

Original article

A COMPARATIVE STUDY OF Ki 67 IMMUNOPROFILE OF BENIGN AND MALIGNANT SALIVARY GLAND TUMOURS

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ABSTRACT

OBJECTIVES: To discriminate between benign and malignant salivary gland tumours, using their Ki-67 immunoreactivity in a South-South Nigerian population.

METHODS: A descriptive and analytical study of 42 formalin fixed paraffin embedded (FFPE) tissue blocks of histologically diagnosed neoplastic salivary gland tumours. FFPE tissue block of 21 benign and 21 malignant tumours were sectioned and stained with Ki-67(MIB1) primary antibody, using the polymer horseradish peroxidase method. The Ki-67 labelling index (LI) of each tumour was assessed. The mean Ki-67 LI of the benign salivary gland tumours was compared with that of the malignant tumours.

RESULTS: There was a generally low reactivity of the salivary gland tumours to Ki 67 antibody, only 31.0% cases showed immunoreactivity for Ki-67 antibody, and the Ki-67 LI ranged from 0.5 to 7 % in both the benign and malignant tumours. The mean Ki-67 LI for the malignant lesions (3.9%) was higher than that of the benign tumours (2.4%), but the difference was not statistically significant [$p=0.280$].

CONCLUSION: This study showed that Ki 67 antibody cannot reliably differentiate a benign from a malignant salivary gland tumour, although the malignant lesions had a higher Ki 67 LI.

Key words: Benign and malignant, salivary gland tumours, Ki 67 immunohistochemistry

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INTRODUCTION

Salivary gland tumours have a special status in human neoplasia and probably have the most complex histopathologic feature of the body organs, and are very heterogeneous.¹⁻² They are relatively rare and account for less than 2% of all human neoplasms^{1,3-5} and 2.8 to 10.5% of head and neck tumours.^{4, 5-7}

As a result of the rarity, the number of histologic subtypes, and the morphologic overlap and heterogeneity among these subtypes, salivary gland tumours often remain diagnostically

challenging even for experienced pathologists.⁸⁻¹⁰

One major challenge of diagnosis of SGT is the tendency for one tumour to mimic others histologically.¹¹ These challenges are further compounded in cases of small oral biopsies or fragmented tissues where the luxury of sampling a wide field and the margin of the tumour is not at the disposal of the pathologist. Histologic mimicry of clinically and prognostically divergent lesions is a daunting challenge in salivary gland pathology.

Salivary gland malignancies more often than not, appear cytologically bland, and may be mistaken for a benign salivary gland tumour.¹²⁻¹⁴ A benign SGT notably the solid myoepithelial cell-rich pleomorphic adenoma could be a great mimicker of various malignant salivary gland tumours.¹¹ Furthermore, lesions that have malignant and benign counterparts such as: myoepithelioma and myoepithelial carcinoma; basal cell adenoma and basal cell adenocarcinoma can further pose a challenge in histologic distinction, especially in small or fragmented biopsy tissue.¹⁰

Because of the frequently bland cytologic features of the malignant salivary gland tumours, they are often distinguished from their benign counterpart by other histological hallmarks, such as their invasive outgrowth (being the most important diagnostic feature), perineural and vascular invasion, and tumour necrosis. However, in cases with limited samples, the morphological appearance may not be sufficient to provide a differential diagnosis between these two groups of lesions.¹¹

Although the gold standard method used for the diagnosis of salivary gland tumours still remain the histopathological examination of H&E stained sections,¹⁵ immunohistochemistry (IHC) can enhance the accuracy of diagnosis and serve as a helpful tool to investigate the features that cannot be assessed by histological examination of H&E stained sections. Cell proliferation, and expression of proteins by the tumour cells are better elucidated by immunohistochemistry.¹⁶ In this way, immunohistochemical markers have provided useful contributions in the salivary gland tumour diagnosis.^{10,17}

