

Proliferative potential in benign mixed salivary gland tumors, using Ki67 marker in relation to different clinicopathological parameters

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Abstract

Background and objective: Pleomorphic adenoma is the most common benign tumor of the salivary gland. It shows a remarkable degree of morphological diversity. Immunohistochemical Ki67 expression is used to predict the proliferative potential of the growth. The aim of this study was to evaluate the expression of the proliferation marker Ki-67 in benign mixed salivary gland tumors.

Methods: Fifty five paraffin embedded tissue blocks were included in the study. After histopathological reassessment of hematoxylin and eosin stained section for each block, an immunohistochemical staining was performed using anti-Ki67 protein.

Results: From total number of 55 cases, 58.1% of patients were in group aged ≤ 40 years. The male to female ratio was 1.037:1. The parotid gland was the most affected (56.37%) gland. The mean duration of lesions before treatment was 17.96 months and the mean size of tumors was 3.76 cm. Histologically, cell-rich subtype constituted 54.54%, followed by stroma-rich (34.55%), and only 10.91% of cases were classic. Ki67 expression was positive in 18.18% predominantly in epithelial variant with a weak score in nine cases (16.36%). Six of positive cases were in parotid gland and two cases in the submandibular gland. Only one case was in minor salivary gland. Moderate Ki67 expression score was seen in one epithelial variant case (14.28 %) associated with parotid gland.

Conclusion: Immunohistochemical expression of Ki-67 in pleomorphic adenoma was characterized by low proliferative rate and predominantly seen in epithelial tumor variants.

Keywords: pleomorphic adenoma, Ki-67, immunohistochemistry.

Introduction

Salivary gland tumors are the second most common neoplasms in the mouth with widely variable histologic characteristics, which make it difficult to determine the pathogenesis.¹ Pleomorphic adenoma (PA), is a benign neoplasm of the salivary gland which shows a remarkable degree of morphological diversity.² PA is found mostly in the parotid gland in middle-aged women.³ When they affect the minor salivary glands, the most frequent site of involvement is the region of the hard palate, followed by the upper lip and buccal mucosa.^{4,2} Local recurrence after surgical treatment is described in 1% to 5% of cases, whereas, malignant changes is reported in 2% to 9% of cases of PA of

salivary gland origin.⁵ Different patterns of malignant changes occur in pleomorphic adenoma, of which carcinoma ex pleomorphic adenoma is one form; the other 2 forms are metastasizing PA and mature malignant mixed tumor (carcinosarcoma).⁶ Clinically, PA presents as a slow growing mass. It affects any age but is more frequent in adults aged 30 - 50 years, with a slight predominance among females. White persons have a slightly higher risk of PA than that of other races.^{7,8} Although it is "benign", it can recur after resection. It invades normal adjacent tissue and distant metastases have been reported after long time intervals.⁷ Histologically a wide variety of structures may be seen. These varied tissues are

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completely disordered in arrangement and the proportions of the different components also vary widely.⁹ Pleomorphic adenoma shows a pronounced morphologic complexity and diversity for this the immunoprofiles and clinical course of PA differed according to cellular differentiation therefore it is important to assess potential biomarkers in diagnostic and therapeutic trials.¹⁰ Ki-67 has been considered to be a potent tool for making an easy and quick evaluation of the proportion of proliferating cells in the tumor.¹¹ The Ki-67 protein is expressed in all phases of the cell cycle except G₀.¹² Its therefore has the potential to be a more sensitive biomarker for cellular proliferation than mitoses.¹³ The expression of Ki-67 has been correlated with mitotic activity, histological grade and clinical behavior of tumor and they found Ki67 proliferation marker has been absent¹⁴ or had lowpositivity¹⁵ in PAs. The aim of this study was to evaluate the expression of the proliferation marker Ki-67 in benign mixed salivary gland tumors in relation to age, sex, primary anatomical site, duration, size of the lesion, and the histological subtype of the tumor using a quantitative approach, and apply these data for further understanding of proliferative peculiarities, pathological characteristics and clinical behavior of this neoplasm.

