

Research Article

Immunohistochemical Expression of p53 in Pleomorphic Adenoma and Carcinoma Ex Pleomorphic Adenoma

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Context. Immunohistochemical stains for p53 are used as a diagnostic marker associated with malignancy in several histologic types of salivary gland tumors. This marker may be useful in differentiating pleomorphic adenoma (PA) from carcinoma ex pleomorphic adenoma (CPA), as these tumors are often difficult to distinguish on the basis of morphology alone. *Objective.* to evaluate whatever inactivation of tumor suppressor gene (p53) increases with the tumor progression from normal salivary tissue to PA and eventually CPA. *Design.* Paraffin blocks of 29 cases of PA, which were surrounded by normal parotid gland, and 27 cases of carcinoma ex pleomorphic adenoma were retrieved and validated. In all cases of carcinoma ex pleomorphic adenoma, a PA “ghost” was identified, and the malignant element was either undifferentiated carcinoma or adenocarcinoma. *Results.* The results showed negative nuclear expression of P53 in normal parotid gland. Nuclear P53 was expressed strongly in 6/29 (20.7%) pleomorphic salivary adenoma and 10/27 (37%) carcinoma ex pleomorphic adenoma. *Conclusion.* Our data suggest that inactivation of p53 may play an important role in the evolution of pleomorphic salivary adenoma and carcinoma ex pleomorphic adenoma.

1. Introduction

Pleomorphic salivary adenoma (PA) is the most common neoplasm of salivary glands [1] and was shown sometimes to undergo malignant transformation in its natural course [2]. Carcinoma ex pleomorphic adenoma (CPA) is considered to be a malignant transformation product of pre-existing pleomorphic adenoma [3]. CPA is the most common malignant mixed tumor and has been estimated to account 10% of all salivary gland malignancy [4]. The pathogenetic mechanisms involved in the progression of pleomorphic adenoma to a carcinoma remain unclear, requiring evaluation of molecular events in both pleomorphic adenoma and carcinoma arising in pleomorphic adenomas [5]. The current studies of the molecular biology of cancers have demonstrated that the loss of function of tumor suppressor gene such as p53 may lead to the development of many different cancer types [6, 7]. The published literature on tumor markers in CPA is limited due to the fact that these tumors are rare. Mutation of P53 tumor suppressor gene, located on the short arm

of chromosome 17, is among the most commonly detected genetic abnormalities in human neoplasia.

Our study aimed to characterise the alterations and aberrations in the expression of p53 and to evaluate whatever inactivation of tumor suppressor gene (p53) increases with the tumor progression from normal salivary tissue to PA and eventually CPA.

2. Materials and Methods

Archived formalin-fixed, paraffin-embedded tissue blocks of 29 cases of pleomorphic adenomas, and 27 cases of carcinoma ex pleomorphic adenoma were obtained from the Department of Oral Pathology at Aleppo Dental School (Tables 1 and 2). Normal tissue of the salivary gland surrounding the tumor was used as a control in 29 cases of pleomorphic adenoma. The immunohistochemical expression of antibodies against p53 was examined in the selected cases.

TABLE 1: Clinical data of pleomorphic adenomas cases.

Case	Age	Gender	Gland type
1	87	F	Parotid gland
2	52	M	Parotid gland
3	63	M	Parotid gland
4	48	F	Parotid gland
5	76	F	Parotid gland
6	47	M	Parotid gland
7	62	F	Parotid gland
8	33	M	Parotid gland
9	49	F	Parotid gland
10	45	M	Parotid gland
11	63	F	Parotid gland
12	53	F	Parotid gland
13	27	F	Parotid gland
14	59	F	Parotid gland
15	33	F	Parotid gland
16	55	F	Parotid gland
17	26	F	Parotid gland
18	65	F	Parotid gland
19	40	M	Parotid gland
20	57	M	Parotid gland
21	34	M	Parotid gland
22	74	F	Parotid gland
23	67	F	Parotid gland
24	32	M	Parotid gland
25	31	M	Parotid gland
26	62	F	Parotid gland
27	76	F	Parotid gland
28	21	F	Parotid gland
29	61	F	Parotid gland

2.1. Inclusion Criteria for Carcinoma Ex Pleomorphic Adenoma. According to the World Health Organization histological classification published in 2005, malignant changes in the PA include three different types: CPA, carcinosarcoma, and metastasizing PA.