One of the most important biological mechanisms in oncogenesis is cell proliferation.¹⁸ Ki-67, encoded by the MKI67 gene,¹⁹ is a nuclear and nucleolar non-histone protein that is associated with cellular proliferation. It is required for the synthesis of ribosomes during the cell cycle.²⁰ The Ki-67 protein is present during all the active stages of the cell cycle (G1, G2, M), but absent during the G0 phase, which is the inactive or resting phase.²¹ This makes it an excellent marker in determining the growth fraction of any given tumour cell population, which correlates with the clinical course or behaviour of the tumour. This

fraction is known as the Ki-67 labelling index.²² Evaluation of proliferation markers are currently used to predict biological behaviour and to differentiate benign from malignant tumours.²³⁻²⁴ There are conflicting reports in the literature as regards the diagnostic benefit of the Ki 67 immunohistochemistry in discriminating benign SGT from the malignant SGTs.^{15,23-30} Whereas some researchers have observed a statistically significant higher Ki 67 LI in the malignant SGTs compared to the benign tumours, others are of the opinion that it may not be a useful tool in the differential diagnosis between the two group of lesions. However, there has been no Nigerian study which compared the Ki 67 immunoreactivity of the benign SGTs and the malignant SGTs. This study therefore aims to compare the benign SGTs and the malignant SGTs, using their Ki-67 immunoprofile in a South-South Nigerian population as a contribution to global study of this subject.

MATERIALS AND METHODS

Ethical approval to perform this descriptive and analytical study was obtained from the University of Benin Teaching Hospital Ethics and Research Committee (Protocol number: ADM/E 22/A/VOL. VII/1031). Patients' records, histopathology reports, and histologic slides of diagnosed cases of salivary gland tumours (SGT) between 1990 and 2015 were retrieved from the archives in the Department of Oral Pathology/Medicine and the Department of Morbid Anatomy, University of Benin Teaching Hospital, Benin City, Nigerian.

Forty two cases were selected among the cases of SGTs whose blocks of formalin-fixed, paraffin embedded (FFPE) tissues were available. The patients' age, gender and site of tumour were obtained from the patients' clinical records. Two (2) sections (5mm-thick) of each FFPE tissue blocks were made: 1 section for histopathologic re-evaluation using Haematoxylin and Eosin (H&E) stain; 1 section for Ki-67 immunohistochemical staining. Each of the sections obtained from FFPE tissue blocks for the immunohistochemical staining were mounted on silanized slides for immunohistochemical staining with monoclonal mouse anti-human

antibodies against Ki-67 using the polymer-horseradish peroxidase method.

Positive and negative tissue controls were obtained according to the antibodies manufacturer's datasheets and added to each run.

Details of antibodies used for the immunohistochemical study

- Antibody: Ki 67
- Type: Mouse Antihuman
- Clonality: Monoclonal
- Clone: MIBI
- Manufacturer: Dako, Denmark
- Dilution: 1:100

The positive control for the Ki-67 was a section from a previously diagnosed case of *invasive carcinoma of the breast* (Fig 1). These sections were added to each run, and incubated simultaneously with the test samples. The negative control was a tissue section of a diagnosed case of *invasive breast carcinoma* incubated with the antibody diluents without the inclusion of the primary antibody.

Immunohistochemical slides were reviewed without reference to initial histologic diagnosis (H&E) to eliminate bias. Immunohistochemical signal specificity was demonstrated by the absence of immunostaining in the negative control slides and the presence of immunohistochemical staining in the positive controls.

Tumour cell positivity was indicated by *brown nuclear* staining. For the cases that showed positivity, the Ki-67 labelling index (LI) was assessed. This was evaluated *semi-quantitatively*. The percentage of the positive tumour cells out of 1000 tumour cells at high magnification (x40) was considered the Ki-67 LI. These cells were counted in five microscopic fields (at x40 magnification) which illustrated more intense staining. The number of positively stained cells (nuclei) counted in these microscopic fields were expressed as a percentage of 1000 cells.²⁴ The cases that showed negative Ki67 expression were excluded.

Data obtained were analyzed using the Statistical Package for Social Science (SPSS) for Windows, version 23 software (IBM Corp., 2015). The

mean Ki-67 LI of positive cases for each tumour type was calculated, and the mean Ki-67 LI of the benign lesions was compared with those of the malignant lesions SGT using the independent samples student's T-test. The level of significance was set at 95% (p -value <0.05).

