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*Original Research Article*

# Immunohistochemical Expression of P53 in Benign Pleomorphic Adenomas of Salivary Glands: A clinicopathological Study

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## Abstract

Pleomorphic adenoma (PA) is the most common benign neoplasm of the salivary gland and shows remarkable morphological diversity. It, malignant transformation is not very rare. Mutation in the tumor suppressor gene (p53) may lead to the progression of this tumor. The purpose of this study was to analyze the immunohistochemical expression of p53 in salivary glands PAs in relation to sex, age, primary anatomical site, the duration, size and histopathological subtype of the tumor.

The materials used in this study consist of (45) paraffin-embedded salivary gland biopsy specimens of benign pleomorphic adenoma of salivary gland. The p53 immunoreactivity was semi-quantitatively evaluated in 1000 cells examined under the microscope at x40 magnification and recorded as percentage of p53 positive tumor cells over the total number of cells examined in the area.

PAs showed negative expression of p53 in 60% of cases. The p53 labeling index was ranging between 6% to 70%. Statistical analysis showed no significant relation of the mean labeling indices with the sex, age, duration of the tumor, size, and histopathological subtype ($p>0.05$), but it showed significant relation with the gland site ($p<0.05$).

**Key Words:** apoptosis; P53; immunohistochemistry; salivary gland tumors.

## الخلاصة

الورم الغدي المتعدد الاشكال هو الأكثر شيوعا فى أورام الغدد اللعابية الحميده ويظهر تعدد شكلى واضح , و تحوله الى سرطان خبيث هو ليس نادر جدا. وطفرة في الجين الكابت للورم (p53 ) قد تودى الى تطور هذا الورم. الهدف من هذه الدراسه هو تحليل التعبير المناعي ل p53 للورم الغدي المتعدد الاشكال الحميد وعلاقته مع الجنس, العمر , الموقع التشريحى الاولى ,الفتره المرضيه ,الحجم, ونوع التشريح النسيجى المرضى للورم.

المواد المستعمله فى الدراسه هى٤٥ قالب بارافين لحاله الورم ألغدي المتعدد الاشكال الحميد والتفاعل المناعى ل p53 تم تقييمه كميا فى 1000 خليه فحصت تحت المايكروسكوب تحت قوه تكبير 40 وتسجل كنسبه لخلايا الورم الموجبه ل p53 على العدد الكلى للخلايا المفحوصة فى المنطقة

الورم الغدي المتعدد الاشكال اظهر تعبير سالب الى p53 فى٦٠ % من الحالات.و معدل مقاييس p53 يتراوح من 6−70% وأظهرت التحليلات الإحصائية عدم وجود علاقة معنوية بين معدل مقاييس p53 مع من الجنس, العمر , مدة الورم ,الحجم , ونوع النسيج المرضى($p>0.05$) , ولكن أظهرت علاقة معنوية مع موقع الغدة ($p<0.05$).

## Introduction

Salivary gland tumors include a large diverse group of tumors with complex and overlapping histologic features [1]. It comprise 1–4% of all human neoplasms [2]. The most common tumor of salivary gland origin is PA [3], and the parotid salivary gland showed the most common site of occurrence [4]. 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 the

females. PA has the capacity to grow to a large size and may undergo a malignant transformation, and this risk can increases with time [5]. Kim *et al*. found that carcinoma ex pleomorphic adenoma is a malignancy that develops from either a long-standing primary or a recurrent PA [6].

Histologically PA characterized by a great diversity of morphological aspects. Its structural pleomorphism is given both by the epithelial component, due to cytological differentiations and the growing patterns, and by the stromal component because of its rich morphological and quantitative diversity. This histomorphologic heterogeneity arises mainly from duct epithelium or myoepithelial cells, and a wide variety of structures can be seen, with disordered arrangement and the proportions [7]. The molecular changes in the progression from PA to early stages of malignancy have not been fully studied. However, the inactivation of tumor suppressor genes and the activation of oncogenes appear to be involved in the early transition phase to malignancy [8]. Mutations in the p53 gene often result in prolonging the half life of the p53 protein, causing it to accumulate within cells nuclei to the extent that it can be easily detected by means of immunohistochemistry, so that it can be detected in benign and malignant tissue by immunohistochemistry [9]. Somes studies postulated that the p53 mutation was an early event in the malignant transformation of PA [10-12]. Al-Rawi et al [13] studied 10 cases of PAs, seven of 10 cases (70%) of these tumors were negative for p53 expression, two of 10 (20%) expressed weak p53 immunostaining and one case only expressed moderate p53immunostaining. The present study attempted to investigate the immunohistochemical localization of p53 in salivary gland PAs in relation to sex, age, primary anatomical site, and the duration, size and the histopathological subtype of the tumor.

