J Med Sci 2022;42(5):206-213
DOI: 10.4103/jmedsci.jmedsci_204_21

ORIGINAL ARTICLE

Expression of Ki-67, P63, P40, and Alpha-Smooth Muscle Actin in Salivary Gland Carcinomas with or without Myoepithelial Differentiation

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Background: Myoepithelial cells are involved in the development of salivary glands. Many studies propose that these cells can prevent cell proliferation. **Aim:** This study aimed to investigate the expression of Ki-67, P63, P40, and alpha-smooth muscle actin (α-SMA) in salivary gland carcinomas with or without myoepithelial differentiation. **Methods:** A panel of myoepithelial markers including P63, P40, α-SMA, and Ki-67 were used for immunohistochemical study in 67 salivary gland carcinomas (33 with and 34 without myoepithelial differentiation). The percentage of positive cells was calculated (in high-power field) from a minimum of 1000 neoplastic cells. SPSS software (version 21) was used. **Results:** There was no statistically significant difference between Ki-67 expression and the presence or absence of myoepithelial cells ($P = 0.6$), but Ki-67 expression was related to the age ($P = 0.032$) and location of carcinomas ($P = 0.001$). All carcinomas with myoepithelial differentiation exhibited consistent P63+/P40+ staining, whereas polymorphous adenocarcinomas showed P63+/P40− immunophenotype. The expression of Ki-67 in adenoid cystic carcinomas was higher than mucoepidermoid carcinomas ($P = 0.020$) and polymorphous adenocarcinomas ($P = 0.002$). **Conclusion:** In the present study, although the decrease in the number of myoepithelial cells was associated with increased proliferation in adenoid cystic carcinomas, no such relationship was found in the overall assessment between the two groups. This can be justified by the fact that the clinical behavior of salivary carcinomas and their cell proliferation may be affected by factors other than the presence of myoepithelial cells or lack thereof. Ki-67 and P63/P40 expressions may be useful to differentiate adenoid cystic carcinomas from polymorphous adenocarcinomas in small biopsies.

Key words: Cancer, immunohistochemistry, myoepithelial, salivary gland tumor

INTRODUCTION

The Ki-67 antigen is present in all cell cycle phases except the G0 stage, and it is considered a proliferative marker that can be easily assessed by immunohistochemical staining.[1,2] It is believed that the overexpression of this marker is associated with the prognosis of patients afflicted by a variety of epithelial cancers and lymphomas.[3] It seems that different subtypes of salivary gland carcinomas harbor a broad range of malignancy potential.[2] Several salivary carcinomas contain myoepithelial cells and others have either a few or none.[4] Researchers have shown that salivary tumors with myoepithelial differentiation are different.[5-7] Myoepithelial cells are contractile cells that are associated with the secretory end piece and the ducts of salivary glands. They are located between the base membrane and the secretory or ductal cells, and their attachment to cells is via desmosomes. These cells have an epithelial origin.[8] Evidence suggests that myoepithelial cells secrete several proteins that suppress tumor activity, and they can make antiangiogenesis factors as well. In addition, these cells are barriers against the invasion of epithelial neoplasms.[7] Primary salivary carcinomas

Received: June 16, 2021; Revised: November 02, 2021; Accepted: November 11, 2021; Published: December 27, 2021; Corresponding Author: Dr. Saede Atarbashi-Moghadam, Department of Oral and Maxillofacial Pathology, School of Dentistry, Shahid Beheshti University of Medical Sciences, Daneshju Blv, Velenjak, Tehran, Iran. Tel: +989122385589; Fax: +9821-22580914. E-mail: dr.atarbashi@gmail.com

How to cite this article: Salehizalani S, Atarbashi-Moghadam S, Mokhtari S, Yazdani F. Expression of Ki-67, P63, P40, and alpha-smooth muscle actin in salivary gland carcinomas with or without myoepithelial differentiation. J Med Sci 2022;42:206-13.