The proposed criteria for defining carcinoma ex pleomorphic adenoma by Nagao et al. [8] were used to select and reclassify our cases of carcinoma ex pleomorphic adenoma.

The use of strict pathological criteria may underestimate the frequency of carcinoma ex pleomorphic adenoma because the malignant cells in some cases may obliterate the original pleomorphic adenoma.

The Inclusion criteria for carcinoma ex pleomorphic adenoma includes the following:

- (i) Major gland primary lesion (parotid or submandibular), and the macroscopic features that suggest malignant transformation in pleomorphic adenoma include poorly defined and/or infiltrative tumor margins, the presence of foci of hemorrhage, and necrosis.
- (ii) The existence of benign and malignant elements are considered as well.

- (iii) Benign element can be pleomorphic adenoma within the tumor mass, biopsy proven history of previous PA (pleomorphic adenoma) indicated that it was in the same location as the subsequent carcinoma.

- (iv) Malignant elements can be undifferentiated carcinoma, adenocarcinoma, and multiple patterns of differentiation including undifferentiated or adenocarcinoma patterns.

2.2. Exclusion Criteria for Carcinoma Ex Pleomorphic Adenoma. The exclusion criteria for carcinoma ex pleomorphic adenoma includes the other well-recognized salivary carcinomas and those of uncertain type included in the current WHO histological classification of tumors [9].

All specimens using hematoxylin and eosin slides were reviewed by two pathologists to confirm the histopathological diagnosis and to reclassify the studied cases. The carcinoma cases classified according to the above-mentioned criteria as undifferentiated carcinoma or adenocarcinoma. The Research Ethics Committee at Aleppo Dental School provided a favourable ethical opinion (Ref: 145/2010).

2.3. Immunohistochemistry. Paraffin-embedded, 4- μ m-thick tissue sections from all 55 specimens were cut. The sections were deparaffinized in xylene and dehydrated through graded alcohols. Sections were processed used streptavidin-biotin-peroxidase method. Briefly, the endogenous peroxidase was blocked by 3% hydrogen peroxidase for 5 minutes followed by TBS wash. Nonspecific immunoreactivity was blocked by incubation with normal goat serum for 20 minutes. A primary antibody TP53 monoclonal mouse anti-human (clone, D-O7: Dako) was diluted to 1 : 25 (40 μ L/mL) in tris buffer saline (TSA) containing 0.1% bovine serum albumin for 2 hours at room temperature. All sections were washed by TBS for 5 minutes. Sections were incubated with the biotinylated secondary antibody reagent for 30 minutes followed by (TBS) wash for 5 minutes. Slides were incubated with streptavidin and horseradish peroxidase for 30 minutes followed by (TBS) tris buffer saline wash for 5 minutes. Incubate with a prepared chromogenic substrate solution (Diaminobenzidine) for 15 minutes. Sections were counterstained with 0.25% methyl green in distilled water for 5 minutes. Sections were dehydrated and mounted in Depax. Squamous cell carcinoma was used as positive control. Negative control was used only with substitution of the primary antibody with TBS. The percentage of P53-positive nuclei was semiquantitatively assessed by two independent observers and scored as negative (0) no expression of nuclear protein, (1) weak staining 0–25% of the total cells shows positive staining in the nucleus, (2) moderate staining >25–75% of the total cells in the test area show positive nuclear staining, (3) strong staining >75–100% cells show positive nuclear staining.

2.4. Statistical Analysis. Cells of the carcinomatous component of the CPA were always scored. The statistical analysis included the use of descriptive statistics, frequencies/proportion, and crossed tabulation. Also, statistical

TABLE 2: Clinical data of cases from carcinomas ex pleomorphic adenomas.