## Materials and Methods

The materials used in this study consist of forurty five formalin fixed, paraffin-embedded salivary gland biopsy specimens of benign PAs. Demographic data and clinical aspects of the tumors, as reported in the forms, were analyzed. Sections were made and stained with hematoxylin and eosin, and additional sections were made for immunohistochemical study. The positive tissue control included in this study was breast ductal carcinoma tissue section, and the negative tissue controls indicates a tissues specimen, processed using a non immune serum and applying the antibody diluents alone, this was done under the same test conditions throughout the work time and run with each batch of stain. 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 p53 expression.

For immunohistochemical staining, a thin tissue sections (4µm) were cut from paraffin blocks and mounted on silanized and placed in oven over night at 55 °C. The sections were deparaffinized in xylene for 5 minutes, then hydrated in100% ethanol, 90% ethanol, 70% ethanol each for 5 minutes respectively, and then rinsed by distilled water for 5 minutes. Slides placed in antigen retrieval and placed in pressure cooker for 50 minutes at 75 ° C. Then the container with the slides was removed from the steamer and allowed to cool slowly for 10-20 minutes at room temperature.The slides were rinsed in PBS solution.The excess buffer was tapped off gently and the sections are wiped around by gauze pad and a circle around the section was made by pap pen. Enough hydrogen peroxidase block`was applied to cover all the tissue and was incubated for 10 minutes in order to block endogenous peroxidase activity. The slides were rinsed in PBS for 5 minutes, incubated with protein block for 5 minutes,washed in PBS for 5 minutes, incubated with primary antibody for 30 minutes at room temperature, washed in PBS

for 5 minutes, incubated with post primary block for 30 minutes, washed in PBS for 5 minutes, incubated with NovoLinkTM Polymer (UK) for 30 minutes, then rinsed in 2 jars of Tris-Buffer Solution (TBS) for 5 minutes each respectively with gentle rocking.

Diaminobenzidine (DAB) working solution was prepared by adding 50µl of DAB Chromogen to1ml of NovoLinkTM DAB substrate buffer, the sections were incubated with this solution for 5 minutes which resulted in a brown colored precipitate at the antigen sites. Slides were rinsed with tap water, hematoxylin was used as nuclear counter stain for 30 seconds, the slides were washed in running water gently, sections then were dehydrated in graded ethanol (70%, 90%,100%) for 2 minutes each respectively, then were transferred to xylene, then slides were mounted, dried and examined under light microscope.

All hematoxylin and eosin stained slides were examined to find the subtype classification of PA (stroma-rich, cell-rich or classic which contains balanced amount of epithelial and stromal components). Five randomly selected fields in each slide were examined to find the mean percentage of stroma-rich or cell-rich area, using microcomputer imaging device (MCID) image analysis software. For quantitative analysis of p53 positive cells, only the number of cells showing nuclear expression of p53( nuclear staining of brown color) was quantified by counting at least 1000 cells in five representative fields at x40 objective in each case. Calculation of the labeling index was based on the ratio of the number of immunopositive cells per 1000 counted cells per case studied, and then divided by 10 to express the index in percentage. The absolute labeling indices were transformed into the following scores:

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

**Statistical Analysis**

The clinicopathological variables and the immune expression detected by immunohistochemical staining were compared by Pearson's chi-square test, t test, and ANOVA test. All statistical analyses were performed using the Statistical Package for Social Sciences software program, version 19, and the results were considered statistically significant when the P value was $\leq 0.05$.

**Results**

Most of the cases of PAs were the males (26 cases) and comprising (57.78%) of the total patients, and (19cases) were seen associated with the females and comprising (42.22%), with a male to female ratio equal to 1.37:1. The age of the individuals ranged between (13-75) years with a mean age of (41.71) years for the total sample (48.38 years for the males and 31.37 years for the females). Age group (21-30) years showed the highest number of cases of PAs (12 patients), (26.67%) prevalence, followed by the age group (31-40) years (11 patients) with (24.44%) prevalence (Table -1). The parotid gland was mostly affected (24 cases, 53.34%), followed by the submandibular salivary gland (14 cases, 31.11%). The duration of the symptoms before treatment varied from 3 months to120 months, with a mean of 23.42 months duration. The tumor sizes varied from (1- 9cm) with a mean of 3.49cm.

