



ORIGINAL ARTICLE

Immunohistochemical evaluation of salivary gland tumors differentiation and proliferation by using calponin and telomerase



Srwsht Saeed Abdulrahman^a, Dena Nadhim Mohammad^{b,*},
Marwa Abdul-Salam Hamied^b, Mardin Othman Abdulqadir^c

^a Ministry of Health, Sulaimani, Iraq

^b Oral Diagnosis Department, College of Dentistry, Sulaimani University, Iraq

^c Technical College of Health-Sulaimani Polytechnic University, Iraq

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KEYWORDS

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Abstract *Background:* Salivary gland tumors are a heterogeneous group of lesions with diverse histological features. Hence they are considered as a diagnostic challenge for the pathologist. Myoepithelial cells are considered as a key in the morphogenetic process, with diverse differentiation in various salivary gland tumors. Calponin is an actin filament-associated protein that represents a sensitive marker of myoepithelial cells. Telomerase is a ribonucleoprotein that adds telomere repeats at the end of chromosomes in order to prevent replicative senescence. It has a key role in cellular immortality and tumorigenesis of various tumors. This study evaluates the immunohistochemical expression of calponin and telomerase in various salivary gland tumors.

Methods: This retrospective study involved 30 formalin fixed paraffin embedded blocks of salivary gland tumors. The immunohistochemical staining and evaluation of subcellular localization, pattern, intensity, and distribution for calponin and immune scoring for telomerase were done. The statistical analyses of data were conducted by Chi-square and ANOVA-test, a P-value of < 0.05 was considered significant.

Results: Calponin showed expression at the periphery of acini and intercalated ducts in the normal salivary gland. It revealed cytoplasmic expression in 83.3% of benign tumors. The pleomorphic adenoma showed a diffuse pattern of staining (85.7%), strong intensity (64.3%), and mixed distributions (57.1%). The diffuse pattern of calponin was seen in all cases of mucoepidermoid, polymorphous low-grade adenocarcinoma and epithelial-myoepithelial carcinoma (100%). Telomerase

* Corresponding author at: College of Dentistry, Sulaimani, Iraq.

E-mail address: Dena.Mohammad@univsul.edu.iq (D.N. Mohammad).

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revealed negative expression in the normal salivary gland. Pleomorphic adenoma illustrated high telomerase expression in score 2 and score 3 (93.3%). Telomerase immune scoring is significantly related to the benign tumors as P value was 0.03. Both polymorphous low grade and epithelial-myoeithelial carcinoma were detected only in score 3. Finally, the mean level of telomerase activity was slightly higher in malignant tumors than benign ones with non-significant relation as P value was 0.6.

Conclusions: Calponin showed high diffuse staining with altered distribution in salivary gland tumors, which might give an additional role for this marker in the identification of luminal immuno-modified neoplastic cells. Telomerase is considered as a useful marker in identifying proliferation capacity of salivary gland tumors and is remarkably more detected in malignant salivary gland tumors.

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1. Introduction

Salivary glands are secretory organs that consist of duct-acinar units. These glands comprise from three main major paired glands, plus hundreds of small minor salivary glands located within the submucosa of the oral cavity and oropharynx that might lead to a wide range of neoplasms (Kamal, 2009; Nagao et al., 2012).

Salivary gland tumors are a heterogeneous group of lesions with significant morphologic diversity (Sarkis et al., 2010). Thus different classifications are noticed. The World Health Organization (WHO) classification is the most widely used one, which classifies them into benign and malignant tumors (Eveson et al., 2005). Pleomorphic adenoma is the most commonly detected benign tumor, while mucoepidermoid carcinoma is the most frequent salivary gland malignancy (Rasheed and Majeed, 2011).

The origin of salivary gland tumors could be from acinar/ductal epithelial cells (luminal cells) and/or myoepithelial/basal cells (abluminal cells) (Nagao et al., 2012). The myoepithelioma, acinic cell carcinoma and salivary duct carcinoma are designated as monophasic tumors, while pleomorphic adenoma, epithelial-myoeithelial carcinoma, and adenoid cystic carcinoma are considered as biphasic tumors (Zhu et al., 2015).

Regarding cell differentiation, myoepithelial cell differentiation is seen in nearly 70% of the salivary gland tumors (Halcy-Kowalik et al., 2016). However, controversy still exists concerning its identification in certain tumors. This could be related to diagnostic difficulty in determining the wide spectrum of morphologic alteration associated with normal and neoplastic myoepithelial cells (Savera and Zarbo, 2004).

Myoepithelial cells can be detected by several immunohistochemical markers (Elias, 2015). Neoplastic myoepithelial cells have been represented specifically by vimentin (De Araújo et al., 2000). Later on, other biomarkers have been applied for myoepithelial cells, which are; calponin, h-caldesmon, smooth muscle actin, muscle-specific actin and smooth muscle myosin (Cavalcante et al., 2007).

Calponin is an actin filament-associated regulatory protein that expressed in smooth muscle, and non-muscle cells, (Wu and Jin, 2008). Both calponin and smooth muscle actin are reported as the most sensitive markers of neoplastic myoepithelial cells (Scarpellini et al., 2001). Cavalcante et al. (2007)

found that calponin and vimentin exhibit strong expression in salivary gland tumors.