Case	Age	Gender	Gland	Histological subtype	Metastasis to lymph nodes*
1	77	F	Parotid	Adenocarcinoma	Yes
2	28	M	Parotid	Adenocarcinoma	No
3	78	M	Submandibular	Undifferentiated	Yes
4	45	M	Parotid	Undifferentiated	Yes
5	76	F	Parotid	Undifferentiated	No
6	82	F	Parotid	Undifferentiated	No
7	71	M	Parotid	Adenocarcinoma	No
8	67	M	Submandibular	Undifferentiated	Yes
9	63	M	Submandibular	Undifferentiated	Yes
10	55	M	Submandibular	Undifferentiated	Yes
11	73	M	Parotid	Undifferentiated	Yes
12	71	M	Parotid	Undifferentiated	No
13	64	M	Parotid	Undifferentiated	Yes
14	60	F	Parotid	Undifferentiated	Yes
15	49	F	Submandibular	Undifferentiated	No
16	39	F	Parotid	Undifferentiated	Yes
17	56	M	Parotid	Undifferentiated	No
18	45	F	Parotid	Undifferentiated	Yes
19	57	M	Parotid	Undifferentiated	Yes
20	66	F	Parotid	Undifferentiated	No
21	86	F	Submandibular	Undifferentiated	Yes
22	17	F	Parotid	Undifferentiated	No
23	78	M	Submandibular	Undifferentiated	Yes
24	26	M	Parotid	Undifferentiated	No
25	31	F	Parotid	Undifferentiated	No
26	71	M	Parotid	Undifferentiated	No
27	71	M	Parotid	Undifferentiated	No

*Metastasis to lymph nodes at the time of tumor resection.

TABLE 3: p53 expression of nuclear staining in the nontumor duct cells.

p53 pattern	Frequency	Percent
Negative staining	12	41.4
Weak staining	13	44.8
Moderate staining	3	10.3
Strong staining	1	3.4
Total	29	100.0

analyses, including Mann-Whitney and Wilcoxon's nonparametric tests, were performed on the data. All statistical tests were two sided and P -values less than .05 were considered to be statistically significant.

3. Results

3.1. p53 Expression in the Normal Tissue of Salivary Gland Surrounding the Pleomorphic Adenoma. Three components, duct, acinar cells, and stroma were examined in the normal tissue adjacent to the pleomorphic adenoma Figure 1. We evaluated the percentage of p53-positive cells in each case,

TABLE 4: p53 expression of nuclear staining in acinar cells surrounding the tumor of pleomorphic adenoma.

P53 pattern	Frequency	Percent
Negative staining	24	82.8
Weak staining	4	13.8
Moderate staining	1	3.4
Total	29	100.0

with the use of the frequency test. The results in Table 3 (nuclear staining of nontumor duct cells) indicated that p53 showed strong positive staining in 1 (3.4%) case out of 29, 3 (10.3%) cases with moderate staining, 13 (44.8%) cases with weak staining, and 12 (41.4%) cases had no expression (Table 3) whereas expression of p53 cytoplasmic staining was present in nontumor duct cells as follows: 3 (10.3%) cases out of 29 with moderate staining, 9 (31.0%) with weak staining, and 17 (58.6%) with negative staining.

p53 (nuclear staining of the acinar cells) showed negative staining in 24 (82.8%) cases out of 29, 4 cases with weak staining and 1 (3.4%) case with moderate staining (Table 4).

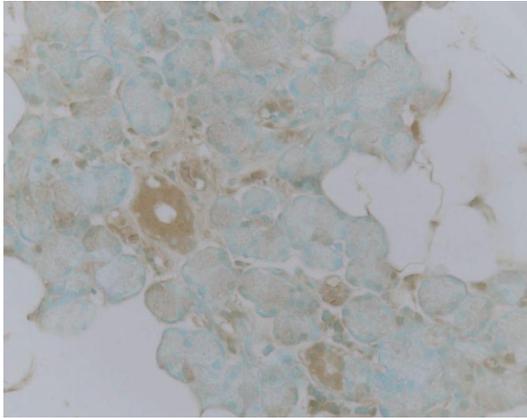


FIGURE 1: Showing negative nuclear staining of p53 in normal salivary tissue. Original magnification x40.

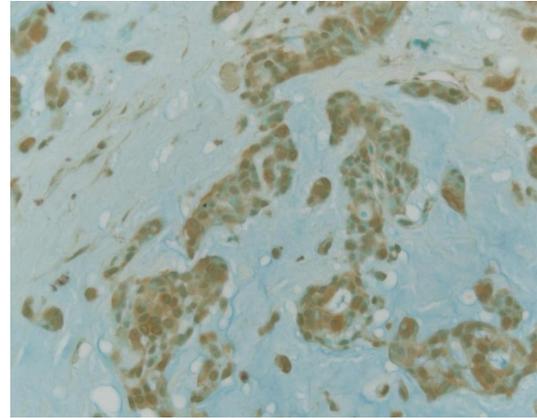


FIGURE 2: Showing strong p53 staining in pleomorphic salivary adenoma. Original magnification x40.

p53 (nuclear staining in stroma) was observed in 1 (3.4%) case out of 29 with strong staining, 8 (27.6%) cases with weak staining, and 20 cases (69.0%) with negative staining (Table 5).