with myoepithelial differentiation include adenoid cystic carcinoma (AdCC), polymorphous adenocarcinoma (PAC), epithelial − myoepithelial carcinoma (EMC), myoepithelial carcinoma (MC), and carcinoma ex pleomorphic adenoma (Cx PA).[9] Several studies have been conducted on the expression of Ki-67 in salivary carcinomas, but none have considered the presence of myoepithelial cells.[1-3,10] P63 is a member of the P53 gene family, which is involved in epithelial development, stem cell biology, and carcinogenesis.[11] In addition, P63 is a good myoepithelial marker for salivary gland tumors. The protein P40 (ΔNP63), an isoform of P63, is expressed in myoepithelial cells and the P63/P40 panel can be a useful tool in the differentiation of neoplasms containing myoepithelial cells.[12] One of the most common immunohistochemical markers that characterize myoepithelial cells is alpha-smooth muscle actin (α-SMA). This marker is also used to detect myofibroblasts, which are specialized actin-containing fibroblasts that are involved in the progression of various malignant neoplasms.[13] The present study aimed to evaluate the expression of Ki-67, P40, P63, and α-SMA markers in these two groups of salivary carcinomas with or without myoepithelial differentiation.

MATERIALS AND METHODS

This study was approved by the ethics committee of Shahid Beheshti University of Medical Sciences (IR. SBMU. RIDS. REC.1395.421). Seventeen normal salivary gland tissues and 67 salivary carcinomas were selected from the archive of the Oral Pathology Department, Shahid Beheshti University of Medical Sciences, Tehran, Iran. Thirty-three carcinomas had myoepithelial differentiation (18 AdCC, 4 PAC, 5 MC, 3 EMC, and 4 Cx PA) and 34 had no myoepithelial differentiation (23 mucoepidermoid carcinomas (MEC), 14 low-grade tumors, 9 high-grade tumors, 3 acinic cell carcinomas (ACC), 2 salivary duct carcinomas (SDC), all high-grade tumors, 4 adenocarcinomas NOS and 1 hyalinizing clear cell carcinoma (HCCC)) were selected. Paraffin blocks of the samples were used for immunohistochemistry (IHC) staining.

Immunohistochemistry

The staining procedure for all four markers was performed by Envision technique on 4-μm-thick sections. The tissue sections were deparaffinized and rehydrated in descending ethanol series. For antigen retrieval, slides were subjected to a 0.01 mM citrate buffer (pH 6.0 for Ki-67, P63 and α-SMA, and pH 8.0 for P40) twice every 10 min in a microwave oven. A distilled water solution was used to block endogenous peroxidase activity for 10 min at room temperature. After rinsing with phosphate-buffered saline (PBS), primary antibodies (all ready to use) including Ki-67 (clone SP6, Rabbit, DBS, USA), P63 (clone DRB16.1, Rabbit, DBS, USA), P40 (BC28, Rabbit, DBS, USA), and α-SMA (clone 1A4, Mouse, DBS, USA) were used (30 min for P63 and Ki-67; 15 min for α-SMA; and 1 h for P40). Sections were washed in PBS for 10 min and were then incubated with a secondary antibody (Mouse/Rabbit PolyVue™ HRP/diaminobenzidine tetrahydrochloride [DAB]) for 30 min at 37°C. After rinsing with PBS, the colorimetric development with the 3,30-DAB substrate was performed, followed by counterstaining with hematoxylin. For the negative control, the primary antibody was replaced with phosphate buffer saline. As positive controls, oral squamous cell carcinoma, normal salivary gland, and prostate adenocarcinoma sections were, respectively, used for Ki-67, P63, α-SMA, and P40 immunostainings. In addition, the α-SMA slides had an internal control (wall of the vessels).

Microscopic evaluation

For histopathologic evaluation of the samples, an optical microscope (Leica DM500) was used by a pathologist (S.A.M). Only cytoplasmic and membranous staining of tumor cells was considered positive for α-SMA and nuclear staining for Ki-67, P63, and P40 proteins. Ten fields were chosen for each section. The percentage of positive neoplastic cells was calculated (in high-power field) from a minimum of 1000 tumoral cells. P63, α-SMA, and P40 markers were also used to prove the myoepithelial nature of the cells.

Data analysis

To compare the effect of different factors on the expression of markers, Pearson Chi-Square test, Mann–Whitney test, multiple linear regression, Spearman's correlation test, and Kruskal–Wallis nonstatistical tests were used. Statistical significance was set at $P < 0.005$.

