopathological findings of pleomorphic adenomas of the salivary glands. Med Oral Patol Oral Cir Bucal 2009; 14:E57–61.

[2] Seifert G, Langrock I, Donath K. [A pathological classification of pleomorphic adenoma of the salivary glands (author's transl)]. HNO 1976; 24:415–26.

[3] Nagao T, Sato E, Inoue R, Oshiro H, Takahashi RH, Nagai T, et al. Immunohistochemical analysis of salivary gland tumors: application for surgical pathology practice. Acta Histochem Cytochem 2012; 45:269–82.

[4] Redder C, Kandagal VS, Vibhute N, Ingaleshwar P, Shetty SJ, Ahamad S. Myoepithelial cell: current perspective in salivary gland tumours. Clin Cancer Investig J 2015; 20:101–5.

[5] Burford-Mason A, Dardick I, van Nostrand P. Salivary gland neoplasms–stem cell histogenesis? J Laryngol Otol 1990; 104:521–3.

[6] Pammer J, Horvat R, Weninger W, Ulrich W. Expression of bcl-2 in salivary glands and salivary gland adenomas. A contribution to the reserve cell theory. Pathol Res Pract 1995; 191:35–41.

[7] Reis JS, Schmitt FC. Taking advantage of basic research: p63 is a reliable myoepithelial and stem cell marker. Adv Anat Pathol 2002; 9:280–3.

[8] Nylander K, Dabelsteen E, Hall PA. The p53 molecule and its prognostic role in squamous cell carcinomas of the head and neck. J Oral Pathol Med 2000; 29:413–25.

[9] Krane JF, McKeon F, Faquin WC. Expression of p63, a p53 homologue, in normal and neoplastic salivary gland (SG). Modern Pathol 2001; 14:151a–a.

[10] Edwards PC, Bhuiya T, Kelsch RD. Assessment of p63 expression in the salivary gland neoplasms adenoid cystic carcinoma, polymorphous low-grade adenocarcinoma, and basal cell and canalicular adenomas. Oral Surg Oral Med Oral Pathol Oral Radiol Endod 2004; 97:613–9.

[11] Tacha D, Yu C, Bremer R, Qi WM, Haas T. A 6-antibody panel for the classification of lung adenocarcinoma versus squamous cell carcinoma. Appl Immunohistochem Mol Morphol 2012; 20:201–7.

[12] Hicks DG. Immunohistochemistry in the diagnostic evaluation of breast lesions. Appl Immunohistochem Mol Morphol 2011; 19:1501–5.

[13] Furuse C, Sousa SO, Nunes FD, Magalhaes MH, Araujo VC. Myoepithelial cell markers in salivary gland neoplasms. Int J Surg Pathol 2005; 13:57–65.

[14] Scarpellini F, Marucci G, Foschini MP. [Myoepithelial differentiation markers in salivary gland neoplasia]. Pathologica 2001; 93:662–7.

[15] van Diest PJ, van Dam P, Henzen-Logmans SC, Berns E, van der Burg ME, Green J, et al. A scoring system for immunohistochemical staining: consensus report of the task force for basic research of the EORTC-GCCG. European Organization for Research and Treatment of Cancer-Gynaecological Cancer Cooperative Group. J Clin Pathol 1997; 50:801–4.

[16] Di Como CJ, Urist MJ, Babayan I, Drobnjak M, Hedvat CV, Teruya-Feldstein J, et al. p63 expression profiles in human normal and tumor tissues. Clin Cancer Res 2002; 8:494–501.

[17] Alves FA, Perez DE, Almeida OP, Lopes MA, Kowalski LP. Pleomorphic adenoma of the submandibular gland: clinicopathological and immunohistochemical features of 60 cases in Brazil. Arch Otolaryngol Head Neck Surg 2002; 128:1400–3.

[18] Bilal H, Handra-Luca A, Bertrand JC, Fouret PJ. p63 is expressed in basal and myoepithelial cells of human normal and tumor salivary gland tissues. J Histochem Cytochem 2003; 51:133–9.

[19] Margaritescu C, Raica M, Simionescu C, Mogoanta L, Surpateanu M, Cionca L, et al. Tumoral stroma of salivary pleomorphic adenomas (PA)-histopathological, immunohistochemical and ultrastructural correlations. Modern Pathol 2006; 19:108–9.