Methods

The materials used in this study consisted of 55 formalin fixed, paraffin-embedded salivary gland biopsy specimens of benign pleomorphic adenomas, which were retrieved from the archives of the Department of Histopathology, Rizgary Teaching Hospital, Erbil, Kurdistan region of Iraq during the period between January 2009 and December 2013. Demographic data and clinical aspects of the tumors, as reported in the forms, were analyzed. These data included the name, age sex, of the patients, as well as anatomical site, size, duration of the lesion...etc. Only patients with biopsy-proven of benign pleomorphic adenoma of salivary gland

were included in the study. The most representative tumor tissue block was chosen for each case and new sections were made and stained with hematoxylin and eosin (H&E) for histological re-evaluation and additional sections were made for immunohistochemical study. Sample collection was authorized by Rizgary Teaching Hospital, Ministry of Health. The study was carried out with the approval of the Human Research Ethics Committee of the hospital. The positive tissue control included in this study was tonsillar tissue section, and it was run with each batch of stain. The negative tissue controls indicates a tissues specimen, processed using a non-immune serum by omitting the primary antibody and applying the antibody diluents alone, this was done under the same test conditions throughout the work time. Five biopsy specimens of normal salivary glands (border of excision biopsy of mucocele in the lip mucosa) of healthy individuals were used as controls for normal ki67 expression. From each paraffin block of the studied samples and control groups, 4µm thick sections were obtained and mounted on a clean glass slides for routine hematoxylin and eosin staining. The hematoxylin stained the cell nucleus blue color, and in contrast, eosin stained the cytoplasm red color. All hematoxylin and eosin stained slides were re-examined to find the classification of the tumor, paraffin block was selected and 4µm sections were used for immunohistochemical study. The streptavidin – biotin – peroxidase method (SABC – streptavidin – biotin complex) with antibodies against proliferation of ki67 proteins was used. The sections were deparaffinized, submitted to antigen retrieval and incubated with the anti – ki67. For inactivation of endogenous peroxidase, the sections were immersed twice in hydrogen peroxide (10 volumes) for 5 min each. Between steps, the samples were washed with Tris–HCl, pH (7.4). The reaction was developed with 0.03% diaminobenzidine (Sigma) as chromogen,

and the material was counterstained with Mayer's Hematoxylin. The slides submitted to immunohistochemistry were analyzed regarding the presence or absence of labeling of the antigens studied. For quantitative analysis of the positive cells, the cells were counted under a light microscope, using a grid, by two independent examiners. The mean obtained was then used to calculate the labeling index based on the ratio of the number of immunopositive cells per 1000 randomly counted cells per case studied, and then divided by 10 to express the index in percentage. The labeling intensity was not considered in this analysis. For qualitative analysis which permitted comparison of the present data with those reported in the literature, the absolute labeling indices were transformed into the following scores according to the criteria of Alves *et al*²⁴

1. (-) negative, ≤ 5%.
2. (+) weak, >5 and ≤ 25%.
3. (++) moderate, >25 and ≤ 50%.
4. (+++) strong, >50%.

Data were analyzed using OpenEpi software.¹⁶ Chi-square test of association was used to compare proportions. A *P* value of ≤0.05 was considered significant.

Results

From total number of 55 cases, 28 (50.91%) cases were males and 27 (49.09%) cases were females. The mean age of the patients was 40.39 years (range 9-75 years). The mean age of male patients was 46.36 years compared to that of female patients 34.19 years. The highest percentage of PA distribution (58.1%) was seen at patients aged ≤40 years (*P*=0.018) as shown in Table 1. The most commonly involved salivary gland was the parotid (31 cases, 56.37%) followed by the submandibular salivary gland (15 cases, 27.27%) and minor salivary gland (9 cases, 16.36%). The mean duration of symptoms before treatment was 17.96 months (range from 1 month to 60 months); 23 cases (41.82%) had symptoms for 12 months or less, 14 cases (25.45%) had symptoms for (13-24) months and 18 cases (32.73%) had symptoms for 25 months and more. The tumor size varied from 1-9 cm with a mean of 3.76 cm. Twenty six cases (47.27%) had a size equal to or less than 3cm in its maximum dimensions and 29 cases (52.73%) had a tumor size more than 3 cm (Table 2).