Human telomerase is a ribonucleoprotein that comprises from human telomerase RNA component (hTR), the human telomerase reverse transcriptase (hTERT) and additional telomerase-associated proteins, that add telomere repeats at the ends of chromosomes in order to keep the telomeric length and prevent replicative senescence (Tefferi et al., 2015). The telomerase is the single-stranded 3'-telomeric overhang that caps the very end of all telomeres. The RNA hTR is used by the telomerase as a template for the synthesis of DNA repeats to the 3'-telomeric overhangs (Burchett et al., 2014). Telomerase showed over-expression in the majority of human cancers. Therefore, it might be considered a unique cancer biomarker (Jafri et al., 2016).

Liao et al. (2000) found telomerase expression in 83.3% of malignant salivary gland tumors by polymerase chain reaction (PCR). Furthermore, Shigeishi et al. (2011) illustrated that telomerase has a significant role in the detection of salivary gland carcinomas, and it could determine the grade of mucoepidermoid carcinomas. Telomerase overexpression is remarked as an early event in oral cancer (Abrahao et al., 2011). Furthermore, its expression showed a significant correlation with the stage of head and neck squamous cell carcinoma (Padhi et al., 2015). The goal of this study is to investigate the immunohistochemical expression of calponin and telomerase in different salivary gland tumors.

2. Materials and methods

2.1. The sample of the study

This cross-sectional retrospective study was involved 30 formalin fixed paraffin embedded blocks previously diagnosed as salivary gland tumors of which 18 cases were benign (15 Pleomorphic adenoma, 1 Basal cell adenoma, 2 Warthin's tumors), 12 cases were malignant (4 Mucoepidermoid carcinoma, 4 Adenocystic carcinoma, 1 Acinic cell carcinoma, 2 Polymorphous low grade adenocarcinoma, 1 Epithelial-myoeithelial carcinoma), and 5 normal salivary glands from cases in surgical samples of mucocele. This study had been conducted in Sulaimani Governorate from September 2016 to October 2017. The study was approved by the Ethical Committee in the College of Dentistry, University of Sulaimani.

2.2. Tissue preparation and evaluation

Three tissue sections of 4 μm were cut from each block; one was stained with Hematoxylin and Eosin in order to confirm histopathological features and identify representing tumor area for immunohistochemical staining. The other two sections were mounted on a positive charge slide and stained immunohistochemically one for calponin, another for telomerase. A biotin-free immuno-enzymatic antigen detection system (expose mouse/rabbit specific HRP/DAB micro polymer detection IHC kit, Abcam; UK) was used. For IHC staining, the slides were put in the oven (60 °C) for 6 h, then they were deparaffinized, rehydrated, and washed with phosphate buffer saline (PBS) for 3 min. Antigen retrieval was done by pressure cooker with citrate buffer (PH 6) for 15 min, then were cooled at room temperature, and washed with PBS twice. The excess buffer was removed gently. Slides were incubated with hydrogen peroxidase in a humid chamber at 37 °C for 10 min and then washed twice with PBS. Protein block was applied and incubated at 37 °C for 10 min, and then washed with PBS. Primary antibodies (polyclonal rabbit anti-human calponin antibody, dilution 1:100, and polyclonal rabbit anti-telomerase reverse transcriptase antibody, dilution 1:100, Abcam; UK) were applied, and slides were incubated for 45 min in a humid chamber at 37 °C, then washed three times with PBS. Slides were incubated for 10 min with complement and then washed twice with PBS. Goat anti-rabbit HRP conjugate was applied on sections (15 min) and then washed with PBS. Finally, the tissue sections were incubated with diaminobenzidine (DAB) chromogen for 5 min in the dark field and then washed with PBS. Slides were counter-stained with hematoxylin for 1 min and then washed with distilled water. Lastly, slides were dehydrated, cleared and mounted with DPX, covered and evaluated. The normal human tonsil tissue was served as positive control for telomerase, while for calponin human gastric carcinoma was used as a positive control as recommended by the manufacturer protocol. While for the negative controls, primary antibodies diluents alone were used for both antibodies.

Sections stained with calponin were evaluated according to the pattern as (focal or diffuse), distribution of staining was either (luminal, abluminal and mixed), subcellular localization and the intensity was arranged as (strong or weak) (Cavalcante et al., 2007).

Semi-quantitative evaluation of telomerase was done by using Image J software, as the immunostained positive cells from 5 high spot field pictures from a light microscope (400X) were taken, then uploaded to the software and counted by using the grid, and the percentage of positive cells/1000 counted cells was graded into 4 scores as follows: score 0: Negative 0–10%. Score 1: Focal positivity 11–25%. Score 2: Regional positivity 26–75%. score 3: Diffuse positivity 75–100% of immunoreactive cells (Maraei et al., 2012).

2.3. Statistical analysis

Data were statistically analyzed by SPSS 22.0 software program. Chi-square (χ^2) was used to test for significance of associations in the categorical variables. A p-value of 0.05 was used as a cut off point for the significance of statistical tests. One-way ANOVA-test was used to determine whether the mean of a dependent variable is the same in two unrelated and independent groups.

