

Immunohistochemical expression of p21 in normal tissues of salivary gland, pleomorphic adenoma and carcinoma ex pleomorphic adenoma- (undifferentiated and adenocarcinoma types)

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Abstract

Objective: Our study aimed to characterize alteration in the immunohistochemical expression of p21 in normal tissue of the salivary gland surrounding pleomorphic adenoma, the tumor cells of pleomorphic adenomas, and carcinoma arising in pleomorphic adenoma.

Study design: A selected series of 29 cases of pleomorphic adenomas, and 27 cases of carcinoma ex-pleomorphic adenoma (undifferentiated and adenocarcinoma types) were examined.

Results: The results showed that p21 expression was negative in the most components of normal tissue of the salivary gland surrounding pleomorphic adenoma, 24 cases out of 29 of the non tumour duct cells (82.8%), and 28 (96.6%) cases out of 29 of the acinar cells shows negative p21 expression. P21 expression in pleomorphic adenomas shows that 2 cases out of 29 (6.9%) strongly expressed in the duct cells. p21 was strongly expressed in carcinoma cells in 9 (33.3%) cases out of 27.

Conclusion: Our data suggest that the strong nuclear staining as an indicator for altered p21, then the alteration of p21 expression would increase from pleomorphic adenoma to carcinoma arising in pleomorphic adenomas (6.9% versus 33.3%).

Key words: p21 expression, pleomorphic salivary adenoma, carcinoma ex pleomorphic adenoma.

Introduction

p21 is a cell cycle regulatory protein encoded by the p21 WAF 1 gene which is located on chromosome 6p21.2, (1-2). It believed that the inhibitory effects on cell division by p21 are related to cancerous change of normal cells, regulation, cell differentiation and induction of apoptosis involving p53 and Rb (3). P21 plays an important role in arresting the cell cycle. Loss of function of p21 may favour tumour growth. Pleomorphic salivary adenoma (PSA), the most frequent tumour in salivary glands, can undergo malignant transformation. Carcinoma ex pleomorphic adenoma (CXPA) is more frequent in the 5-6th decades of life (4-5). It has been suggested that a carcinoma may develop within a recurrent PSA or when it remains untreated over an extended period of time (6-7). Despite the recognized clinical importance

of CXPA, little is known about its biology, therefore the diagnosis of CXPA is a challenge for pathologists. The purpose of this study was to determine, with the use of immunohistochemistry, to characterize alteration in the immunohistochemical expression of p21 in normal tissue of the salivary gland surrounding pleomorphic adenoma, the tumor cells of pleomorphic adenomas, and carcinoma arising in pleomorphic adenoma.

Materials and Methods

Case selection

A selected series tissue 29 cases of pleomorphic adenomas, and 27 cases of carcinoma ex-pleomorphic adenoma were retrieved from the archive of the oral pathology departments in the North West region of England (Table 1). Normal tissue of the salivary gland surrounding the

Table 1. Clinical data of 29 pleomorphic adenomas cases (PA) and 27 carcinomas ex-pleomorphic adenomas cases (CXPA).