Table 1: Distribution of the total sample by age and sex.

Age groups (years)	Pleomorphic adenoma of salivary glands		Total No. (%)	P value
	Male No. (%)	Female No. (%)		
≤ 40	12(42.9)	20(74.1)	32(58.1)	0.0189
> 40	16(57.1)	7(25.9)	23(41.8)	
Total	28(100)	27(100)	55(100)	

Table 2: The clinical characteristics of the cases.

Clinical parameter		No.	%
Site	Parotid	31	56.37
	Other sites	24	43.63
	Total	55	100
Duration (months)	(1-12)	23	41.82
	(13- 24)	14	25.45
	≥25	18	32.73
	Total	55	100
Size(cm)	≤3	26	47.27
	>3	29	52.73
	Total	55	100

The histopathological examination showed that the cell-rich subtype constitutes 54.54 % of the cases, the stroma-rich formed 34.55%, and only 10.91% were classic (Table 3). There were significant correlations between the sex and both the age of patients and the location of the

tumor (more lesions found in females at age ≤ 40 and in parotid gland). The size of the lesion was highly correlated with the location of the tumor (parotid gland had larger size lesions). The histopathological subtypes did not correlate with the studied clinical parameters (Table 4).

Table 3: The histopathological subtypes of salivary glands pleomorphic adenoma.

Histopathological subtype	No. (%)
Cell-rich	30(54.54)
Stroma-rich	19(34.55)
Classic	6(10.91)
Total	55(100.00)

Table 4: The correlation coefficients between clinicopathological parameters.

		Age	Site	Duration	Size	Type
Sex	Pearson Correlation	-.367**	-.269*	-.050	.141	-.093
	Sig. (2-tailed)	.006	.047	.719	.306	.499
Age	Pearson Correlation		-.039	.090	.103	.188
	Sig. (2-tailed)		.780	.511	.456	.170
Site	Pearson Correlation			-.038	-.388**	.123
	Sig. (2-tailed)			.784	.003	.371
Duration	Pearson Correlation				-.077	.135
	Sig. (2-tailed)				.576	.324
Size	Pearson Correlation					-.151
	Sig. (2-tailed)					.271

Immunohistochemical expression of Ki-67 was negative in normal salivary glands (Figure 1). The expression was positive in 10 cases of pleomorphic adenoma (18.18%). These positive cases were more in males and in the parotid gland (70%), but equally distributed between age groups. However, all of them were of cell-rich variants (P = 0.02), (Table 5). The stromal variants of pleomorphic adenoma showed negative expression (Figure 2).

Concerning Ki67 labeling index score, analysis indicated that Ki-67 expression was weak (>5 and ≤ 25%) in nine cases (16.36%), all of them were of epithelial (cell-rich) subtype (Figures 3). They were predominately seen in parotid gland (six cases). Moderate Ki67 expression (>25 and ≤ 50%) was seen in one epithelial variant case (14.28 %) associated with parotid gland (Table 5 and Figure 4).

Table 5: Percent distribution of labeling scores for Ki67 in pleomorphic adenoma in relation to sex, age group and tumor site.