3. Results

Positive controls showed expressions for both calponin and telomerase as recommended by the manufacturer protocols (Fig. 1A and B). Calponin revealed positive expression at the periphery of acini and intercalated duct in normal salivary glands (Fig. 2A and B). In benign tumors, it revealed positive cytoplasmic expression in 15 cases (83.3%). The pattern showed a significant relation to different categories of benign tumors ($P = .02$) (Table 1). Pleomorphic adenoma had diffuse expression in 85.7%, mixed distribution in 57.1%, solid distribution (group of cells) in 28.6% (Tables 1 and 2) and (Fig. 3A–C). While the single case of basal cell adenoma had diffuse, mixed expression and weak intensity (Fig. 3D). Warthin's tumor showed negative immune reactivity (Table 1). All the malignant salivary gland

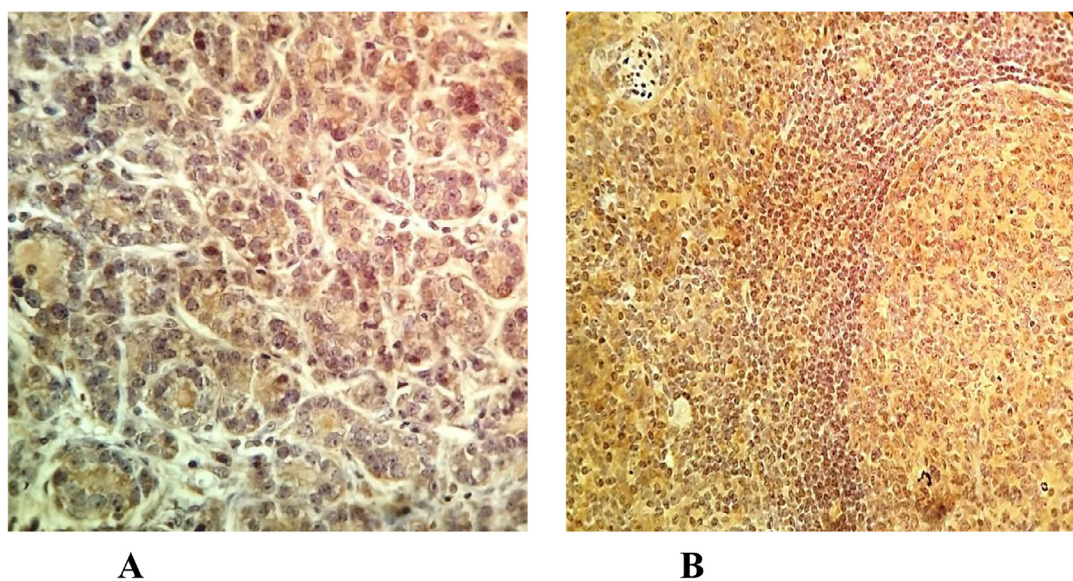


Fig. 1 Cytoplasmic expression in positive controls. A – Calponin expression in human gastric carcinoma. B – Telomerase expression in normal human tonsil tissue.

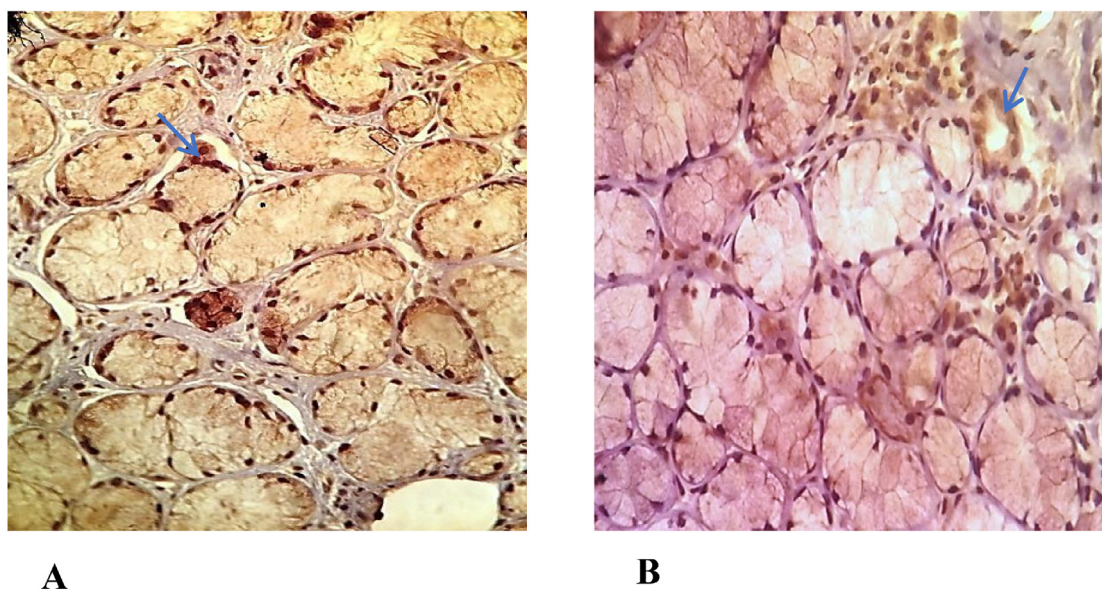


Fig. 2 Calponin immune expression in normal salivary glands X400. A – Periphery of acini (arrow). B – Periphery of the intercalated duct (arrow).