RESULTS

In the present study, the age of patients ranged from 11 to 94 years (mean = 50.67), and the female-to-male ratio was 1.5. Twenty-one carcinomas were found in major salivary glands and 38 were in minor glands.

Evaluation of markers

Ki-67

Ki-67 is not expressed in normal salivary tissues and its expression was considered to be associated with the lymphocytic inflammatory cells in the area. Ki-67 was expressed in all examined carcinomas (ranging from 1% to 40%). The expression of Ki-67 was assessed concerning age, sex, and location. Ki-67 expression was correlated with

the tumor's location. Tumors of major salivary glands had higher proliferative activity than those of minor glands. Ki-67 expression in AdCC was more common in the solid islands, but cells in the tubular and cribriform areas were also positive. All PACs showed a low percentage of proliferation (1%–8%), but EMC, MC, and CxPA showed high percentages of this marker (15%–40%). In MEC, epidermoid and intermediate cells exhibited this marker differently (ranging from 1% to 40%), and the mucous or clear cells were almost negative. In ACC, two cases showed a low proliferation rate (4%), but one displayed a high proliferation rate (25%). In the adenocarcinoma NOS, a wide range of proliferation (10%–40%) was observed. Both SDCs showed high expression of this protein, and finally, HCCC had a very low tumor growth rate (3%) [Figure 1].

P63 and P40

The staining patterns of P63 and P40 were similar in normal tissue. Normal myoepithelial and basal cells around the ducts were positive for these markers. In carcinomas, ductal cells were negative in all cases. Furthermore, the staining pattern of P63/P40 was similar in carcinomas with and without myoepithelial cells, except for PAC. For example, in ACC, SDC, and adenocarcinoma NOS, both markers were negative (P63−/P40−) and in AdCC, EMC, CxPA, MEC, and MC, both were positive in the same cells (P63+/P40+). But in PAC, the staining pattern of P63+/P40− was observed. The expression of P63/P40 was also assessed concerning age, sex, and location. In AdCC, the cells of the cribriform regions and around solid islands were positive for P63/P40. In MEC, clear and mucous cells were negative, and epidermoid and intermediate cells were differently positive for P63/P40. MC was positive for these markers diffusely, and in the EMC, ductal cells were negative. In HCCC, P63/P40 was also diffusely positive [Figure 2].

Alpha-smvooth muscle actin

Regarding α-SMA, normal myoepithelial cells showed staining in normal salivary glands. In carcinomas with myoepithelial cells, α-SMA's presence was relatively variable (from 0% to 100%), and in carcinomas without myoepithelial differentiation, α-SMA was negative. All cases of MCs were positive for α-SMA [Figure 3].

Multiple linear regression showed that there was no statistically significant difference between Ki-67 expression and the presence of myoepithelial cells ($P = 0.6$), but the Ki-67 expression was related to the patients' age ($P = 0.032$) and carcinomas' location ($P = 0.001$) [Table 1]. Kruskal–Wallis test showed the expression of Ki-67 in subtypes containing myoepithelial cells to be significant, with the highest levels of