P A Cases	Age	Gender	Gland type	CXPA Cases	Age	Gender	Gland	Histological sub-type	Metastasis to lymph nodes*
1	87	F	Parotid gland	1	77	F	Parotid	Adenocarcinoma	Yes
2	52	M	Parotid gland	2	28	M	Parotid	Adenocarcinoma	No
3	63	M	Parotid gland	3	78	M	Submandibular	Undifferentiated	Yes
4	48	F	Parotid gland	4	45	M	Parotid	Undifferentiated	Yes
5	76	F	Parotid gland	5	76	F	Parotid	Undifferentiated	No
6	47	M	Parotid gland	6	82	F	Parotid	Undifferentiated	No
7	62	F	Parotid gland	7	71	M	Parotid	Adenocarcinoma	No
8	33	M	Parotid gland	8	67	M	Submandibular	Undifferentiated	Yes
9	49	F	Parotid gland	9	63	M	Submandibular	Undifferentiated	Yes
10	45	M	Parotid gland	10	55	M	Submandibular	Undifferentiated	Yes
11	63	F	Parotid gland	11	73	M	Parotid	Undifferentiated	Yes
12	53	F	Parotid gland	12	71	M	Parotid	Undifferentiated	No
13	27	F	Parotid gland	13	64	M	Parotid	Undifferentiated	Yes
14	59	F	Parotid gland	14	60	F	Parotid	Undifferentiated	Yes
15	33	F	Parotid gland	15	49	F	Submandibular	Undifferentiated	No
16	55	F	Parotid gland	16	39	F	Parotid	Undifferentiated	Yes
17	26	F	Parotid gland	17	56	M	Parotid	Undifferentiated	No
18	65	F	Parotid gland	18	45	F	Parotid	Undifferentiated	Yes
19	40	M	Parotid gland	19	57	M	Parotid	Undifferentiated	Yes
20	57	M	Parotid gland	20	66	F	Parotid	Undifferentiated	No
21	34	M	Parotid gland	21	86	F	Submandibular	Undifferentiated	Yes
22	74	F	Parotid gland	22	17	F	Parotid	Undifferentiated	No
23	67	F	Parotid gland	23	78	M	Submandibular	Undifferentiated	Yes
24	32	M	Parotid gland	24	26	M	Parotid	Undifferentiated	No
25	31	M	Parotid gland	25	31	F	Parotid	Undifferentiated	No
26	62	F	Parotid gland	26	71	M	Parotid	Undifferentiated	No
27	76	F	Parotid gland	27	71	M	Parotid	Undifferentiated	No
28	21	F	Parotid gland						
29	61	F	Parotid gland						

F: female M: male, * Metastasis to lymph nodes at the time of tumour resection.

tumour was used as a control in 29 cases of pleomorphic adenoma. The immunohistochemical expression of antibodies against p21 was examined in the selected cases. 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.

Inclusion criteria for carcinoma ex-pleomorphic adenoma:

- Major gland primary lesion (parotid or submandibular)
- Co-existent benign and malignant elements

Benign element can be:

- Pleomorphic adenoma within the tumor mass
- Biopsy proven history of previous PSA (pleomorphic salivary adenoma) indicated that it was in the same location as the subsequent carcinoma.

Malignant elements can be:

- Undifferentiated carcinoma
- Adenocarcinoma
- Multiple patterns of differentiation including undifferentiated or adenocarcinoma patterns

Exclusion criteria for carcinoma ex-pleomorphic adenoma includes any other type of tumor.

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 local NHS Research Ethics Committee provided a favourable ethical opinion (Ref: 02/104).

Immunohistochemistry

Paraffin-embedded, 4- μ m-thick tissue sections from all 56 specimens were cut. The sections were deparaffinized in xylene and rehydrated through graded alcohols. Sections were processed used streptavidin-biotin-peroxidase method. Briefly, the endogenous peroxidase was blocked by 3% hydrogen peroxidase for 5 min followed by TBS wash. Nonspecific immunoreactivity was blocked by incubation with normal goat serum for 20 minutes. A purified mouse anti-human monoclonal antibody p21 (PharMingen, San Diego) was diluted to 10 μ /ml in 20 μ /ml tris buffer saline containing 0.1% bovine serum albumin for one hour and fifteen minutes at the 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 chromog-

enic 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 the primary antibody with TBS (Fig. 1a). The percentage of P21 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.

Statistical analysis

Cells of the carcinomatous component of the CXPA were always scored. The statistical analysis included the use of descriptive statistics; frequencies proportion and crossed tabulation. Also, statistical 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 0.05 were considered to be statistically significant.