Table 1 Calponin immune positivity in benign salivary gland tumors in relation to pattern and intensity.

Benign salivary gland tumors	No. of positive cases (%)	Pattern		P value	Intensity		P value
		Focal No. (%)	Diffuse No. (%)		Strong No. (%)	Weak No. (%)	
Pleomorphic adenoma (15)	14 (93.3)	2 (14.3)	12 (85.7)	0.02*	9 (64.3)	5 (35.7)	0.1
Basal cell adenoma (1)	1 (100)	0	1 (100)		0	1 (100)	
Warthin's tumor (2)	0	0	0		0	0	
Total (18)	15 (83.3)	2 (13.3)	13 (86.7)		9 (60)	6 (40)	

* P < 0.05 significant difference.

tumors showed positive cytoplasmic expression for calponin marker (Table 3). Mucoepidermoid carcinoma, polymorphous low-grade adenocarcinoma, and epithelial-myoeplithelial carcinoma revealed diffuse pattern (100%); (Table 3) and (Fig. 4A–C), while 75% of adenocystic carcinoma had a diffuse pattern (Fig. 4D) and (Table 3). All mucoepidermoid carcinoma had a mixed expression (100%) (Fig. 4E), while the staining of adenocystic carcinoma was equally distributed among both mixed and solid distribution (50%) (Fig. 4F and G) and (Table 4).

Telomerase revealed negative expression in normal salivary glands (Fig. 5A). 83.3% of benign tumors had cytoplasmic telomerase immunopositivity (Table 5). Pleomorphic adenoma showed high dif-

fuse (score 3) (53.3%) (Fig. 5B), while the single basal cell adenoma had a focal expression (score 1) (Fig. 5C). Warthin's tumor revealed negative immune reactivity (score 0). There was a significant relation of telomerase expression to various benign tumors (P = .03). Ten out of twelve cases of malignant salivary gland tumors had a positive cytoplasmic expression (Table 5). Mucoepidermoid carcinomas were equally seen in score 3 and score 0 (50% each) (Fig. 5D and E). While 50% of adenocystic carcinoma revealed regional (score 2) (Fig. 5F). Lastly, both polymorphous low-grade adenocarcinoma and epithelial-myoeplithelial carcinoma showed (score 3) (100%) (Fig. 5G and H) and (Table 5). Telomerase revealed slightly higher mean of

Table 2 Calponin immune positivity in benign salivary gland tumors in relation to distributions.

Benign salivary gland tumors	No. of positive cases (%)	Distribution				P value
		Luminal positivity No. (%)	Abluminal positivity No. (%)	Luminal & abluminal positivity No. (%)	Solid tumor (no duct) No. (%)	
Pleomorphic Adenoma (15)	14 (93.3)	1 (7.1)	1 (7.1)	8 (57.1)	4 (28.6)	0.1
Basal cell adenoma (1)	1 (100)	0	0	1 (100)	0	
Warthin's tumor (2)	0	0	0	0	0	
Total (18)	15 (83.3)	1 (6.7)	1 (6.7)	9 (60)	4 (26.7)	