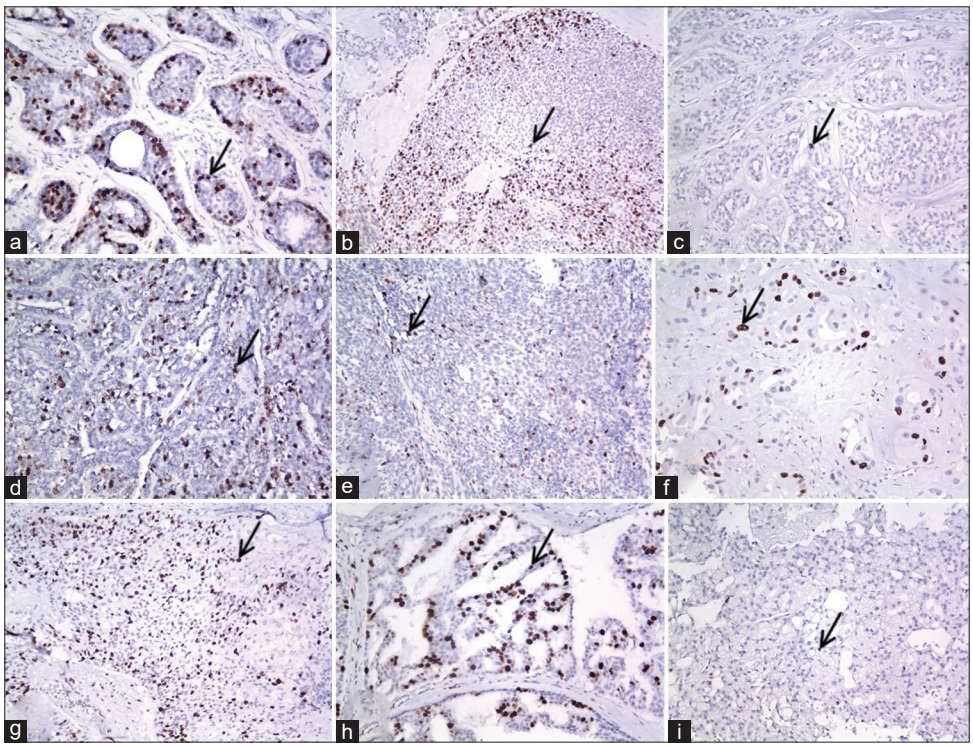

Figure 1: Ki-67 expression presenting as nuclear staining. (a) (×200), (b) (×100): Adenoid cystic carcinoma. (c) (×200): Polymorphous adenocarcinoma. (d) (×200): Epithelial–myoepithelial Carcinoma. (e) (×100): Myoepithelial carcinoma. (f) (×200): Carcinoma ex-pleomorphic adenoma. (g) (×100): Mucoepidermoid carcinoma. (h) (×200): Salivary duct carcinoma. (i) (×100): Acinic cell carcinoma