Clinical Parameter	Scores of labeling indices						
	Total No.	Negative No. (%)	Positive No. (%)	P value	Weakly Positive No. (%)	Moderately Positive No. (%)	Strongly Positive No. (%)
Gender							
Male	28	21(46.7)	7(70)	0.325*	6(85.71)	1(14.28)	0
Female	27	24(53.3)	3(30)		3(100)	0	0
Age group (years)							
≤ 40	32	27(60)	5(50)	0.813*	5(100)	0	0
>41	23	18(40)	5(50)		5(100)	0	0
Site							
Parotid	31	24(53.3)	7(70)	0.548*	6(85.71)	1(14.28)	0
Other sites	24	21(46.7)	3(30)		3(100)	0	0
Histopathological subtype							
Cell –rich	30	20	10(18.18)	0.02	9(90)	1(10)	0
Stromal- rich	19	19	0				
Classic	6	6	0				

*Fisher exact test

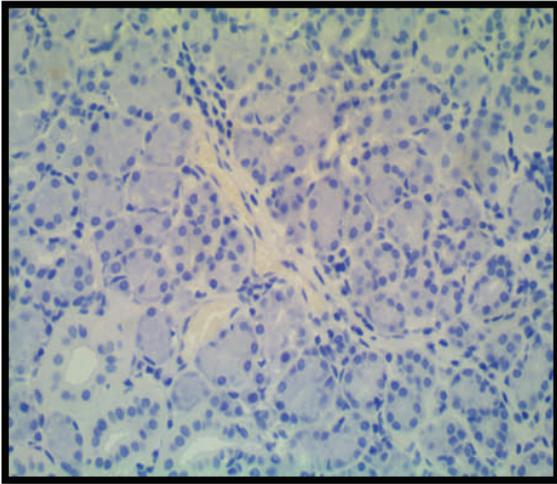


Figure 1: Negative ki67 immunohistochemical expression in normal salivary gland stroma (IHC, X40).

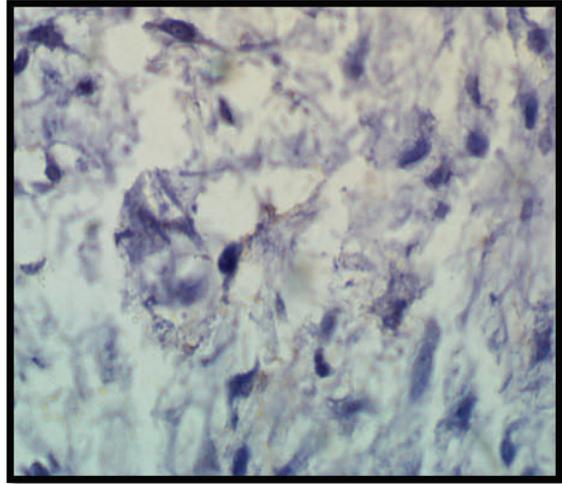


Figure 2: Negative type of PA (IHC, X40).

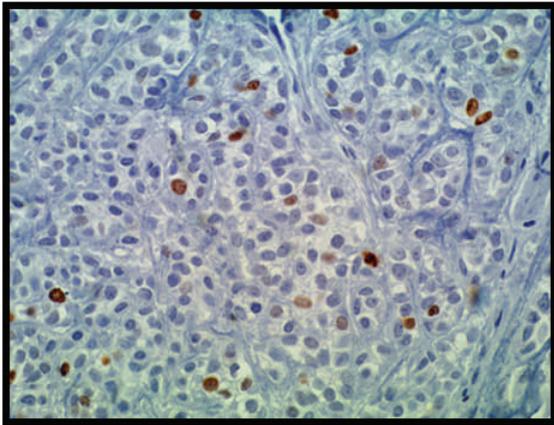


Figure 3: Weak ki67 immunohistochemical expression in epithelial type PA of salivary gland (IHC, X40)

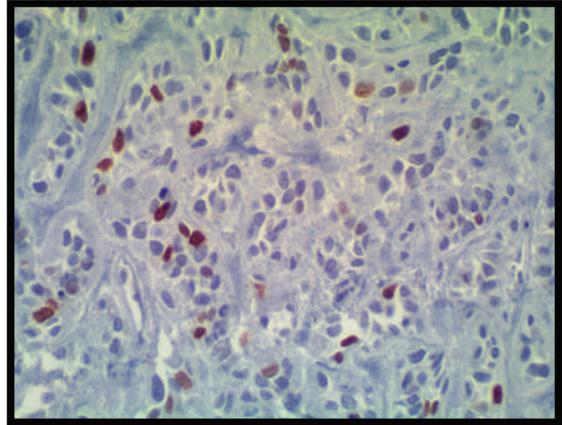


Figure 4: Moderate ki67 immunohistochemical expression in epithelial type of PA in parotid salivary gland (IHC, X40)