RESULTS

Of the 42 cases of SGTs that were selected for this study, there were 13 (31.0%) males and 29 (69.0%) females, giving a male to female ratio of 1:2.2. The age range between 12 and 70 years and a mean age of 41.6 ± 15.6 years. Most of the cases were in the palate (n=20, 47.6%) and parotid region (n=13, 31.0%) [Table 1].

There were 21 benign and 21 malignant lesions including 2 cases of acinic cell carcinoma (ACC), 6 adenocystic carcinoma (ADCC), 6 polymorphous low grade adenocarcinoma (PLGA), 6 mucoepidermoid carcinoma (MEC), 1 epithelial myoepithelial carcinoma (EME CA), 19 pleomorphic salivary adenoma (PSA), and 2 cases of basal cell adenoma (BCA).

Ki67 positive cells were detected as brown nuclear staining. Of the 42 cases of benign and malignant salivary gland tumours studied, only 13(31.0%) cases showed reactivity to Ki67 antibody consisting of 6 (14.3%) malignant SGTs and 7 (16.7%) cases of benign SGTs. These were seen in adenocystic carcinoma (Fig 2), mucoepidermoid carcinoma (Fig 3), pleomorphic adenoma (Fig 4), and epi-myoepithelial carcinoma (Fig 5) [Table 2]. The mean Ki67 labelling index for mucoepidermoid carcinoma was 4.3%, 5% for adenocystic carcinoma, 0.5% for epi-myoepithelial carcinoma and 2.4% for pleomorphic adenoma.

Using the independent samples t- test, the mean Ki-67 LI for the malignant tumours (3.92 ± 1.05 SEM) was slightly higher than that of the benign tumours (2.43 ± 0.81 SEM), but the difference was not statistically significant ($p = 0.280$) [Figure 6].

DISCUSSION

Salivary gland tumours are a group of heterogeneous lesions, with a significant variation and overlap of histo-architecture within tumour types and between different tumour types.³

Table 1: The clinico-demographic characteristics of cases selected for the immunohistochemical study

Tumour Histology	Mean Age $\pm$ SD (Years)	Gender		Site/no.
		Male	Female	
ACC	54.5 $\pm$ 7.8	1	1	Palate-1, Gingiva-1
ADCC	42.2 $\pm$ 15.2	1	5	Parotid-1, Submand-2 Palate-1, Subling-1 Antrum-1
MEC	46 $\pm$ 16.2	1	5	Parotid-4, Palate-2
PLGA	44.8 $\pm$ 17.3	2	4	Palate-5, Submand-1
EME CA	47	0	1	Parotid-1
PSA	35.6 $\pm$ 15.2	7	12	Parotid-4, Submand-2 Palate-11, Cheek-1 Lip-1
BCA	59 $\pm$ 5.7	1	1	Parotid-2
Total	41.6$\pm$15.6	13	29	42

Key: ACC=Acinic cell carcinoma, ADCC=Adenocystic carcinoma, MEC=Mucoepidermoid carcinoma, PLGA=Polymorphous low grade adenocarcinoma, EME CA=Epithelial myoepithelial carcinoma, PSA=Pleomorphic salivary adenoma, BCA=Basal cell adenoma

Table 2: The number of cases of the salivary gland tumours that showed positive and negative expression of Ki-67 antigen

Tumour Histology	No. of cases studied (%)	No. of cases with positive reactivity (%)	No. of cases with negative reactivity (%)
ACC	2(100%)	0(0%)	2(100%)
ADCC	6(100%)	2(33.3%)	4(66.7%)
MEC	6(100%)	3(50%)	3(50%)
PLGA	6(100%)	0(0%)	6(100%)
EME CA	1(100%)	1(100%)	0(0%)
PSA	19(100%)	7(36.8%)	12(63.2%)
BCA	2(100%)	0(0%)	2(100%)
Total	42	13(31.0%)	29(69%)