The results showed in those tables that p53 expression was negative or low with positive nuclear staining in most elements of the control group (duct, acinar cells, and stroma).

3.2. p53 Expression in Pleomorphic Adenoma. p53 was strongly expressed in tumor duct cells in 6 (20.7%) cases out of 29. 16 (55.2%) cases showed moderate staining, and 7 (24.1%) cases expressed weak staining (Table 6). The expression of p53 in pleomorphic adenoma was shown in Figures 2, 3, and 4.

p53 nuclear staining in the myxochondroid tissue was identified in 1 (3.4%) case with strong staining, 10 (34.5%) cases with moderate staining, and 18 (62.1%) cases with weak staining (Table 7).

The p53 expression in pleomorphic adenoma was clarified in cross-tabulation tables (Table 8) to compare p53 expression in myxochondroid and duct cells.

Wilcoxon's test showed significant differences between p53 expression of p53 nuclear staining in myxochondroid and tumor duct cells, P value $< .001$.

3.3. p53 Expression in Carcinoma Arising in Pleomorphic Adenoma. p53 was strongly expressed in carcinoma cells in 10 (37%) cases out of 27. Moderate staining was seen in 2 (7.4%) cases and 15 (55.6%) cases expressed negative staining Figures 5, 6, 7, and 8.

3.4. Comparison between p53 Expression in Pleomorphic Adenoma and Carcinoma Arising in Pleomorphic Adenoma. The Mann Whitney test showed no significant difference between p53 expression in pleomorphic adenoma (tumor duct cells) and carcinoma arising in PA (P value $.08 > .05$). 6 cases out of 29 showed strong p53 staining in tumor duct

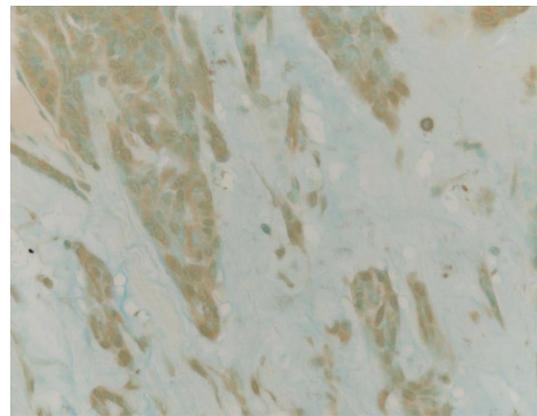


FIGURE 3: Showing moderate p53 staining in pleomorphic salivary adenoma. Original magnification x40.

TABLE 5: p53 expression of nuclear staining in stroma (normal tissue surrounding the tumor) in pleomorphic adenoma.

p53 pattern	Frequency	Percent
Negative staining	20	69.0
Weak staining	8	27.6
Strong staining	1	3.4
Total	29	100.0

TABLE 6: p53 expression in the nucleus of the tumor duct cells.

p53 pattern	Frequency	Percent
Weak staining	7	24.1
Moderate staining	16	55.2
Strong staining	6	20.7
Total	29	100.0

cells in pleomorphic adenoma, but 10 cases out of 27 showed the same expression in carcinoma cells.

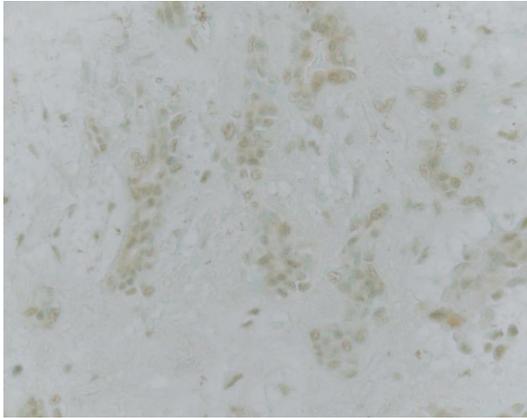


FIGURE 4: Showing low nuclear staining of p53 in pleomorphic salivary adenoma. Original magnification x40.