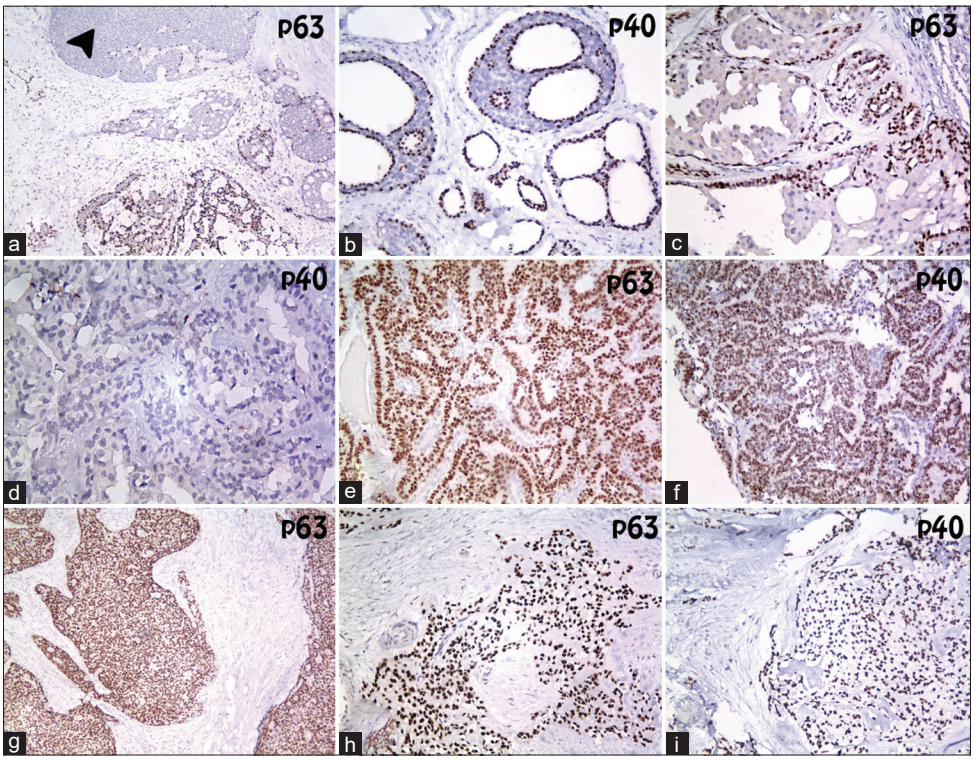

Figure 2: P63/P40 expression presenting as nuclear staining in (a) (×100), (b) (×200): Adenoid cystic carcinoma (c and d) (×200): Polymorphous adenocarcinoma, (e and f) (×200): Epithelial–myoepithelial carcinoma, (g) (×100): Myoepithelial carcinoma and (h) (×100), (i) (×100): Hyalinizing clear cell carcinoma. Only polymorphous adenocarcinoma was negative for P40. Note that a large solid island (arrow head) is almost negative for p63

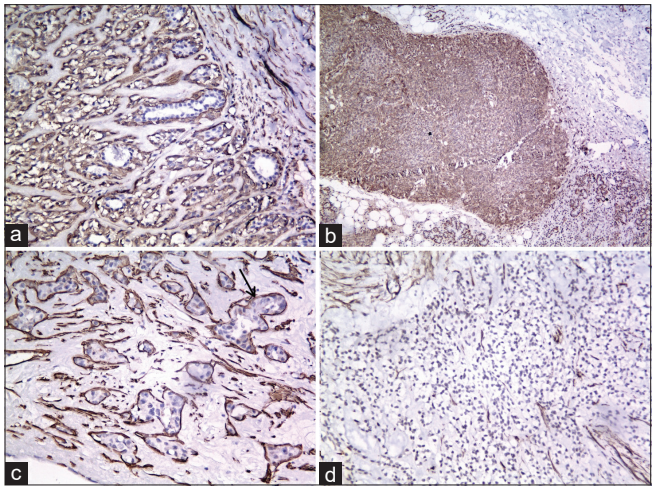

Figure 3: Alpha-smooth muscle actin expression presenting as cytoplasmic/membranous staining (×200). (a) Epithelial–myoepithelial carcinoma, (b) Myoepithelial carcinoma, (c) Carcinoma ex-pleomorphic adenoma and (d) Hyalinizing clear cell carcinoma which was negative for alpha-smooth muscle actin

staining observed in CxPA and EMC and the lowest expression in PAC (P = 0.003) [Table 2]. Kruskal–Wallis statistical test showed that the expression of Ki-67 in nonmyoepithelial subgroups was significant, with the highest expression observed in the SDC and NOS and the lowest in HCCC and MEC (P = 0.047). Moreover, the comparison of PAC and AdCC was statistically significant (P = 0.002). On the other hand, the expression of Ki-67 in AdCC was significant with MEC (P = 0.020). Using the Mann–Whitney test, it was shown that the Ki-67 expression was significantly different in MECs classified as high grade and low grade (P = 0.000). Using Spearman's correlation test, it was found that the expression of Ki-67 was not associated with any other markers. Although, in AdCC, an increase in the expression of Ki-67 was significantly associated with the decrease of P63 (P = 0.034) and P40 (P = 0.039) positive cells as proved by the Spearman test.

DISCUSSION

One of the most common methods to investigate the proliferative activity of neoplasms is to detect the positivity of tumor cells for Ki-67.[1] Various studies have shown that the presence of myoepithelial cells can lead to various natures in salivary tumors.[5-7] Sternlicht and Barsky stated that the proliferation rate of myoepithelial cells is lower than epithelial cells of the basal type.[14] The myoepithelial cell distribution is different in various salivary tumors.[4] Currently, α-SMA, P63,

Table 1: Statistical indicators of Ki-67 in relation to myoepithelial cells, sex, age, and location of the neoplasms using multiple linear regressions

Myoepithelial cells	Mean	*n*	SD	Minimum	Maximum	Age (SD)	Sex (SD)		Location (SD)	
							Male	Female	Major	Minor
Presence	22.03	34	11.687	1	40	56.26 (15.412)	16 (47.1)	18 (52.9)	27.50 (10.326)	15.88 (10.164)
Absence	16.00	33	14.107	1	40	45.09 (19.306)	11 (33.3)	22 (66.7)	21.55 (15.227)	13.23 (12.987)
Total	19.06	67	13.191	1	40	50.76 (18.198)	27 (40.3)	40 (59.7)	25.54 (12.498)	14.34 (11.805)