Key: ACC=Acinic cell carcinoma, ADCC=Adenocystic carcinoma, MEC =Mucoepidermoid carcinoma, PLGA=Polymorphous low grade adenocarcinoma, EME CA=Epithelial myoepithelial carcinoma, PSA=Pleomorphic salivary adenoma, BCA=Basal cell adenoma

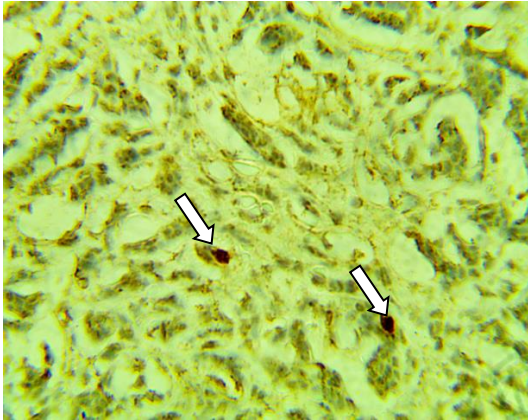


Figure 1(a): Ki-67 IHC expression in invasive breast carcinoma, with brown nuclear staining in positive cells (POSITIVE CONTROL) x400

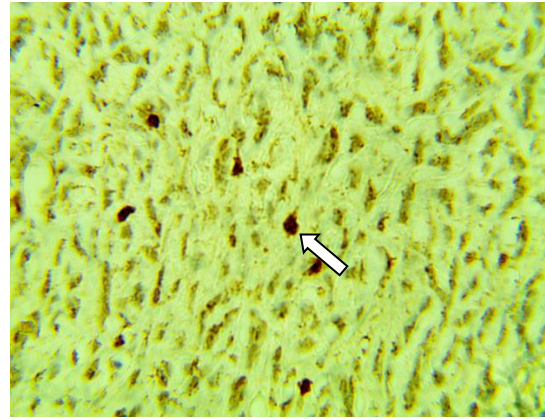


Figure 1(b): Ki-67 IHC expression in invasive breast carcinoma (POSITIVE CONTROL) x400

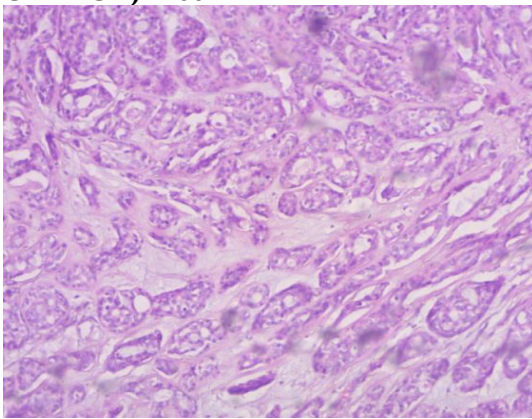


Figure 2(a): Adenocystic carcinoma (tubular pattern), H&Ex100.

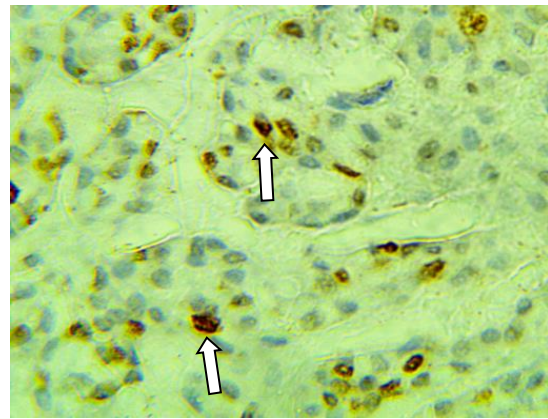


Figure 2(b): Ki-67 IHC expression in adenocystic carcinoma, X400.