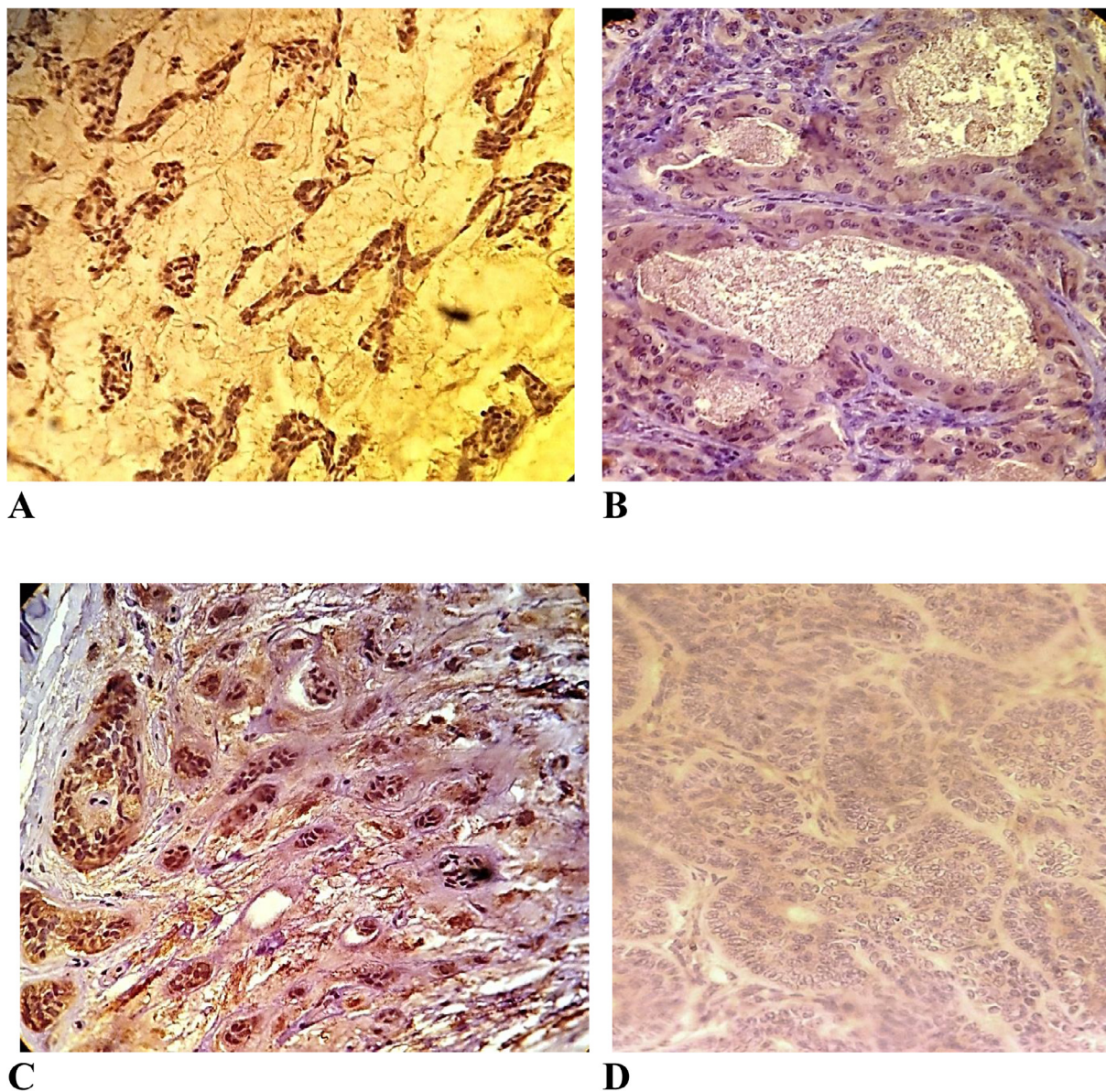


Fig. 3 Calponin expression in benign salivary gland tumors X400. A – Diffuse expression in pleomorphic adenoma. B – Mixed distribution in pleomorphic adenoma. C – Solid distribution in pleomorphic adenoma. D- Diffuse weak mixed distribution in basal cell adenoma.

Table 3 Calponin expression in malignant salivary gland tumors in relation to pattern and intensity.

Malignant salivary gland tumors (12)	No. of positive cases (%)	Pattern		P value	intensity		P value
		Focal No. (%)	Diffuse No. (%)		Strong No. (%)	Weak No. (%)	
Mucoepidermoid carcinoma (4)	4 (33.3)	0	4 (100)	0.5	3 (75)	1 (25)	0.5
Adenocystic carcinoma (4)	4 (33.3)	1(25)	3 (75)		4 (100)	0	
Acinic cell carcinoma (1)	1 (8.3)	1(100)	0		1 (100)	0	
Polymorphous low-grade adenocarcinoma (2)	2 (16.7)	0	2 (100)		1 (50)	1 (50)	
Epithelial-myoepithelial carcinoma (1)	1 (8.3)	0	1 (100)		1 (100)	0	
Total (12)	12 (100)	2 (16.7)	10 (83.3)		10 (83.3)	2 (16.7)	