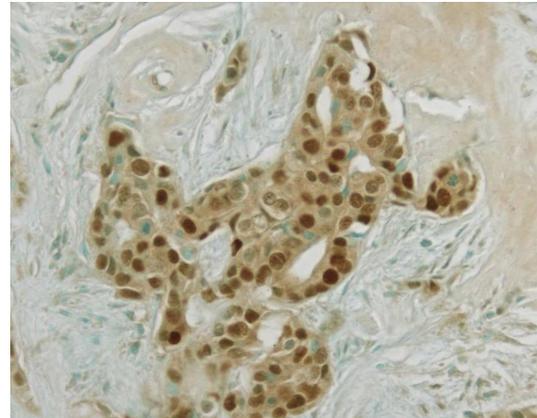


FIGURE 5: Showing strong p53 staining of nuclei in CPA. Original magnification x40.

TABLE 7: p53 expression of the nucleus staining in myxochondroid (tumor area).

p53 pattern	Frequency	Percent
Weak staining	18	62.1
Moderate staining	10	34.5
Strong staining	1	3.4
Total	29	100.0

TABLE 8: Cross-tabulation p53 nuclear staining in myxochondroid tissue and duct cells of pleomorphic adenoma.

	p53 in duct cells			Total
	Weak Staining	Moderate staining	Strong staining	
p53 expression in Myxochondroid				
Weak staining	6	12	0	18
Moderate staining	1	4	5	10
Strong staining	0	0	1	1
Total	7	16	6	29

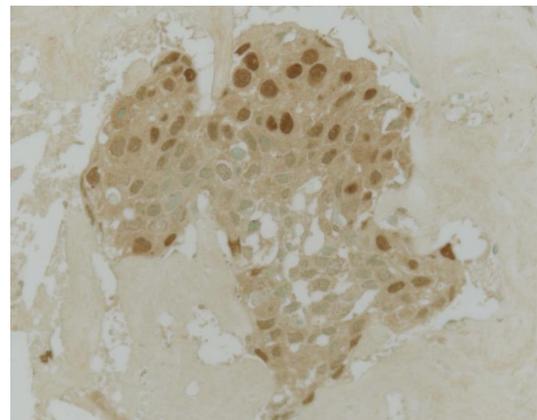


FIGURE 6: Showing moderate p53 nuclear staining in CPA. Original magnification x40.

p53 expression in either pleomorphic adenoma or carcinoma cases arising in pleomorphic adenoma is shown in Figures 2–7.

4. Discussion

4.1. *p53 and Normal Tissue of the Salivary Glands.* p53 expressed negative or weak nuclear staining in normal salivary glands (duct and acinar cells of the control group), and these results are consistent with Deguchi et al. [10] who stated that no p53 immunostaining was found in the normal salivary gland tissues.

Soini et al. [11] mentioned that the p53 wild-type protein had a short half life less than the p53 mutated protein allowing p53 mutated protein to be detected in

the cells. The authors of [12] observed that there is no evidence that normal thymic epithelial cells expressed p53. Ogden et al. [13] studied p53 expression in specimens from normal, benign oral mucosa. Biopsies were obtained from 54 specimens of nonmalignant tissues (normal mucosa, lichen planus, papillomas, keratosis). p53 protein was not observed in the above lesions. Ogden et al. [14] detected overexpression of p53 in normal oral mucosa of oral cancer patients. The p53 positivity in normal mucosa may be due to stabilization of the wild-type p53. The tumor suppressor protein p53 is present in a wide variety of cells. In the normal cell, the concentration of wild-type p53 protein is generally below the detection level of immunohistochemical methods. However, in the gene coding for the p53 protein, point mutation occurs frequently, leading to the accumulation of mutant protein. Thus, in 22–70% of malignancies such as cancer in the colon, stomach, bladder, breast, lung, thyroid, p53 protein can be demonstrated by immunohistochemistry [15, 16].

It is concluded that wild-type p53 in normal tissues is generally not detectable by immunochemistry, owing

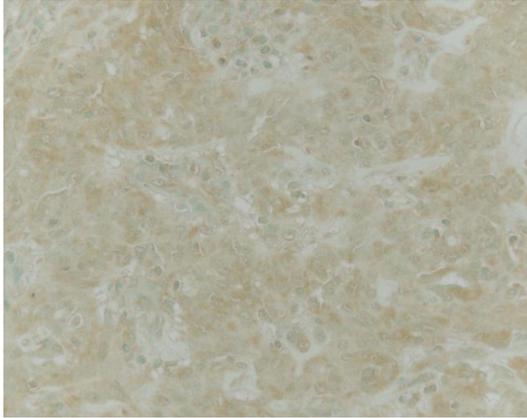


FIGURE 7: Showing low nuclear staining of p53 in CPA. Original magnification x40.