Results

p21 expression in the control group (normal tissue) surrounding the pleomorphic adenoma

Of the 29 cases, p21 expression of the non tumour duct cells was negative in 24 (82.8%), and weak in 5 cases (17.2%) cases with weak staining. P21 (nuclear staining of the acinar cells) showed negative staining in 28 (96.6%) cases out of 29, 1 (3.4%) case expressed weak staining (Fig. 1b). P21 (nuclear staining in stroma) was negative in 29 (100%) cases out of 29.

P21 expression in pleomorphic adenoma components p21 nucleus staining was moderate in tumour duct cells in 13 (44.8%) cases out of 29 (Fig. 1c), 13 (44.8%) cases showed weak staining (Fig. 1d), 1 (3.4%) case negative staining, and 2 cases (6.9%) strong staining (Table 2), (Fig. 1e). p21 nuclear staining in myxochondroid tissue was identified in 20 (69.0%) cases with weak staining, 4 (13.8%) cases with moderate staining, and 5 (17.2%) cases with negative staining (Table 2). Also the cross-tabulation table showed p21 expression in tumour duct cells and myxochondroid tissue (Table 2).

Comparison between staining of different components in pleomorphic adenoma and the adjacent tissue around this tumour

Wilcoxon test showed a significant difference (p value < 0.001) between p 21 expression in the nuclei of duct cells in normal tissue surrounding the tumour and the tumour area. The non tumour duct cells showed 24 cases with p21 negative staining, whereas there were 5 cases with weak staining but in the tumour duct cells, 1 case showed negative staining, 13 weak staining, 13 moderate