Discussion

Salivary glands tumors comprise significant proportion of oral tumors and are the next common neoplasm of the mouth after squamous cell carcinoma.¹⁷ Pleomorphic adenoma is the most common neoplasm of the salivary glands, accounts for 54 to 65% of all salivary gland tumors and was shown sometimes to undergo malignant transformation in its natural course.¹⁸ Primary PAs is more common in female than in male,³ but in our study it was nearly equally distributed between both sexes (male to female ratio equal to 1.037:1). Parotid gland was the mostly affected gland. This comes in agreement with²¹ who found that the tumors developed mainly in the major salivary glands (74% in the parotid gland) and (82% of the cases found in the parotid gland).²³ Pleomorphic adenoma presents as painless slow growing mass with variable duration of symptoms. The duration before treatment, in this study, varied from 1 month to 60 months, in comparison to the results reported by Alves et al.⁴ They reported longer disease duration with a range of 2 to 240 months and a mean of 54.6 months. On the other hand, Anna Kazanceva et al²³ reported that most common surgically removed benign salivary gland tumors are of small size (1 to 3 cm) followed by those measured from 3 to 5cm. Nevertheless, more frequent tumors in the present study were in the group sized > 3cm. Our results showed no significant correlation between the duration of symptoms and the tumor size. This comes in agreement with the study of Anna Kazanceva et al²³ and Chau and Radden¹⁹ who showed no significant correlation between the size and the duration of the tumor^{19, 20} The histopathological results in the present study showed that the cell-rich histological subtype was predominant with no significant correlation with the tumor size. Margaritescu et al studied the relative proportion between the stromal and the epithelial component of PA.²¹ They found that 55% of their cases were type I (PAs with 30% to 50% of the

tumoral stroma), 24% were of type II (with 80% of the stromal component), 12% were of type III (with 20% to 30% of the tumoral stroma) and 9% were of type IV represented tumoral stroma in a similar proportion to that from subtype III but with focal monomorphic epithelial differentiations.¹⁹ PAs were also classified by Ito et al²² as stroma-rich in 99 cases (52.4%), cell-rich in 69 (36.5%) and classic in 21 cases (11.1%). Chau and Radden¹⁹ studied 53 cases of intraoral PA, and found that 29 (54.7%) cases were stroma poor. The present study showed no significant difference in Ki-67 labeling index in regard to the age, sex and site, however, a high percentage of positive cases were seen in parotid gland (70%). The total Ki-67 expression was relating to the histopathological subtype (more in cell-rich type). A study²³ showed that Ki-67- positive cells were present in both stromal type and epithelial rich epithelial variant of tumor and this disagree with our result since Ki-67 expression was observed in the epithelial tumor variants only. According to the results obtained by Eveson et al,⁸ less than 5% of Ki-67 positive cells were present in case of PA. Gimotty et al¹³ reported that the Ki-67 value was significantly higher in large salivary gland tumors. Alves et al⁴ results showed that all pleomorphic adenomas were negative for Ki-67 and the expression of Ki 67 was more in malignant tumors of salivary glands. These results nearly come in agreement with our study indicating that these mixed tumors have low proliferative rate.

Conclusion

Pleomorphic adenomas were nearly equally distributed in both sexes and more frequently at the parotid gland (53.34%) and in patients aged ≤40years (58.1%). Female patients were significantly at younger age than males. Non-significant correlation was found between histopathological subtypes and the tumor size and between the duration and the tumor size. The results showed that (18.18%) of the

cases were positive for Ki-67 expression and all were of epithelial (cell rich) tumor variants. The highest percentages of Ki67 positive cases were seen associated with males (70%), and parotid salivary glands (70 %).

Conflicts of interest

The authors report no conflicts of interest.

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