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Immunohistochemical expression of pRb in pleomorphic adenoma and carcinoma ex pleomorphic adenoma

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Abstract

Objective: This study aimed to characterize alteration in the immunohistochemical expression of pRb 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 pRb expression was negative in the components of normal tissue of the salivary gland surrounding pleomorphic adenoma. PRb expression in pleomorphic adenomas shows that 2 cases out of 29 (6.9 %) strongly expressed in the duct cells, 7/29 (24.1 %) cases showed moderate staining. pRb nuclear staining in myxochondroid was identified in 10/29 (34.5 %) cases of pleomorphic adenomas with weak staining, 6/29 (20.7 %) with moderate staining. pRb was strongly expressed in carcinoma cells in 19 out of 27 cases (70.4 %).

Conclusion: This study suggests the alteration of pRb expression would increase from pleomorphic adenoma to carcinoma arising in pleomorphic adenomas (6.9 % versus 70.4 %).

Key words: *pRb expression, pleomorphic adenoma; carcinoma ex pleomorphic adenoma.*

Introduction

Many human tumours contain abnormalities in one or more of the genes responsible for regulating cell cycle progression. The retinoblastoma protein (pRb) is one of the key cell-cycle regulating proteins and its inactivation leads to neoplastic transformation and carcinogenesis (1). This protein regulates critical G1-to-S phase transition through interaction with the E2F family of cell-cycle transcription factors repressing transcription of genes required for this cell-cycle check-point transition. Its activity is regulated through network sensing intracellular and extracellular signals which block or permit phosphorylation (inactivation) of the Rb protein. The product of the RB gene is pRb, its function is to prevent the cell from entering S phase, whereas phosphorylated or mutated forms of this protein are incapable of arresting the cell in G1 phase of the cell cycle (1-3).

Phosphorylation of pRB is mediated by complexes comprised of a D-type cyclin and cyclin dependent protein kinases (CDK4)/ (CDK6). The activity of these kinases is in turn negatively regulated by cyclin kinase inhibitors including p16. The cascade composed of pRB, cyc-

lin D1,CDK4/CDK6 and p16 plays a central role in cell cycle control. p16 controls cell proliferation through maintenance of a hypophosphorylated state of pRb. The loss of p16 function by gene deletion, methylation and mutation within the reading frame have been found in various cancers (4,5).

Pleomorphic adenoma (PA) is the most common neoplasm of salivary glands (6) and has shown sometimes tendency to undergo malignant transformation in its natural course (7). Carcinoma ex-pleomorphic adenoma (CXPA) is considered to be a malignant transformation of pre-existing pleomorphic adenoma (8). Carcinoma ex-pleomorphic adenoma has been estimated to account 10% of all salivary gland malignancy (9). 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 (10). 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 the alterations in the immunohistochemical expression of pRb 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 of 29 cases of pleomorphic adenomas, and 27 cases of carcinoma ex-pleomorphic adenoma were retrieved from the files of two Oral Pathology Departments in Liverpool, and Manchester Dental School (Table 1). Normal tissue of the salivary gland surrounding the tumour was used as a control in the 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. (11) were used to select and reclassify our cases of carcinoma ex-pleomorphic adenoma.

According to the World Health Organization histological clasification published in 2005, malignant changes in the PA include three different types: CXPA, carcinosarcoma, and metastasizing PA The Inclusion criteria for carcinoma ex-pleomorphic adenoma compromised 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. Also the co-existent benign and malignant elements are considered as well. 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. Malignant elements can be undifferentiated carcinoma, adenocarcinoma, and multiple patterns of differentiation including undifferentiated or adenocarcinoma patterns.

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 (12).

Microscopic slides stained with hematoxylin and eosin were reviewed by two pathologists to confirm the histopathological diagnosis and to reclassify the studied cases. The carcinoma cases were classified according to the above mentioned criteria as undifferentiated carcinoma or adenocarcinoma. The ethical approval was provided from research ethics committee (Ref: 02/104).