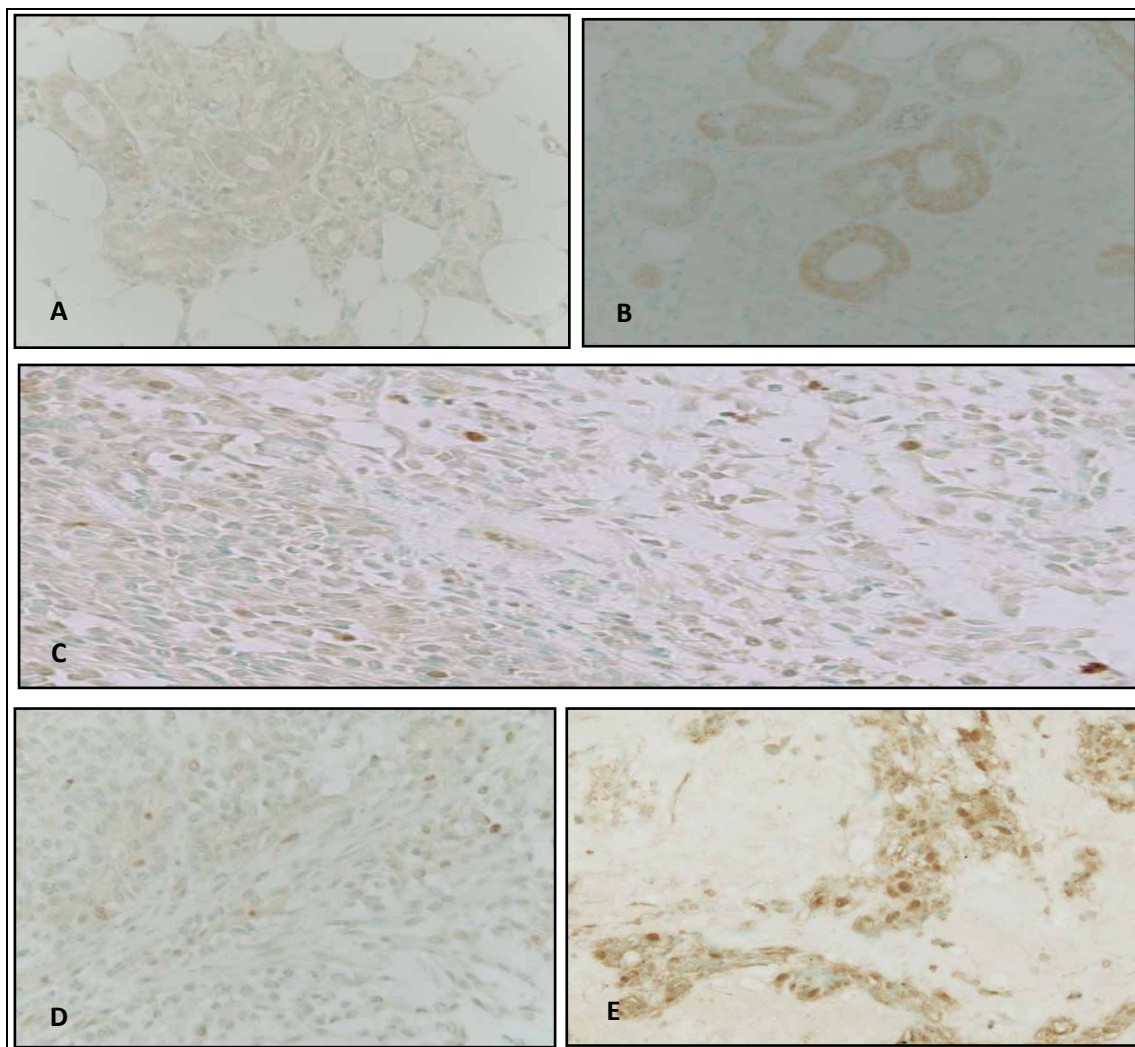


Fig. 1. Showing nuclear staining of p21 in normal tissue of parotid gland and pleomorphic adenoma components (Original magnification x40), A showing p21 negative control, B showing negative nuclear staining of p21 in ductal and acinar cells in parotid gland, C showing moderate nuclear staining of p21 in pleomorphic salivary adenoma, D showing low nuclear staining of p21 in pleomorphic salivary adenoma, E strong nuclear staining of p21 in pleomorphic salivary adenoma.

staining, and 2 cases strong staining. We conclude that there was a greater alteration of p21 expression in the tumour duct cells compared with non tumour duct cells. The Wilcoxon test identified a significant difference (p value = 0.002) between p21 expression in the nuclear staining in myxochondroid and tumour duct cells because the tumour duct cells showed negative staining in 1 case, 13 showed weak staining, 13 moderate staining, and 2 strong staining but in the myxochondroid tissue, 5 cases had negative staining, 20 weak staining, and 4 expressed moderate staining (Table 2). This means that the incidence of overexpressed p21 in tumour duct cells was higher than in tumour myxochondroid tissue.

p21 expression in carcinoma arising in pleomorphic adenoma

p21 was strongly expressed in carcinoma cells in 9 (33.3%) cases out of 27 (Fig. 2a). 5 (18.5%) cases showed moderate staining (Fig. 2b), and 5 (18.5%) cases expressed low staining (Fig. 2c), 8 cases (29.6%) negative staining.

Comparison between p21 expression in pleomorphic adenoma and carcinoma arising in pleomorphic adenoma

The Mann Whitney test showed no significant difference between p21 expression in pleomorphic adenoma (tumour duct cells) and carcinoma arising in PSA (p value 0.939 > 0.05). p21 nuclear staining was moderate in tumour duct cells (pleomorphic adenoma) in 13 (44.8%) cases out of 29, 13 (44.8%) cases showed weak staining, 1 (3.4%) case negative staining, and 2 cases (6.9%) strong staining but p21 in carcinoma cells

Table 2. Crosstabulation table shows comparison between p21 expression in myxochondroid tissue and tumour duct cells in pleomorphic adenoma.