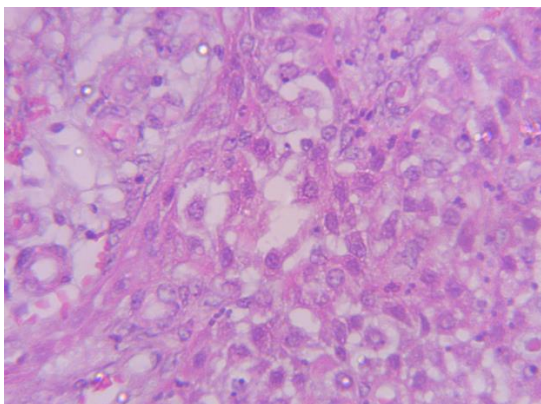


Figure 3(a): Mucoepidermoid carcinoma, H&E x400.

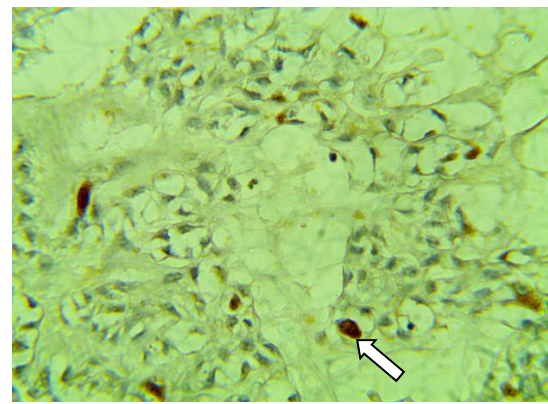


Figure 3(b): Ki-67 IHC expression in mucoepidermoid carcinoma x400

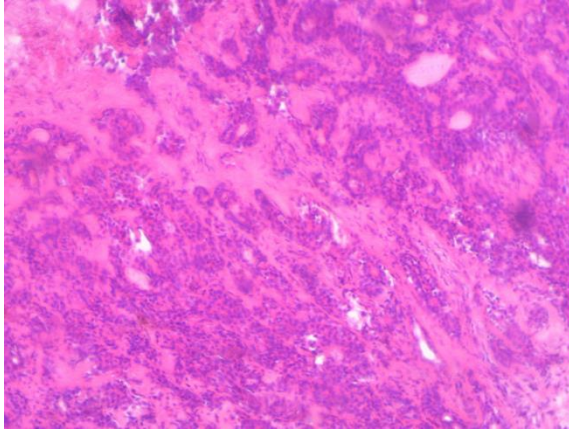


Figure 5(a): Pleomorphic adenoma, H&E x100

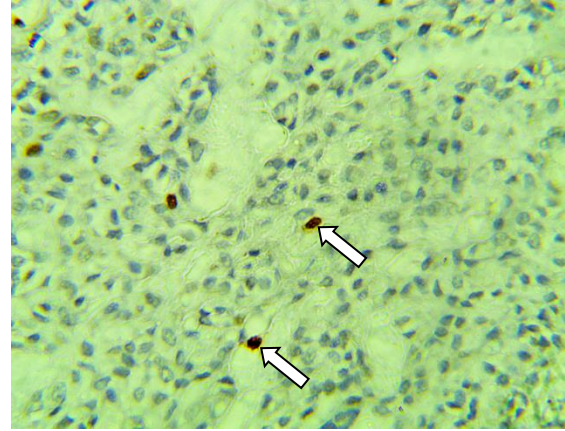


Figure 5(b): Ki-67 IHC expression in pleomorphic adenoma x400

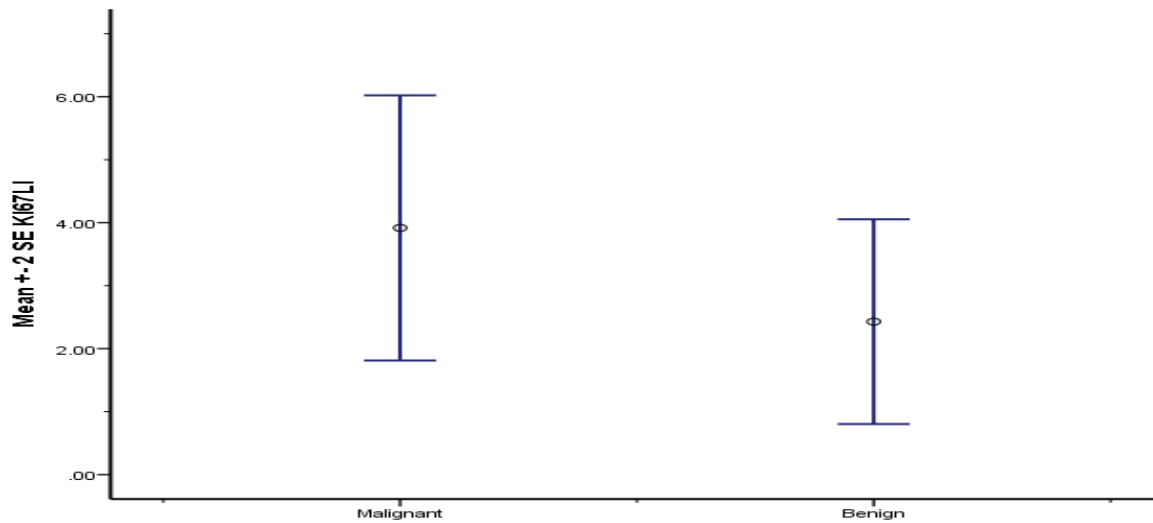


Figure 6: The error bars of the mean Ki-67 Labelling Index, of malignant and benign salivary gland tumours. (Independent Samples T-test, $p = 0.280$)

They have a high tendency for histologic mimicry of different tumours with differing clinical course and prognosis. They can sometimes pose a considerable challenge in their histopathologic diagnosis, even for the experienced pathologist.^{3,17} The cytologically bland nature of most malignant salivary gland tumours could be deceptive in some cases especially in small or fragmented tissue samples. Though reports are conflicting, the role of proliferation markers like Ki 67 as ancillary tool in resolving this diagnostic difficulties in equivocal cases has previously been documented.²³⁻³⁰ This study compared the Ki 67 immunoprofile in the benign SGTs and malignant SGTs.

Generally, low levels of Ki-67 antigen was expressed in the SGTs in this study. Only 13 (31.0%) cases showed immunoreactivity for Ki-67 antibody, with the Ki-67 LI ranging from 0.5% to 7% in both benign and malignant tumours. This was a relatively lower level of Ki-67 positive expressivity compared to previous studies which recorded a Ki-67 LI ranging from 1% to 23%.