Immunohistochemistry

Paraffin-embedded tumor samples stored in pathology laboratory files were used in this study. A serial 4-μm- sections were consecutively cut from all 56 specimens. 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 (Tris buffered saline) wash. Nonspecific immunoreactivity was blocked by incubation with normal goat serum for 20 minutes. A primary mouse anti-human retinoblastoma protein RB (Pharmingen, San Diego) was diluted to 5 μ/ml in 10 μ/ml tris buffer saline (TSA) containing 0.1 % bovine serum albumin for 1 hour 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 and incubated with a prepared chromogenic substrate solution (Diaminobenizidine) 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 pRb positive nuclei was semi-quantively 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 PA, and carcinomatous component of the CXPA

Table 1. Clinical data of 29 pleomorphic adenomas cases (PA) and 27 carcinomas ex-pleomorphic adenomas cases (CXPA) from Oral Pathology Departments in Liverpool, and Manchester Dental schools. F: female, M: male, * Metastasis to lymph nodes at the time of tumour resection.

P A Cases	Age	Gender	Gland type		CXPA Cases	Age	Gender	Gland	Histological subtype	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							

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 (ordinal data), 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

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

The results of nuclear staining of duct cells, acinar cells, and stroma indicated that pRb was not expressed in any of the 29 (Fig. 1b).

pRb expression in pleomorphic adenoma cell components

pRb weak nuclear staining was noted in duct cells in 12/29 (41.4 %) cases (Fig. 1c), 7/29 (24.1 %) cases showed moderate staining (Fig. 1d), 8/29 (27.6 %) had negative staining, and 2/29 cases (6.9 %) exhibited strong staining (Fig.1e). pRb nuclear staining in myxochondroid was identified in 10/29 (34.5 %) cases with weak staining, 6/29 (20.7 %) with moderate staining, 13/29 (44.8 %) cases with negative staining. The crosstabulation table showed pRb expression in tumour duct cells and myxochondroid tissue (Table 2).

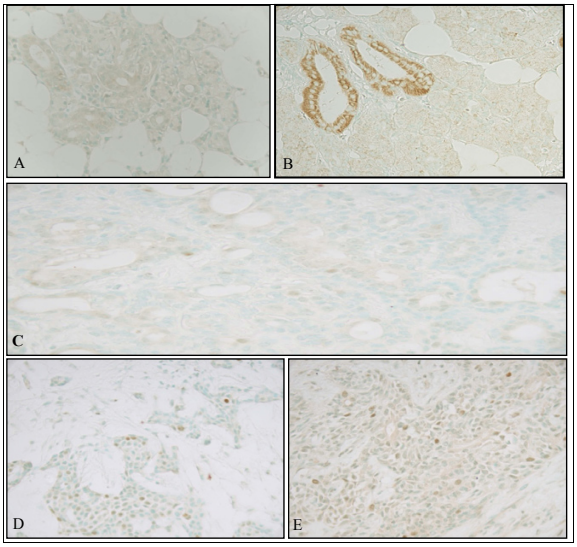

Fig. 1. Showing nuclear staining of pRb in normal tissue of parotid gland and pleomorphic adenoma components (Original magnification x40).
A showing pRb negative control.
B showing positive cytoplasmic and negative nuclear staining of pRb in ductal cells in parotid gland.
C showing low nuclear staining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

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

	pRb in tumour duct cells				
	Negative staining	Weak staining	Moderate staining	Strong staining	Total
pRb expression in Myxochondroid					
Negative staining	7	5	1		13
Weak staining	1	5	4		10
Moderate staining		2	2	2	6
Total	8	12	7	2	29

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

The Wilcoxon test was used to show that there was a significant difference ($p < 0.001$) between pRb expression in both duct cells of normal tissue surrounding the tumour and the tumour area itself. In normal tissue, the duct cells were pRb negative in 29 cases but in tumour duct cells, 8 cases showed negative staining, 12 expressed weak staining, 7 moderate staining, and 2 had strong staining. Wilcoxon test showed a significant difference ($p = 0.01$) between pRb expression in the nucleus in myxochondroid and tumour duct cells because pRb in tumour duct cells showed strong staining in 2 cases, 8 negative staining, 7 moderate staining, 12 weak staining but in myxochondroid tissue, pRb was not expressed in 13 cases, 6 had moderate staining, and 10 showed weak staining.

pRb expression in carcinoma arising in pleomorphic adenoma

pRb showed negative staining in 5 cases (18.5 %), 1 case had low staining (3.7 %) (Fig. 2a), 2 moderate staining (7.4 %) (Fig. 2b), 19 cases strong staining (70.4 %) (Fig. 2c).