	P21 in duct cells				Total
	Negative Staining	Weak staining	Moderate staining	Strong staining	
P21 expression in Myxochondroid					
Negative staining	0	4	1	0	5
Weak staining	1	7	10	2	20
Moderate staining	0	2	2	0	4
Strong staining	0	0	0	0	0
Total	1	13	13	2	29

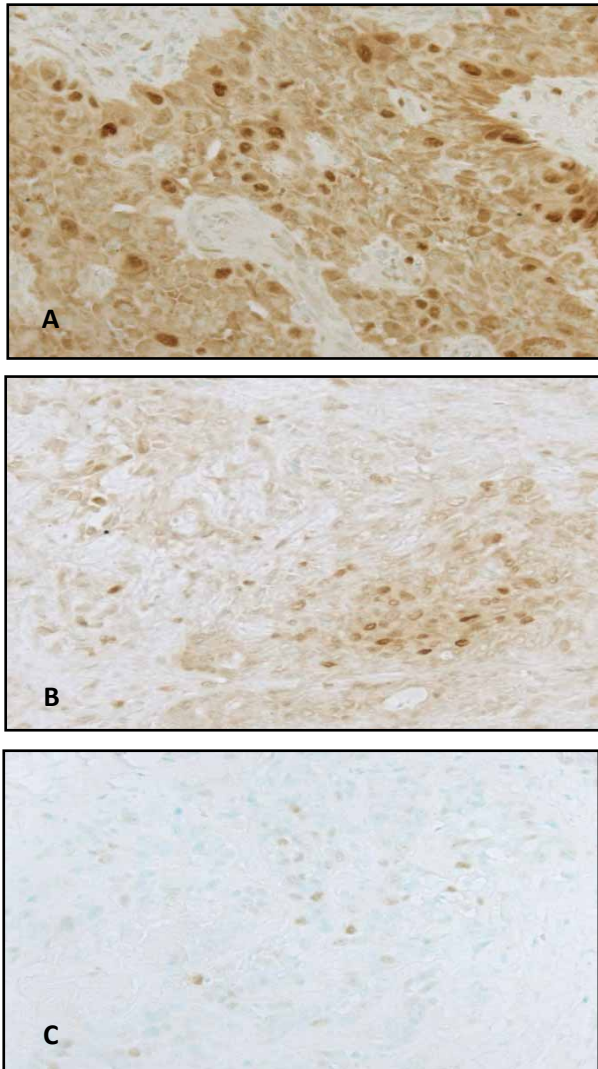


Fig. 2. Showing nuclear staining of p21 in carcinomas ex-pleomorphic adenomas, (Original magnification x40), A showing strong nuclear staining of p21 in CXPA. B showing moderate nuclear staining of p21 in CXPA, C showing low nuclear staining of p21 in CXPA.

showed 9 (33,3%) strong staining, 5 cases moderate staining (18,5%), 5 cases (18,5%) low staining, 8 cases (29,6%) negative staining. These results showed that mutation of p21 increased from pleomorphic adenoma to carcinoma arising in pleomorphic adenoma.

Discussion

p21 belongs to a class of tumour suppressors including p16 and p27, which control progression through the cell cycle by inhibiting the activity of cyclin-cdk complexes.

No study has been published regarding p21 expression in salivary glands and particularly in carcinoma arising in pleomorphic adenoma.

P21 and normal tissue of salivary glands

In the normal salivary glands (control group) p21 was negative in the non tumour duct cells or the acini. These results are consistent with Rey et al. (9) who studied p21 expression in breast cancer. The normal ducts and acinar cells did not express p21 and the same negative p21 nuclear staining was observed in the five non-neoplastic cases.

Clasen et al. (10) analysed the expression of p21 in urothelial carcinoma. Their results revealed that no p21 was expressed in normal mucosa. Jung et al. (11) analysed the expression of p21 in normal and reactive brain in gliomas of various malignancy grades. Immunohistochemical assay showed that the p21 detected in normal and reactive brain tissue with at very low expression.