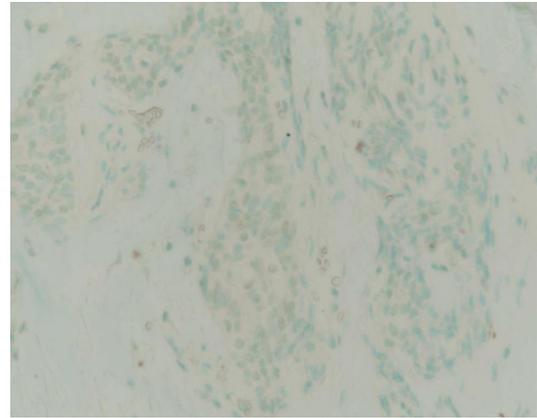


FIGURE 8: Showing p53 negative control for CPA. Original magnification x40.

to a combination of low expression and short half life. Mutation of the p53 gene results in a conformation change in the protein, which becomes stabilised, thus allowing for immunohistochemical detection.

4.2. p53, Pleomorphic Adenoma and Carcinoma Arising in Pleomorphic Adenoma. p53 strong and moderate nuclear staining was classified as positive, and the indicator for existing altered p53 in pleomorphic adenoma and carcinoma arising in PA. Low and negative nuclear staining was classified as negative, and the indicator for existing wild-type (nonaltered p53). The proportion of p53-positive nuclear stained cells (strong and moderate) was (75.9%) in tumor duct cells of pleomorphic adenoma cases (22 cases out of 29 expressed strong or moderate staining). These results are consistent with Azuma et al. [17] who found p53 accumulation in 75% (3/4) of pleomorphic adenoma cell lines (using Ab 1801 and Ab 2401 antibodies). Deguchi et al. [10] found that six cases out of 33 (18%) with benign pleomorphic adenoma were p53 positive (using the CM-1 antibody). Yamamoto et al. [5] analysed tissue specimens containing three morphological components including adenoma, transitional foci, and carcinoma for 8 cases of carcinoma arising in pleomorphic adenoma. The immunohistochemical analysis of p53 protein (using ab-6, clone DO-1 antibody) revealed that p53 was expressed in only one case (13%) out of 8 of pleomorphic adenoma. Li et al. [18] analyzed the numeric aberrations of chromosome 17 and p53 gene deletion in pleomorphic adenoma. Monosomy 17 was shown in (29.6%) of pleomorphic adenoma cells. p53 was expressed in 2 out of 75 cases of pleomorphic adenoma. They confirmed that chromosome aberrations started early in pleomorphic adenoma.

Yamamoto et al. [19] found no mutations in p53 observed in the 7 cases of pleomorphic adenoma (control group). Nordkvist et al. [20] studied p53 expression (using Do-7 antibody) in 68 cases of benign pleomorphic adenoma. Only 8 cases out of 68 expressed p53 with weakly positive staining. Gallo et al. [21] found that three cases showed

positive p53 (11%) in benign parotid gland tumors out of 26 cases. Kärjä et al. [22] reported that p53 expression (using Ab 1801) was expressed in 41% of pleomorphic adenomas.

The results of carcinoma arising in pleomorphic adenoma showed that 12 cases (44.4%) expressed moderate or strong staining of p53. This is comparable to the frequencies of 67% [10] and 75% [5], reported previously. Li et al. [18] studied the numeric aberrations of chromosome 17 and p53 gene deletion carcinomas arising in pleomorphic adenoma. Polysomy was observed in 19.6% of carcinoma cells, and monosomy 17 was shown in (30.8%) of CPA cells (carcinoma in pleomorphic adenoma). Immunohistochemical staining showed p53 was expressed in 6 out of 9 CPA cases (66.7%).

Yamamoto et al. [19] found a high rate of mutations (loss of heterozygosity) as the p53 gene was detected in cases of carcinoma arising in pleomorphic adenoma (58%). Nordkvist et al. [20] studied 24 cases of carcinoma ex pleomorphic adenoma. 17 cases showed p53 expression. 9 cases with positive p53 staining had 1–10% of positive cells, 5 cases showed positive p53 staining in 11–50% of the cells, and 3 cases showed very strong staining of p53 in 51–100% of the cells.

Kärjä et al. [22] studied 12 cases of carcinoma arising in PA, 6 (50%) cases showed p53-positive nuclear staining.