SD=Standard deviation

Table 2: Mean rank of Ki-67, P63, P40, alpha-smooth muscle actin markers among the subtypes of tumors with/without myoepithelial cells

Myoepithelial cells	Tumors	Mean rank for Ki-67	Mean rank for - SMAα	Mean rank for P40	Mean rank for P63
Present	AdCC	15.72	19.39	16.33	14.86
	PAC	3.25	8.75	2.50	11.50
	MC	23.90	23.10	31.80	31.70
	EMC	25.50	15.33	16.83	15.67
	CxPA	25.75	12.38	20.38	19.00
Absent	MEC	15.41	17.00	21.04	21.04
	ACC	13.17	17.00	5.00	5.00
	SDC	31.00	17.00	5.00	5.00
	NOS	25.00	17.00	5.00	5.00
	HCCC	5.00	17.00	32.00	32.00

SMAα=Smooth muscle actin alpha; AdCC=Adenoid cystic carcinoma; PAC=Polymorphous adenocarcinoma; EMC=Epithelial–myoepithelial carcinoma, MC=Myoepithelial carcinoma, MEC=Mucoepidermoid carcinomas, ACC=Acinic cell carcinomas, SDC=Salivary duct carcinomas, NOS: Not otherwise specified, HCCC: Hyalinizing clear cell carcinoma, CxPA: Carcinoma ex pleomorphic adenoma

and calponin are antibodies that can prove the myoepithelial nature of cells, and P40 has recently been added to this group.[7]

Ye *et al*. found the decrease in myoepithelial cells in CxPA to be associated with invasion and metastasis.[7] Du *et al*. hypothesized that the absence of myoepithelial differentiation leads to poor prognosis in the solid subgroup of AdCC.[6] Contrary to these results, Alves *et al*. found no relationship between cellular pattern and Ki-67 expression in AdCC.[15] In the present study, no statistically significant difference was observed in the proliferation rate of carcinomas with or without myoepithelial cells. Both groups included carcinomas with very low and very high Ki-67 rates (such as PAC and ACC in comparison with SDC and MC). This difference can be justified by the fact that the tumoral variation in our study was high and the clinical behavior of carcinomas and their cell proliferation may be affected by factors other than the presence or absence of myoepithelial cells. For example, the MC with high myoepithelial content in the present study had a high proliferation rate. Although in the AdCC subgroup an increase of Ki-67 expression was associated with a decrease in the number of positive P63 cells.

In the current research, the expression of Ki-67 was associated with increasing age and major salivary gland location. In the study of Bussari *et al*., the expression of Ki-67 was associated with aging, and the highest expression was present in the 50–59 years age group, but it was not associated with sex, location, and size of the tumor.[16] Alves *et al*. concluded that submandibular MECs had a higher degree of proliferation than parotid and minor glands.[15]

One of the noticeable results of the present study is the low expression of the Ki-67 in PAC, which can help diagnose small or incisional biopsies and differential diagnosis of cases with similarity to AdCCs. These results are similar to several other studies,[17-19] However, some reports do not support such an association between these two carcinomas.[1,20] Comparing the expression of Ki-67 between MECs and AdCCs, the rate of proliferation in AdCC (with myoepithelial differentiation) was significantly higher which contradicts the results of do Prado[1] and agrees with that of Fonseca.[21] One possible cause for this difference is the presence of a large number of mucus cells in the MECs evaluated in the study.