Expression of p21 in the tumour duct cells and myxochondroid tissue in pleomorphic adenomas

Wilcoxon test showed a significant difference between p21 expression of the nuclear staining in myxochondroid and tumour duct cells, (p value <0.001).

p21 expression in pleomorphic adenoma showed that the incidence of aberrant expression of this protein was higher in tumour duct cells than in myxochondroid tissue. Although p21 showed alteration in expression in

myxochondroid tissue, there is evidence that cells in myxochondroid tissue show low levels of proliferation Zhu et al. (12).

P21, pleomorphic adenoma, and carcinoma arising in pleomorphic adenoma

It was considered that p21 strong and moderate nuclear staining was positive, and an indicator for altered p21 in pleomorphic adenoma and carcinoma arising in PSA. Low and negative nuclear staining was classified as negative, and indicated wild type (non altered p21). P21 showed positive nuclear staining (moderate and strong) in 15 (51.7%) out of 29 cases in tumour duct cells of pleomorphic adenoma. In carcinoma arising in pleomorphic adenoma, p21 staining was positive in 14 (51.8%) cases out of 27.

De lima et al. (13) reported that no expression of p21 was found in adenoid cystic carcinomas. Marques et al. (14) reported that p21 expression was considered absent in pleomorphic adenomas and myoepitheliomas. Harada et al. (3) reported that p21 expression was increased in non small lung carcinoma, hepato cellular carcinoma, and head and neck cancers. Rey et al. (9) studied p21 expression in breast cancer. Expression of p21 (using anti p21 clone, EA10 from Oncogene Science) was observed in preinvasive lesions, and in infiltrating carcinomas (57%). P21 expression was associated with high histological grade (II-III). P21 positivity correlated with increased proliferation. Jung et al. (11) used an immunohistochemical assay and demonstrated that p21 (using mAb obtained from Oncogene Science) was positive in both astrocytoma (low grade) and glioblastoma (high grade) in the tested paraffin blocks of brain tumours. Ogawa et al. (15) observed that loss of p21 expression correlated with disease progression in gastric carcinoma. Brunotto et al. (16) observed that the Ck14, p53, p21 and Bcl-2 proteins were found modified in the leukoplakia, oral lichen planus and squamous cell carcinoma.

P21 strong nuclear staining as a strong indicator for altered p21

Using strong nuclear staining as a indicator for altered p21, the proportion of p21 positive tissues would be 6.9 % in pleomorphic adenoma cases. In my sample, only 2 cases out of 29 showed strong positive staining in the tumour duct cells of pleomorphic adenomas. In carcinoma arising in PSA, 9 (33.3%) cases out of 27 showed positive strong staining.

The interpretation of the variations in the detection of p21 staining

This differences may have resulted from the following reasons:

1. The use of different antibodies
2. Different classifications e.g (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 tumour to carcinoma. It was surprising that the results showed that the proportion of altered p21 in pleomorphic adenomas was comparable with in carcinoma cases, 15 (51.7%) out of 29 cases versus 14 (51.8%) cases out of 27, after combining moderate and strong staining together, and negative and low staining together. If only strong positive staining (p21) was used as indicator for the alteration in the expression of tumour suppressor protein, then alteration in expression of p21 was detected in two cases out of 29 (6.9%) in pleomorphic adenoma and increased in carcinoma arising in pleomorphic adenoma in nine cases out of 27 (33.3%).

Immunostaining 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 e.g (Ploymerase Chain Reaction, Western Blotting) to detect and confirm existence of a mutation. Many studies used criteria such as negative, low, moderate, and strong staining. This study comprised 29 cases of pleomorphic adenoma and 27 cases of carcinoma ex pleomorphic adenoma, although the sample size is limited but it is bigger compared to other published studies. We have concluded that the alteration of p21 expression has increased from pleomorphic adenoma to carcinoma arising in pleomorphic adenoma depending on use the p21 strong nuclear staining as a strong indicator for altered p21.

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