The histopathological evaluation showed that the chief component of most PAs was cell-rich (26 case, 57.78%), fourteen cases (31.11%) were stroma-rich, and only five cases (11.11%) were classic. Statistical analysis showed no significant correlation between histopathological subtypes with the tumor size and duration of the tumor ($p>0.05$) as seen in Table-2 and Table -3 respectively, and no significant correlation between the duration and the tumor size ($p>0.05$) as seen in Table-4.

The results showed that 27 cases (60%) showed p53 negative expression, and 18 cases (40%) showed positive p53 expression (five cases were strong positive, four cases were moderate positive, and nine cases were weak positive). The highest percentages of p53 positive cases were seen associated with males (46.15% males), the age group (41-50) years (100%), upper lip mucosa for the minor salivary glands (50%) and the parotid (45.83%) for the major salivary glands, ≥25 months duration(46.66%), ≤3 cm (45.83%) and in the cell-rich histopathological subtype (50%) as seen in Table-5 and Table-6 respectively.

The p53 labeling index for p53 positive salivary glands PAs was ranging between 6% to 70%. Statistical analysis showed no significant relation of the mean labeling indices ± standard deviation with the sex, age, duration of the tumor (months), and size(cm), ($p>0.05$), but it showed significant relation with the gland site ($p<0.05$). A highest percentages of p53 mean labeling index was seen associated with the submandibular salivary gland (57.6 % ±0.149) as seen in Table-7. Statistical analysis also showed no significant relation of the mean labeling indices ± standard deviation with the histological type($p>0.05$) as seen in Table-8. Figure-1 shows negative, weak, moderate, and strong p53 positive nuclear immunostaining in the salivary pleomorphic adenomas of present study.

**Table 1:** Clinical characteristics of the pleomorphic adenoma

| Clinical parameter | | No. | % |
|---|---|---|---|
| Sex | Male | 26 | (57.78) |
| | Female | 19 | (42.22) |
| | Total | 45 | (100) |
| Age group (year) | ≤20 | 2 | (4.44) |
| | 21-30 | 12 | (26.67) |
| | 31-40 | 11 | (24.44) |
| | 41-50 | 5 | (11.11) |
| | 51-60 | 8 | (17.78) |
| | ≥ 61 | 7 | (15.56) |
| | Total | 45 | (100) |
| Site | Parotid | 24 | (53.34) |
| | Submandibular | 14 | (31.11) |
| | Palate | 3 | (6.67) |
| | Upper lip mucosa | 2 | (4.44) |
| | Check mucosa | 2 | (4.44) |
| | Total | 45 | (100) |
| Duration | (1-12) | 21 | (46.66) |

| | | | |
|---|---|---|---|
| **(months)** | **(13- 24)** | 9 | (20) |
| | **≥25** | 15 | (33. 34) |
| | **Total** | 45 | (100) |
| **Size(cm)** | **≤3** | 24 | (53.34) |
| | **>3** | 21 | (46.66) |
| | **Total** | 45 | (100) |

**Table 2 :** The correlation between the histopathological subtypes of pleomorphic adenomas and the size

| **Histopathological subtype** | **Tumor Size** | | **Total ( No. & %)** | **p-value** |
|---|---|---|---|---|
| | **≤3cm ( No. & %)** | **>3cm ( No. & %)** | | |
| Cell-rich | 14(53.8) | 12(46.2) | 26(100) | 0.176 |
| Stroma-rich | 7(50) | 7(50) | 14(100) | |
| Classic | 3(60) | 2(40) | 5(100) | |
| Total | 24(53.3) | 21(46.7) | 45(100) | |

**Table 3:** Correlation between the duration and the histopathological subtypes of pleomorphic adenomas

| **Duration (months)** | **Histological subtypes** | | | **Total** | **p-value** |
|---|---|---|---|---|---|
| | **Cell-rich** | **Stroma- rich** | **Classic** | | |
| (1-12) | 13 | 6 | 2 | 21(46.7%) | 0.550 |
| (13-24) | 6 | 4 | 0 | 10(22.2%) | |
| (≥25) | 7 | 4 | 3 | 14(31.1%) | |
| Total | 26(57.8%) | 14(31.1%) | 5(11.1%) | 45(100%) | |