Statistical significant differences between pRb expression in pleomorphic adenoma and carcinoma arising in pleomorphic adenoma

Mann Whitney test showed significant difference between pRb expression in pleomorphic adenoma (tumour duct cells) and carcinoma arising in PA (p value <0.001). 2 cases out of 29 showed pRb strong staining in tumour duct cells in pleomorphic adenoma but 19 cases showed the same expression in carcinoma cells.

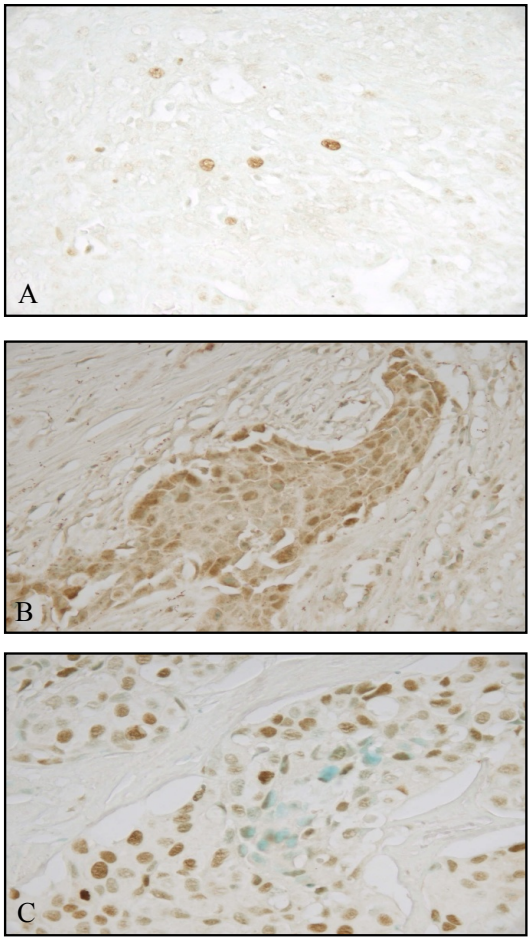

Fig. 2. Showing nuclear staining of pRb in carcinomas ex-pleomorphic adenomas
(Original magnification x40).
A showing low nuclear staining of pRb in CXPA.
B showing moderate nuclear staining of pRb in CXPA.
C showing strong nuclear staining of pRb in CXPA.

Discussion

pRb expression and normal tissue of salivary glands

The results failed to identify pRb expressed in normal salivary glands. Shintani et al. (13) indicated that pRb was expressed in myoepithelial and duct cells of the normal salivary glands but not in the acinar cells. Etges et al. (14) have indicated that pRb was abundant in normal salivary gland samples. Weber et al. (15) reported that pRb alteration was not detected in parotid tissue surrounding pleomorphic adenoma samples. Russo et al. (16) have indicated that pRb2/p130 showed a clear nuclear immunoreactivity in normal salivary glands.

pRb expression was not detected in normal salivary glands, this may be due to the poor sensitivity of the technique used (e.g antibody used, antibody concentration and other factors may all be important). pRb positive expression indicated existing altered pRb.

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

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

pRb expression in pleomorphic adenoma showed that the incidence of aberrant expression of these proteins was higher in tumour duct cells than in myxochondroid tissue. Although pRb showed alteration in expression in myxochondroid tissue, there is evidence that cells in myxochondroid tissue show low levels of proliferation (17). Zhu et al. (17) indicated that PCNA and MIB-1 indices are reliable markers for discriminating between benign and malignant tumors of the parotid gland, and the parameters PI, MI, NPI and NMI may have prognostic applications.

Also it may be concluded that pRb is overexpressed in some cases of pleomorphic adenoma as positive nuclear staining was detected at a level of immunocytochemical sensitivity too low to detect wild type normal expression. The overexpression may be due to mutation or up regulation by another mechanism.

pRb, pleomorphic adenoma and carcinoma arising in pleomorphic adenoma

pRb positive is used as an indicator of overexpression pRb but pRb negative is used as indicator for existing wild type pRb (non altered pRb).

In pleomorphic adenoma, pRb positive nuclear staining was noted in tumour duct cells in 9/29 (31 %) cases. 20/29 (69 %) cases showed negative staining.

In carcinoma arising in pleomorphic adenoma, pRb showed negative staining in 6/27 cases (22.2 %), and 21/27 (77.8 %) cases expressed positive staining.