In the present study, comparative statistical analysis of different patterns of AdCCs was not possible due to the disproportion between the number of cases with cribriform, tubular, and solid patterns. However, the decrease in the number of P63/P40 positive cells was associated with increased proliferation in AdCCs. Some researchers have reported high expression of Ki-67 in solid tumors containing basal cells and its inverse relationship with cell differentiation.[22] In our study, solid islands showed a high proliferation rate as well. Although these cells were morphologically similar to basal cells, they were negative for the P63/P40 marker. This fact questions their basal nature because cells with basal phenotype in the salivary glands are positive for P63/P40. Rooper *et al*. found that the pattern of P63/P40 expression in AdCC is P63+/P40+ and less likely to be P63−/P40−, which is seen in high-grade and solid forms, but in PAC, P63+/P40− can be seen. They suggested that P40 is a valuable new marker to evaluate salivary tumors by myoepithelial differentiation.[23]

Argyris *et al*. stated that the P63 isoform contains both ΔNP63 and TAP63. However, P40 only detects the ΔNP63 portion. P63 alone will not help detect these two neoplasms, but the P63/P40 markers can be useful in the diagnostic process.[24] Studies on skin carcinomas have suggested that P40 exhibits better specificity than P63;[25] however, this advantage was not seen in salivary carcinomas in our study. Teixeira *et al*. stated that the expression of P63/P40 is not compatible.[12] In the study of Larsen *et al*., the highest Ki-67 expression was in adenocarcinoma NOS and the lowest in PAC, ACC, and CxPA.[2] In our study, adenocarcinoma NOS and CxPA were among tumors with high Ki-67 expressions (10%–40%).

EMC accounts for about 1% of salivary tumors and contains myoepithelial cells with clear cytoplasm and ductal structures similar to intercalated ducts. Although it is considered a low-grade tumor, high-grade and dedifferentiated types have also been reported.[26] In the present study, the expression of the Ki-67 marker was assessed to be between 20% and 40%, which is similar to high-grade tumors. Ettl *et al*. also suggested that salivary tumors with Ki-67 index above 10%–20% are considered as high-grade tumors.[27]

HCCC is a rare tumor of minor salivary glands, in which myoepithelial markers including SMA, S100, and calponin are negative, but P63 is positive. The expression of Ki-67 in its classical form is about 5%.[28] In our study, the level of cell proliferation in the case of HCCC was as low as 3%. This tumor was negative for SMA. HCCC cells were similarly positive for P63 and P40. SMA negativity in HCCC can help differentiate this tumor from a clear variant of MC, which shows positive immunostaining for SMA.

MC is easily mistaken with many tumors due to its cellular morphology and rarity. It could also be recognized by IHC and ultrastructural studies.[29] MCs are positive for P63, α-SMA, and S-100. In our study, all MCs were positive for α-SMA, the pattern of expression of P63/P40 was similar, and the rate of Ki-67 was like high-grade tumors (15%–40%), which is consistent with the classification of MC as an invasive neoplasm.[29]

Nagao *et al*. also stated that for the differentiation of MC from myoepithelioma, an expression of Ki-67 more than 10% would be helpful.[8]

SDC is a highly invasive salivary carcinoma that may be mistaken with high-grade MEC. One of the possible markers for this differential diagnosis is P63, which is positive in high-grade MEC but negative in SDC.[30] In our study, the P40 marker was similarly negative as P63 in this tumor. Ki-67 expression was also high (40%) in SDC which is consistent with the invasive behavior of this tumor.

ACC is a low-grade salivary tumor that may show common microscopic characteristics with MEC.[31] The P63 marker is negative in all ACCs, while it was positive in MECs, and in cases where ACC showed the expression of P63, it should probably be placed in the category of mammary analog secretory carcinoma, since MEC is always positive for this marker and ACC is always negative. In our study, the same results were obtained. In addition, similar to P63, negative P40 was also observed in ACC. One case of ACC showed a high expression of Ki-67 (25%). In this research study, Ki-67 expression in salivary gland tumors had no association with P40, P63, and SMA.

CONCLUSION

The myoepithelial cell count decrease in AdCC was associated with increased proliferation, but no similar relationship was found in the overall assessment between the two groups of salivary gland tumors with/without myoepithelial differentiation. This can be justified by the fact that the clinical behavior of salivary carcinomas and their cell proliferation may be affected by factors other than the presence of myoepithelial cells or lack thereof. P40 is considered a useful marker for the detection of myoepithelial cells, and its pattern of expression is similar to P63 in all carcinomas except PAC. The P63/P40 can be used to differentiate the MEC from the SDC as well as the MEC from the ACC.

Financial support and sponsorship

Nil.

Conflicts of interest

There are no conflicts of interest.

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