**Table 4:** Correlation between the duration and size of pleomorphic adenomas.

| **Duration(months)** | **Tumor size** | | **Total ( No. & %)** | **p-value** |
|---|---|---|---|---|
| | **≤3cm ( No. & %)** | **>3cm ( No. & %)** | | |
| (1-12) | 11(50) | 11(50) | 22(100) | 0.87 |
| (13- 24) | 6(60) | 4(40) | 10(100) | |
| ≥25 | 7(53.8) | 6(46.2) | 13(100) | |
| Total | 24(53.3) | 21(46.7) | 45(100) | |

**Table 5:** Percent distribution of the p53 expression in pleomorphic adenoma in relation to different clinical features

| Clinical Parameter | | Total Negative (No.&%) | Total Positive (No.&%) | Weak Positive (No.&%) | Moderate Positive (No.&%) | Strong Positive (No.&%) | *Total* (No.&%) |
|---|---|---|---|---|---|---|---|
| sex | Male | 14(53.85) | 12(46.15) | 7(26.92) | 2(7.69) | 3(11.54) | *26(100)* |
| | Female | 13(68.41) | 6(31.59) | 2(10.53) | 2(10.53) | 2(10.53) | *19(100)* |
| Age group (years) | < 20 | 2(100) | 0 | 0 | 0 | 0 | *2(100)* |
| | 21-30 | 11(91.67) | 1(8.33) | 0 | 0 | 1(8.33) | *12(100)* |
| | 31-40 | 5(45.45) | 6(54.55) | 3(27.28) | 3(27.27) | 0 | *11(100)* |
| | 41-50 | 0 | 5(100) | 3(60) | 0 | 2(40) | *5(100)* |
| | **51-60** | 4(50) | 4(50) | 3(37. 5) | 0 | 1(12. 5) | *8(100)* |
| | **≥61** | 5(71.42) | 2(28.58) | 0 | 1(14.29) | 1(14.29) | *7(100)* |
| **Site** | **Parotid** | 13(54.17) | 11(45.83) | 8(33. 33) | 2(8.33) | 1(4.17) | *24(100)* |
| | **Submandibular** | 9(64.29) | 5(35.71) | 0 | 1(7.14) | 4(28.57) | *14(100)* |
| | **Palate** | 2(66.67) | 1(33.33) | 1(33.33) | 0 | 0 | *3(100)* |
| | **Upper lip** | 1(50) | 1(50) | 0 | 1(50) | 0 | *2(100)* |
| | **Check** | 2(100) | 0 | 0 | 0 | 0 | *2(100)* |
| **Duration (month)** | **1-12** | 14(66.67) | 7(33.33) | 4(19.04) | 1(4.76) | 2(9.53) | *21(100)* |
| | **13-24** | 5(55.55) | 4(44.45) | 2(22.23) | 1(11.11) | 1(11.11) | *9(100)* |
| | **≥25** | 8(53.34) | 7(46.66) | 3(20) | 2(13.13) | 2(13.13) | *15(100)* |
| **Size(cm)** | **≤3** | 13(54.17) | 11(45.83) | 5(20.83) | 2(8.33) | 4(16.67) | *24(100)* |
| | **>3** | 14(66.67) | 7(33.33) | 4(19.05) | 2(9.52) | 1(4.76) | *21(100)* |

**Table 6 :** Distribution of the p53 expression in pleomorphic adenoma in relation to different histopathological subtype

| Histopatho-logical subtype | No. | Total Negative No.(%) | Total positive No.(%) | Weak Positive No.(%) | Moderate Positive No (%) | Strong Positive (No.&%) | Total No. (%) |
|---|---|---|---|---|---|---|---|
| Cell-rich | 26 | 13(50) | 13(50) | 7(26.92) | 3(11.54) | 3(11.54) | 26(100) |
| Stroma-rich | 14 | 10(71.43) | 4(28.57) | 1(7.14) | 1(7.14) | 2(14.29) | 14(100) |
| Classic | 5 | 4(80) | 1(20) | 1(20) | 0 | 0 | 5(100) |
| Total | 45 | 27(60) | *18(40)* | 9(20) | 4(8.9) | 5(11.1) | 45(100) |