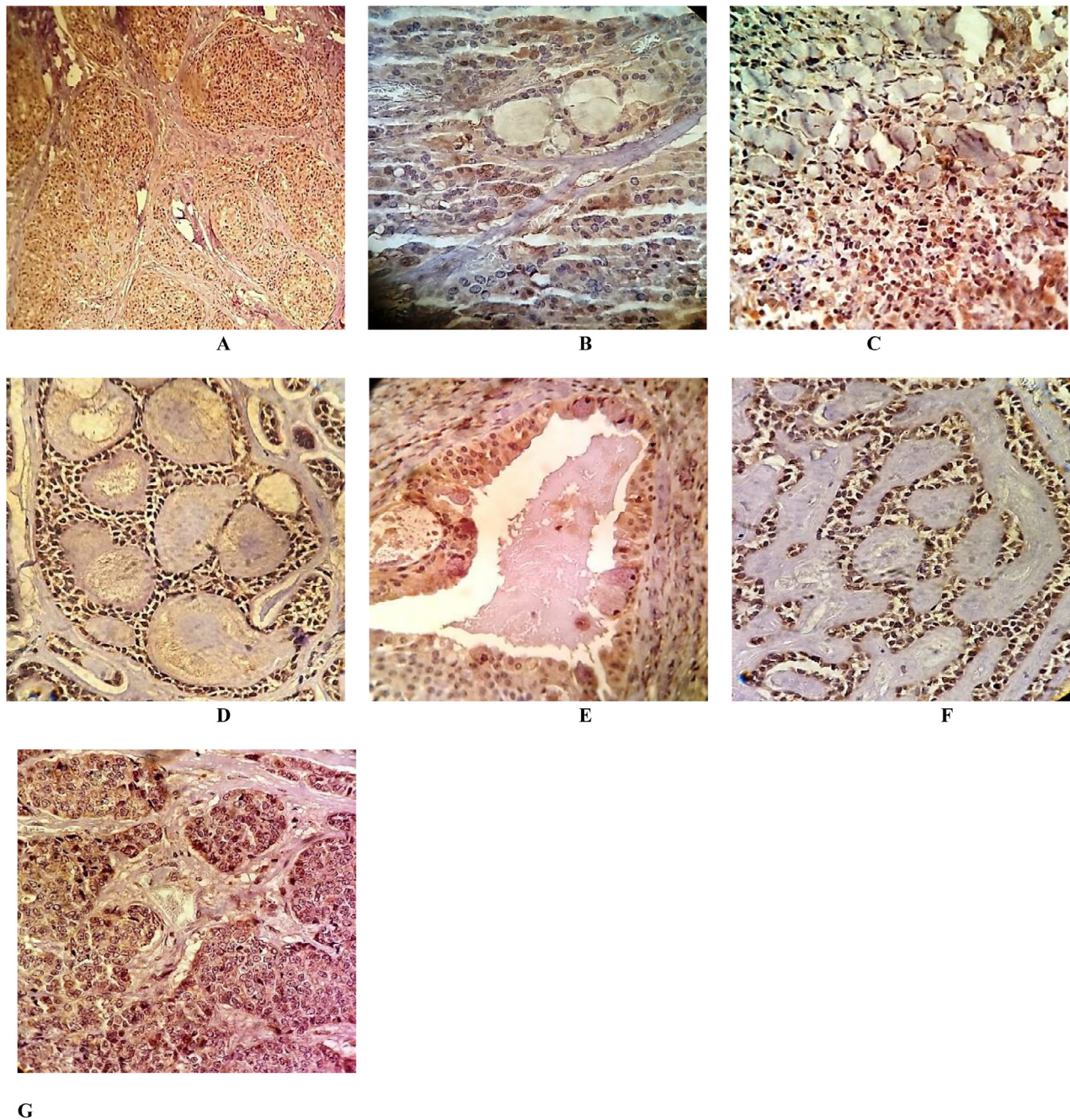


Fig. 4 Immunohistochemical expression of calponin in malignant salivary gland tumors X400. A – Diffuse expression in mucoepidermoid carcinoma X100. B – Diffuse expression in polymorphous low-grade adenocarcinoma. C – Diffuse expression in epithelial-myoepithelial carcinoma. D – Diffuse expression in adenocystic carcinoma. E – Mixed distribution of mucoepidermoid carcinoma. F – Mixed distribution in adenocystic carcinoma. G – Solid distribution in adenocystic carcinoma.

expression in malignant tumors (62.35 ± 37.27) than benign ones (52.26 ± 34.28). One-way ANOVA-test showed no significant relation ($P = .6$). Finally, the relation between calponin pattern and telomerase expression did not reach the significant level both in benign and in malignant tumors as P values were 0.08 and 0.06 respectively.

4. Discussion

Calponin is considered the most useful marker that is used to identify myoepithelial cells differentiation. Neoplastic

myoepithelial cells have diverse architectural patterns in salivary gland tumors (Chitturi et al., 2015).

The expression of calponin in normal salivary glands in this study was similar to that detected by other studies (Nagao et al., 2012; Prasad et al., 1999; Zhu et al., 2015). Despite that previous studies had indicated the myoepithelial participation in salivary gland tumors; this study demarcated the altered distribution of calponin in a certain subset of these tumors, which might have a role in their biological behaviors.

Table 4 Calponin expression in malignant salivary gland tumors in relation to the distribution.

Malignant salivary gland tumors (12)	Distributions				P-value
	Luminal positivity	Abluminal positivity	Luminal & abluminal positivity	Solid Tumor (no duct)	
	No. (%)	No. (%)	No. (%)	No. (%)	
Mucoepidermoid carcinoma (4)	0	0	4 (100)	0	0.09
Adenocystic carcinoma (4)	0	0	2 (50)	2 (50)	
Acinic cell carcinoma (1)	0	0	1 (100)	0	
Polymorphous low grade adenocarcinoma (2)	0	0	0	2 (100)	
Epithelial–myoepithelial carcinoma (1)	0	0	0	1 (100)	
Total (12)	0	0	7(58.3)	5(41.7)	

Pleomorphic adenoma revealed great positive expression (93.3%) with marked diffuse distribution. This finding was in agreement with other studies previously done by [Cavalcante et al. \(2007\)](#) and [Savera et al. \(1997\)](#), as they revealed positivity in 100%, and 98% respectively of pleomorphic adenoma. This finding supports the fact that calponin is considered a useful marker in the diagnosis of pleomorphic adenoma. Furthermore, pleomorphic adenoma revealed mixed distribution in 57.1%. A similar distribution was found by [Ogawa, \(2003\)](#) in 75% of pleomorphic adenoma, while other studies showed only abluminal expression of calponin ([Cavalcante et al., 2007](#); [De Araújo et al., 2000](#); [Savera et al., 1997](#)). The altered expression of calponin in the present study could be explained by altered architecture pattern of neoplastic myoepithelial cells in comparison to the normal counterpart. Furthermore, co-expression of this marker could be seen by other adjacent immune altered tumor cells.