²⁵⁻²⁹ The relatively lower level of Ki-67 positive expressivity observed in this study may be due to the technique and storage duration of antibodies, extent of fixation of the tissue, and the duration of storage of the tissue. Attempt was made to overcome the influence of some limiting factors by repeating the staining of the samples with Ki-

67 immunohistochemical marker in another laboratory using antibody from a different manufacturer. However, a similarly low immunoreactivity for Ki-67 was observed. This may be explained by the nature of malignant SGTs in which mitotic figures are rarely found.

Among the 13 (31.0%) cases that showed reactivity for Ki-67 antibody, there were 6 (14.3%) malignant SGTs, consisting of ADCC (n=2, 4.8%), mucoepidermoid carcinoma (n=3, 7.1%), and epi-myoeplithelial carcinoma (n=1, 2.4%). All the 7 (16.7%) cases of benign SGTs that were positive for Ki-67 were pleomorphic adenoma. In this study, 50.0% of cases of mucoepidermoid carcinoma showed positive expression for Ki-67. This is comparable to the 46.7% positive expression for Ki-67 in mucoepidermoid carcinoma reported by Alves *et al.*,³¹ and the 40.0% positive expression reported by Qureshi *et al.*³² However, a much higher positive expression for Ki-67 in mucoepidermoid carcinoma have been reported by Ashkavandi *et al.*²⁴ (100%) and Tadbir *et al.*²⁸ (98%). The mean Ki-67 LI of mucoepidermoid carcinoma in this study was 4.3%, whereas, Ashkavandi *et al.*²⁴ reported a higher mean Ki-67 LI of 21.89% for mucoepidermoid carcinoma. These studies agree that there is a high positive expression for Ki-67 in mucoepidermoid carcinoma.