Mann Whitney test showed a significant difference between pRb expression in pleomorphic adenoma (tumour duct cells) and carcinoma arising in PA ($p < 0.001$). 2 cases out of 29 showed pRb strong staining in tumour duct cells in pleomorphic adenoma but 19 cases out of 27 showed a strong positive expression in carcinoma cells. The sample size of this study is limited but these results indicated that pRb can used to differentiate between pleomorphic adenoma and carcinoma arising in pleomorphic adenoma. Etges et al. (14) reported that pRb expression was absent in three cases of pleomorphic adenoma, two of epithelial myoepithelial carcinoma and one of carcinoma ex pleomorphic adenoma. They concluded that pRb pathway deregulation in salivary gland neoplasms is unrelated to their biological behavior, Weber et al. (15) reported that pRb was detected immunohistochemically in 40/42 pleomoprphic adenomas. They indicated that alterations of p14 (ARF) and p16 (INK4a), and also p53 mutations, occurred exclusively in the epithelial and transitional components of pleomorphic adenoma supports the theory that these areas are prone to malignant transformation to carcinoma in adenoma. Shintani et al. (13) reported that alterna-

tions of Rb pathway were infrequent events in adenoid cystic carcinoma of salivary glands and inactivation of p16INK4A, cyclin D1 overexpression may be related to the high cell proliferating activity of . adenoid cystic carcinoma of salivary glands.

Liu et al. (18) investigated the expression of Rb pathway-related proteins in salivary gland acinic cell carcinoma, including Rb, Rb proteins phosphorylated at serine 780 and 795 (pRb-S780 and pRb-S795, respectively. Their results indicated that serine 795 but not serine 780 is the preferred phosphorylation site induced by cyclin D1. This phosphorylation appeared to be critical for inactivation of Rb-mediated growth suppression and may play an important role in the pathogenesis of acinic cell carcinoma of salivary gland.

Pavelic et al. (19) reported the loss of pRb in oral cavity tumours. The study included 182 patients with primary squamous cell carcinoma, 75 diagnosed as T1, and 107 as T3 and T4. Normal tissues adjacent to carcinoma area were used as a control in 55 cases. Normal cells including endothelial cells, stromal cells, and lymphocytes were shown to have pRb positive nuclear staining. 9/75 (7 %) T1 and 40/107 (37 %) T3 and T4 lesions appeared to have negative pRb staining. Their results indicated that a high rate of loss pRb expression was identified in advanced oral tumours and suggested that tumours in which pRb expression was reduced were more aggressive than tumours in which the pRb protein was presented. do Prado et al. (20) reported that the loss of beta-catenin adhesion molecule in the development of pleomorphic adenoma, and that the cytoplasmic accumulation of the molecule takes part in the malignant transformation of pleomorphic adenoma into carcinoma in pleomorphic adenoma. Tarakji et al. (21) reported that p21 expression in pleomorphic adenomas shows that 2 cases out of 29 (6.9%) strongly expressed in the duct cells but p21 was strongly expressed in carcinoma cells in 9 (33.3%) cases out of 27. They concluded that the alteration of p21 expression would increase from pleomorphic adenoma to carcinoma arising in pleomorphic adenomas (6.9% versus 33.3%). Tarakji et al. (22) reported that 27 (100 %) of 27 cases had negative nuclear staining for either estrogens or progesterone receptors. They concluded that carcinomas arising in pleomorphic adenoma were not dependent on endocrine function.

pRb nuclear strong staining as indicator for existing aberration pRb

Strong pRb staining was used to indicate aberrant expression of pRb, which was present in 2 cases (6.8 %) of pleomorphic adenoma, and 19 cases (70.4 %) of carcinoma arising in PA.

The interpretation of the variations in the detection of pRb staining

This differences may have resulted from the following reasons: The use of different antibodies, 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), fixation times and concentrations of antibodies, and the sensitivity of the technique used.

Mutation is usually increased as cells progress from benign tumour to carcinoma. If only strong positive staining (pRb) was used as indicator for the alteration in the expression of tumour suppressor protein, then alteration in expression of pRb was detected in 2 cases out of 29 showed pRb strong staining in tumour duct cells in pleomorphic adenoma increased to 19 cases out of 27 showed a strong positive expression in carcinoma cells. 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. The immunostaining technique is used only combined with another technique e.g (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. 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 much bigger compared to other published studies. The conclusion of this study that the alteration of pRb expression has increased from pleomorphic adenoma to carcinoma arising in pleomorphic adenoma depending on use the pRb strong nuclear staining as a strong indicator for altered pRb.

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