In this study, the single case of basal cell adenoma expressed diffuse and mixed distribution of calponin. Similar diffuse staining was reported by [Zarbo et al., \(2000\)](#). This might support the previous study that demonstrated calponin role in the histogenesis of basal cell adenoma ([Dardick et al., 1986](#)). But, it disagreed with [Jung et al., \(2013\)](#) and [Huang \(2014\)](#), who revealed that luminal cells of basal cell adenoma lack immune reactivity for calponin. This difference could be related to the difference in a number of samples and laboratory standardization method.

All mucoepidermoid carcinomas showed remarkable staining of calponin in the present study, while [Prasad et al. \(1999\)](#) showed negative calponin expression. Still, controversy exists regarding whether this malignant tumor arising from intercalated duct cell or that myoepithelial cell might have a role in its histogenesis ([Redder et al., 2013](#)). The result of this study might be explained by the counterpart role of intermediate cells in the histogenesis of mucoepidermoid carcinoma.

Calponin in adenoid cystic carcinoma revealed high diffuse expression (75%). This was in agreement with [Elias \(2015\)](#) and [Prasad et al. \(1999\)](#). This study illustrated the mixed distribution of calponin in two cases of adenocystic carcinoma (1 cribriform pattern and 1 tubular pattern), while studies were done by [Cavalcante et al. \(2007\)](#) and [Furuse et al. \(2006\)](#) reported the abluminal expression of cribriform and tubular patterns. The findings of this study were dissimilar with [Prasad et al. \(1999\)](#) as they showed only abluminal distributions in tubular pattern and luminal distributions in the cribriform pattern.

This difference could be related to immunophenotype modification of the neoplastic myoepithelial cells and limited numbers of the cases in this study.

Although the single case of acinic cell carcinoma had only focal pattern staining of calponin, other researchers demonstrated negative expression of this marker in acinic cell carcinoma ([Prasad et al., 1999](#); [Zhu et al., 2015](#)). Finally, the single case of epithelial-myoeplithelial carcinoma had diffuse distribution. This was in agreement with [Elias \(2015\)](#) and [Prasad et al. \(1999\)](#).

Telomerase plays essential roles in the regulation of the lifespan of human cells. Most human cancers typically express high levels of telomerase and show unlimited cell proliferation ([Palani et al., 2011](#)). Normal salivary gland showed negative expression of telomerase in this study, a similar finding was reported by [Chen et al. \(2002\)](#).

This is the first study that evaluates immunohistochemical expression of telomerase in salivary gland tumors. Pleomorphic adenoma showed high expression in score 2 and 3. This disagreed with other studies done by [Chen et al. \(2002\)](#) and [Shigeishi et al. \(2011\)](#); as they revealed a negative expression of telomerase in all cases of pleomorphic adenoma. The high scoring of telomerase in pleomorphic adenoma could be attributed to the prominent role of these active proliferating cells in the expanding tumors, although the exact origin of these proliferating cells is difficult to be determined structurally and might need other immunohistochemical markers.

The single basal cell adenoma expressed focal telomerase staining. A similar result was detected by single case stained for Ki 67 by [Kudoh et al. \(2014\)](#). While [Singh et al. \(2015\)](#) in their study found slightly higher Ki 67 expression in basal cell adenoma. However, an exact conclusion on this difference could not be done due to limited cases in this study.

Telomerase showed a high frequency of expression in malignant tumors (83.3%). This was in agreement with studies done by [Liao et al. \(2000\)](#), and [Shigeishi et al. \(2011\)](#); as they showed the majority of malignant salivary tumors exhibit telomerase expression. Only two cases of mucoepidermoid carcinoma (50%) had positive telomerase expression. This disagreed with other two studies done with PCR by [Liao et al. \(2000\)](#), and [Shigeishi et al. \(2011\)](#); as they reported higher telomerase expression (75%, 100%) respectively of their samples. The reduced telomerase expression in mucoepidermoid carcinoma in this study might indicate that certain tumor cells