**Table 7:** Distribution of the mean and standard deviation of the percentages of p53-positive nuclei in relation to the clinical picture (p53 immuno-negative cases were excluded from the analysis)

| Clinical parameter | | Mean p53 labeling index | | | |
|---|---|---|---|---|---|
| | | No. | p53mean (%) | ± SD (%) | P -value |
| Sex | Male | 12 | 27 | 0.24 | 0.385 |
| | Female | 6 | 38.5 | 0.24 | |
| Age group (years) | ≤ 20 | 0 | …… | …… | 0.46 |
| | 21-30 | 1 | 62 | …… | |
| | 31-40 | 6 | 20.67 | 0.153 | |
| | 41-50 | 5 | 33.2 | 0.296 | |
| | 51-60 | 4 | 26 | 0.296 | |
| | ≥ 61 | 2 | 48 | 0.240 | |
| Site | Parotid | 11 | 20 | 0.198 | 0.006 |
| | Submandibular | 5 | 57.6 | 0.149 | |
| | Minor salivary glands | 2 | 21.5 | 0.19 | |
| Duration (months) | 1-12 | 8 | 30.13 | 0.244 | 0.98 |
| | 13-24 | 4 | 29.25 | 0.292 | |
| | ≥25 | 6 | 32.33 | 0.261 | |
| Size(cm) | ≤3 | 11 | 35.5 | 0.254 | 0.30 |
| | >3 | 7 | 22.27 | 0.226 | |

**Table 8:** Distribution of the mean and standard deviation of the percentages of p53-positive nuclei in relation to different histopathological subtype (p53 immuno-negative cases were excluded from the analysis)

| Histopatho-logical subtype | Mean p53 labeling indices | | | |
|---|---|---|---|---|
| | No. | p53 Mean (%) | ± SD (%) | P - value |
| Cell-rich | 13 | 0.28 | 0.238 | 0.564 |
| Stroma-rich | 4 | 0.41 | 0.297 | |
| Classic | 1 | 0.17 | ….. | |
| Total | 18 | 0.30 | 0.57 | |

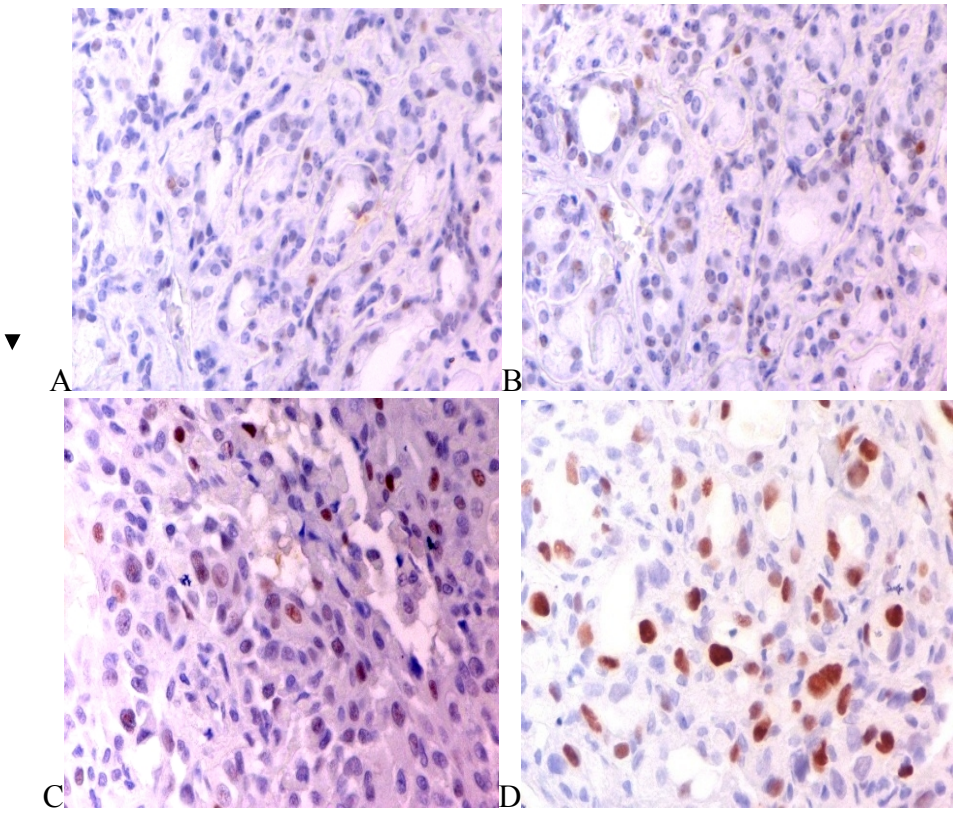


**Figure 1:** (A) Negative p53 immune expression in pleomorphic adenoma of parotid salivary gland in 24 years old female. (B)Weak p53 immune expression in pleomorphic adenoma of palatal minor salivary gland in 42 years old male.(C) Moderate p53 immune expression in pleomorphic adenoma of minor salivary gland of the upper lip in 33 years old male.(D) Strong p53 immune expression in pleomorphic adenoma of submandibular salivary gland in 43 years old male (IHC, x 40).