4.3. p53 Strong Nuclear Staining as a Strong Indicator for Altered p53. If we considered the p53-positive strong staining alone as a strong indicator for altered p53 (>75–100 positive cells), then, the results would show that p53 was expressed strongly in 6 (20.7%) cases (tumor duct cells) out of 29 in pleomorphic adenoma but in 10 cases (37%) out of 27 cases of carcinoma arising in PA. The results demonstrated that the incidence of p53 alteration increased from pleomorphic adenoma to carcinoma arising in pleomorphic adenoma.

The results showed the progressive alteration of p53 expression in pleomorphic adenoma, in a progression to carcinoma arising in PA. It is likely that transcription of a mutated protein that accumulated in the nucleus and was

readily detected immunohistochemically accounted for the majority of these cases.

4.4. The Interpretation of the Variations in the Detection of p53 Staining. This differences in the expression of p53 in our study and the others mentioned above may have resulted from the following reasons.

- (1) The use of different antibodies.
- (2) Different classifications, for example, (0 = negative staining, 1 = low, 2 = moderate, 3 = strong or 0–3 = negative and 4 = positive or 0–2 = negative, and 3–4 = positive or negative and positive staining).
- (3) Fixation times and concentrations of antibodies.
- (4) The sensitivity of the technique used.

Mutation is usually increased as cells progress from benign tumor to carcinoma. It was surprising that the results showed that the proportion of altered p53 in pleomorphic adenomas was higher than in carcinoma cases after combining moderate and strong staining together, and negative and low staining together. If only strong positive staining was used as indicator for the alteration in the expression of tumor suppressor protein p53, then alteration in expression of p53 was increased in pleomorphic adenoma compared to carcinoma arising in pleomorphic adenoma.

Immunostaining technique has been used only to detect the alteration of expression of p53 in our study. This technique is an easy method to carry out, but the assessment of the positive or negative nuclear staining cells is controversial. Many authors used different criteria, so the results cannot be compared. In the present study, the use of negative and positive staining for the assessment of staining avoided any confusion in the interpretation of the results. Ideally the immunostaining technique is used only combined with another technique for example, (Polymerase Chain Reaction, Western Blotting) to detect and confirm existence of a mutation. Many studies used criteria such as negative, low, moderate, and strong staining.

4.5. Expression of p53 in the Tumor Duct Cells and Myxochondroid Tissue in Pleomorphic Adenomas. Wilcoxon's test showed a significant difference between p53 expression of the nuclear staining in myxochondroid and tumor duct cells, (P value $< .001$).

p53 expression in pleomorphic adenoma showed that the incidence of aberrant expression of these proteins was higher in tumor duct cells than in myxochondroid tissue. Although p53 showed alteration in expression in myxochondroid tissue, there is evidence that cells in myxochondroid tissue show low levels of proliferation [23].

4.6. Comparative Statistical Analysis of p53 in Pleomorphic Adenoma and Carcinoma Arising in Pleomorphic Adenoma. The Mann Whitney test showed no significant difference between p53 expression in pleomorphic adenoma (tumor duct cells) and carcinoma arising in PA (P value $> .05$).

Unfortunately, p53 cannot be used as indicator to differentiate between pleomorphic adenomas and carcinoma arising in PA.

5. Conclusion

The conclusion can be summarised in the following points.

- (i) The sample of carcinoma arising in pleomorphic adenoma cases is large (27 cases) compared with others, though further research is required to increase the sample size to determine the role of tumor suppressor proteins in the pathogenesis of malignant transformation of pleomorphic adenoma.
- (ii) p53 was altered in PA and with increased frequency in CPA.
- (iii) The use of one criterion as positive and negative nuclear staining to assess the expression of tumor suppressor proteins appears to give more convincing results than the use of different categories such as negative, low, moderate, and strong staining.
- (iv) The tumor suppressor protein p53 shows aberrant expression in tumor duct cells more frequently than cells in myxochondroid tissue, consistent with the concept that the latter may not be the stem tumor cells responsible for malignant progressions.
- (v) Expression of p53 is altered in PA and CPA. Further research to extract DNA from the studied cases to detect mutations as a probable main cause of inactivation and to identify other causes of inactivation such as methylation or loss of heterozygosity is recommended.

Abbreviations

PA: Pleomorphic salivary adenoma
CPA: Carcinoma ex pleomorphic adenoma.

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