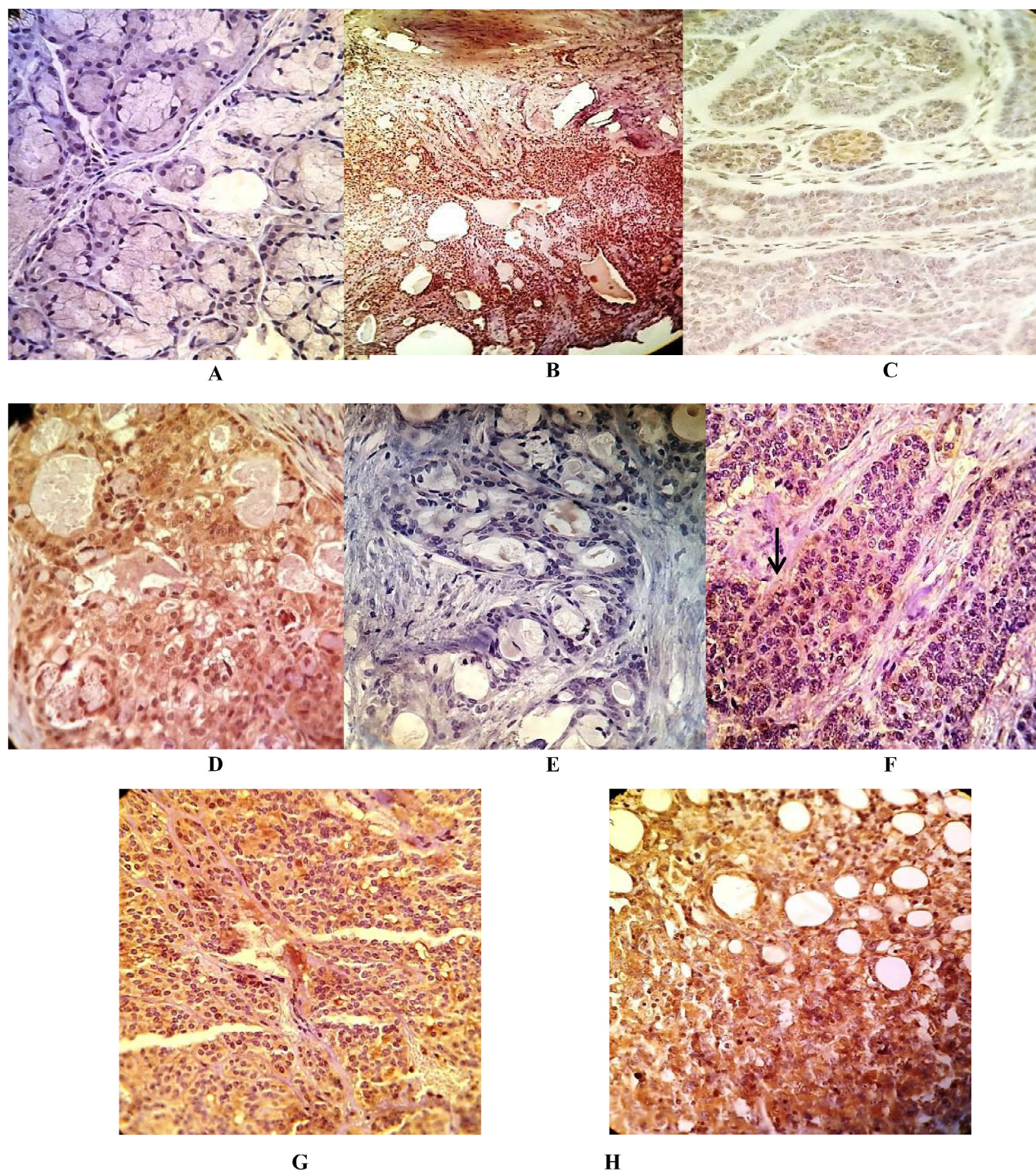


Fig. 5 Expression of telomerase in salivary gland tumors X400. A – Negative expression in normal salivary glands. B – Diffuse expression in pleomorphic adenoma X100. C – Focal expression in basal cell adenoma. D – Diffuse expression in mucoepidermoid carcinoma. E – Negative expression in mucoepidermoid carcinoma. F – Regional expression in adenocystic carcinoma (arrow). G – Diffuse expression in polymorphous low-grade carcinoma. H – Diffuse expression in epithelial-myoepithelial carcinoma.

were found in the non-proliferative stage or terminal differentiation (Shigeishi et al., 2011).

Both adenocystic carcinoma and acinic cell carcinoma revealed 100% positive expression. This was in accordance with the study conducted by Shigeishi et al. (2011).

Finally, the mean level of telomerase activity was higher in malignant tumors than benign ones in this study. This was in agreement with studies done by Liao et al. (2000), and Shigeishi et al. (2011). This finding remarks the valuable role of telomerase in identifying the proliferative capacity of the tumor.

Table 5 Telomerase expression of all salivary gland tumors in relation to immune scoring.

Salivary gland tumors (30)		Score 0	Score 1	Score 2	Score 3	P-value
		Negative No. (%)	Focal No. (%)	Regional No. (%)	Diffuse No. (%)	
Benign	Pleomorphic adenoma (15)	1 (6.7)	0	6 (40)	8 (53.3)	0.03*
	Warthin's tumor (2)	2 (100)	0	0	0	
	Basal cell adenoma (1)	0	1 (100)	0	0	
	Total (18)	3 (16.7)	1 (5.6)	6 (33.3)	8 (44.4)	
Malignant	Mucoepidermoid carcinoma (4)	2 (50)	0	0	2 (50)	0.3
	Adenocystic carcinoma (4)	0	1 (25)	2 (50)	1 (25)	
	Acinic cell carcinoma (1)	0	0	1 (100)	0	
	Polymorphous low-grade adenocarcinoma (2)	0	0	0	2 (100)	
	Epithelial-myoepithelial carcinoma (1)	0	0	0	1 (100)	
Total (12)		2 (16.7)	1 (8.3)	3 (25)	6 (50)	

* P < 0.05 significant difference.

5. Conclusions

Calponin is expressed diffusely in salivary gland tumors but with remarkable altered distribution. Telomerase has a valuable role in determining the proliferation ability of salivary tumors and might be used to differentiate malignant salivary gland tumors from the benign counterpart.

Conflict of interest and source of funding statement

The authors declare that they do not have a conflict of interest. This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Ethical statement

This study was approved by the College of Dentistry Ethical Committee/ Faculty of Medical Science at the University of Sulaimani. Informed consent was obtained from Histopathological Department of Shorsh Hospital in Sulaimani before sample collection.

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