## Discussion

The sample used in this study consist of 45 cases of histologically proven salivary gland primary benign PAs. Most of the cases were common among the males, with a male to female ratio equal to 1.37:1.The increase in occupational risks in addition to cigarette smoking may be implicated as a cause. These result disagree with that of Margartesscu *et al* [14] study which showed females predominance(65 cases, representing 63.1%), with a female to male ratio of 1.71:1.

The individuals with PA had age ranged between (13-75) years with a mean age of (41.71) years for the total sample, and the age group (21-30) years showed the highest number of cases of PAs. Alves *et al* [2] found that most of the patients with PAs in their study were between the third and fifth decades of life (64%), with a mean age of 36.3 years. The result also showed that the parotid gland was mostly affected site, Margaritesscu *et al* [14] also found the same results.

Regarding the duration of the symptoms before treatment, it was varied from 3months to 120 months, with a mean of 23.42 months. This result disagree with that of Alves *et al* [2], they found that the duration of the symptoms before treatment varied from 2 to 240 months, with a mean of 54.6 months.

Our results showed no significant correlation between the duration and the tumor size ($p>0.05$). This comes in agreement with the study of Chau and Radden [15], they studied 53 cases of salivary gland PAs and their statistical analysis showed no correlation between the size and the duration. The histopathological results in the present study showed that the cell-rich histological subtype was predominant (26 case, 57.78%). This result disagree with that of Ito *et al* [16] in which the stroma-rich was predominant and constitutes (52.4%).

The results showed that 18 cases (40%) showed positivity for p53 (five cases strong positive, four cases moderate positive, and nine cases weak positive), and 26 cases (60%) showed p53 negative expression. These results nearly comes in agreement with the study of Kärjä *et al* [17], they reported that p53 expression was expressed in 41% of PAs, but disagree with that of Deguchi *et al* [18], they found that six cases out of 33 (18%) with benign PAs were p53 positive. Some studies observed no expression of p53 in any case studied [2,19, 20], while others found that most of the cases of PAs were positive for the p53 protein[21,22], and according to these authors, this indicating a tendency of PAs toward malignant transformation.

In the present study, the p53 mean labeling index for p53 positive staining in PAs of salivary glands was ranging between 6% to 70%. Nordkvist *et al* [23] studied p53 expression in 68 cases of benign PAs and only 8 cases out of 68 expressed p53 with weakly positive staining and none had nuclear staining exceeding 10%. The differences in mean index results obtained could be due to the methodology employed, type and dilution of primary antibody, or due to the type of tissue analyzed. The statistical analysis of the present study showed no significant relation of the mean labeling indices with the sex, age, duration of the tumor, size, and histopathological subtype , but it showed significant relation with the salivary gland site ( more in submandibular glands). Ohtake *et al* [22] found that the malignant transformation increases with duration of these lesions. Tarakji *et al* [24] found that the expression of p53 was significantly affected by histopathological subtype and it is higher in tumor epithelial part especially the tumor duct cells than mesenchymal part. Al-Rawi *et al* [13] found that p53 immuno reactivity was associated with larger tumor size.

## Conclusion

Most of the cases of PAs were common among the males with a male to female ratio equal to 1.37:1 and a mean age of

(40.74) years, the age group (21-30) years showed the highest number of cases of PAs (26.67%), and the parotid gland was mostly affected (53.34%). The duration of the symptoms before treatment varied from 3months to 120 months, with a mean of 23.42 months. The tumor sizes varied from (1-9cm) with a mean of 3.49 cm. The p53 labeling index for salivary glands PAs was ranging between 6% to 70%. Statistical analysis showed no significant relation of the mean labeling indices ± standard deviation with the sex, age, duration of the tumor, size, and histopathological subtype ($p>0.05$), but it showed significant relation with the gland site ($p<0.05$) and appear more in submandibular glands.

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