There was positive expression of Ki-67 in 33.3% of *adenoid cystic carcinoma* (ADCC) in this study. Similarly, a low (42%) reactivity of Ki-67 was reported by Carlinfante *et al.*,³³ in ADCC. However, a higher positive reactivity of 68%, 100%, 60% and 84% were reported respectively in adenocystic carcinoma by Amoueian *et al.*³⁴, Ashkavandi *et al.*²⁴, Alves *et al.*³¹, and Tadbir *et al.*²⁸ The mean Ki-67 LI of adenocystic carcinoma in this study was 5%. However, higher mean labelling indices of 22.62%, 13.9%, 21.4%, and 11.8% have been reported respectively by Ashkavandi *et al.*²⁴, Darling *et al.*³⁵, Skalova *et al.*³⁶, Lazarro and Cleveland.³⁰ Overall, these findings show a relatively lower positive expression of Ki-67 in ADCC compared with most of the previous studies.

The only case of *epi-myoeplithelial carcinoma* seen in this study also showed a positive reactivity for Ki-67, with a Ki-67 LI of 0.5%.

However, Angiero *et al.*³⁷ reported a range of 5-10% Ki-67 LI for epi-myoeplithelial carcinoma, indicating a possible lower positive reactivity for Ki-67 in epi-myoeplithelial carcinoma, although further studies with more samples is required to ascertain the actual level of reactivity for Ki-67 in epi-myoeplithelial carcinoma in our environment. *Polymorphous low grade adenocarcinoma* did not express reactivity for Ki-67 in this study. This agrees with previous report of 0% reactivity for Ki-67 by Lazarro and Cleveland.³⁰ However, more studies have reported positive expression of Ki-67 in PLGA, with a mean LI ranging from 0.5% to 10%.^{38,39} Findings from this study further underscores the indolent nature of this tumour.

There were 36.8% cases of pleomorphic adenoma with positive reactivity for Ki-67 antibody, with a mean Ki-67 LI of 2.4%, in this study. This was relatively higher than the mean Ki-67 LI of 1% and 1.73% respectively reported for pleomorphic adenoma by Murakami *et al.*,²⁵ and Ashkavandi *et al.*²⁴ This could be due to the predominant histologic variants studied. Pleomorphic adenoma is a benign tumour that has varied histopathologic features. Further studies which will categorise pleomorphic adenoma into the cell- rich types and the stroma- rich types will be needed to truly evaluate and compare the Ki- 67 LI indices of these histopathologic variants.

Malignant SGTs are often distinguished from their benign counterpart by histological hallmarks, such as their invasive outgrowth, perineural and vascular invasion, and tumour necrosis. However, in cases with limited samples, the morphological appearance may not be sufficient to provide a differential diagnosis between malignant and benign lesions, especially in SGT that have both benign and malignant counterparts.¹⁰ The solid myoeplithelial cell-rich pleomorphic adenoma could mimic any other malignant SGT.¹¹ Ki-67 LI has been adjudged useful in distinguishing benign from malignant SGTs in equivocal cases.²⁵⁻²⁹ In this study, the mean Ki-67 LI of the benign SGTs was 2.43± 0.81%, while the malignant tumours had a mean Ki-67 LI of 3.92± 1.05%. Although the malignant tumours had a higher mean Ki-67 LI compared to the benign tumours, this observed

difference was not statistically significant. This observation is consistent with findings from a previous study, which reported non-significant higher Ki-67 LI in malignant lesions than the benign.³⁰ However, some studies have reported a statistically significant difference in the mean Ki-67 LI between malignant and benign SGTs.²⁵⁻²⁹ Furthermore, Ashkavandi et al.²⁴ reported a statistically significant difference between malignant and benign SGTs and recommended a cut-off point of 8% Ki-67 LI to differentiate malignant from benign SGTs.

In conclusion, this study observed a generally low reactivity of salivary gland tumours to Ki 67 antibody. This study showed that Ki 67 antibody cannot reliably differentiate a benign from a malignant salivary gland tumour although the malignant lesions had a higher Ki 67 LI. This study should continue with developed antibodies to the cell cycle proteins.

No conflict of interest is declared in this study.

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