Original

**Immunohistochemical Features of an Atypical Pleomorphic Adenoma of the Salivary Gland - Overexpression of Ki-67 and p53**

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**Abstract**: Atypical pleomorphic adenoma (APA) is a premalignant condition of pleomorphic adenoma (PA), and APA with hypercellularity (h-APA) is seldom positive for HER2/neu. Whether h-APA cells show higher mitotic activity and oncogenic mutations remains unclear. Thirteen cases of h-APA, 10 cases of carcinoma (Ca)-ex-PA, and 19 cases of PA were immunohistochemically examined for overexpression of the proliferation marker protein, Ki-67 and the tumor suppressor protein, p53. Ki-67 expression is higher in Ca-ex-PA than in other groups, and it is also higher in h-APA than in PA. p53 expression is also higher in Ca-ex-PA than in other groups, but there is no difference between h-APA and PA. We hypothesize the sequence of events during carcinogenesis of PA as follows: first there is an increase in proliferation in PA, which leads to h-APA, secondly, there is an amplification of the HER2/neu oncogene in h-APA and finally, p53 overexpression occurs in h-APA and leads to Ca-ex-PA. This hypothesis explains the increase in both patients’ age and tumor size in each group, in the sequence from PA to Ca-ex-PA via h-APA.

**Key words**: salivary gland tumor, atypical pleomorphic adenoma, Ki-67, p53

Introduction

The concept of atypical pleomorphic adenoma (APA), including a description of its properties including, hypercellularity, capsule violation, hyalinization, necrosis and cellular anaplasia, as a premalignant condition of pleomorphic adenoma (PA) has been presented by Auclair & Ellis. Recently we used immunohistochemical staining to show that the HER2/neu oncogene, which is an important marker for carcinoma (Ca)-ex-PA, appears in 40% of cases of APA with hypercellularity (h-APA). Therefore, h-APA indicates a greater potential for malignant transformation, but whether h-APA cells have higher...
mitotic activity and oncogenic mutations as a carcinoma remains unclear. In this study, we examined additional cases and used immunohistochemical staining for the presence of the proliferation marker protein, Ki-67 and the tumor suppressor protein, p53 in APA cells, in comparison with Ca-ex-PA and PA.

**Materials and Methods**

**Tissues**

Thirteen cases of h-APA (Fig. 1a, b) and 10 cases of Ca-ex-PA (Fig. 2a) were retrieved from our stored records of resected salivary gland tumors from 2004 to 2008. In addition, 19 cases of PA (Fig. 3a) were also studied for comparison. There was insufficient detailed clinical data, such as the clinical duration or recurrence after the operation.

All of the tissues had been fixed in 10% formalin, routinely processed, embedded in paraffin wax, sectioned at 3 µm, and stained with hematoxylin and eosin. The histology of all tumors was examined by two independent pathologists (T.K. and T.M.). Ca-ex-PA was defined as a malignant epithelial neoplasm arising in association with a primary PA.
Fig. 2. Histopathology of Ca-ex-PA
A: Ca cells show obvious cytological abnormalities, such as eosinophilic cytoplasm, atypical nuclei, prominent nucleoli, and mitosis. (H & E stain, 400 ×)
B: 33.3% of the tumor cells were positive for Ki-67. (Immunohistochemistry, 400×)
C: 66.7% of the tumor cells were positive for p53. (Immunohistochemistry, 400×)

Fig. 3. Histopathology of hypercellularity of PA
A: Ordinal pleomorphic adenoma is seen. (H & E stain, 40 ×)
B: 2.7% of the tumor cells were positive for Ki-67. (Immunohistochemistry, 400×)
C: 1.3% of the tumor cells were positive for p53. (Immunohistochemistry, 400×)
Immunohistochemistry

Representative blocks were chosen for each case and 3-μm thick sections were cut and immunohistochemical staining for Ki-67 (MIB-1, Dako, Denmark), p53 (DO-7, Dako, Denmark) was performed. Sections were deparaffinized in xylene and dehydrated in descending grades (100–50%) of ethanol, and then subjected to antigen retrieval by microwave treatment in 10 mM citrate pH 6.0 for 25 min. After the slides had cooled to room temperature, the labeled streptavidin-biotin-peroxidase detection technique was used. The sections were incubated to quench endogenous peroxidase activity with 1% hydrogen peroxide in ethanol for 30 min. Nonspecific immunoreactivity was blocked by incubation with normal donkey serum for 30 min whereupon the sections were incubated with the primary antibody. After washing with phosphate-buffered saline (PBS) three times for 5 min each, the sections were incubated for 60 min with a multi-link biotinylated anti-immunoglobulin antibody. They were then re-washed with PBS before and after being incubated with the streptavidin-peroxidase reagent for 30 min. The reactions were visualized with diaminobenzidine (Dako, Denmark) as a chromogen. All steps were immediately followed by washes with PBS. Finally, sections were counterstained with hematoxylin, dehydrated and mounted.

Evaluation of immunohistochemical results

Sections stained immunohistochemically for Ki-67 and p53 were viewed at a magnification of 400 × using a light microscope. The labeling index (LI) represented the cell staining ratio, which was determined by counting the positive-staining cells among all tumor cells in five randomly selected views.

For statistical analyses, we used the t-test and Spearman’s correlation coefficient by rank test. Data were considered statistically significant if the P value was < 0.05.

Results

Clinical data

The h-APA patients in this study ranged in age from 21 to 67 years (mean, 39.1 years), in Ca-ex-PA, 45 to 72 years (mean, 58.7 years), and in PA, 15 to 67 years (mean, 38.7 years) (Table 1). Statistically, the patients’ age was higher in Ca-ex-PA than in other groups, but there was no difference in age between the h-APA and PA cases.
The tumor size ranged from 10 to 100 mm (mean, 33.5 mm) in h-APA, 25 to 60 mm (mean, 35.9 mm) in Ca-ex-PA, and 10 to 65 mm (mean, 28.4 mm) in PA. While the tumor size appeared to be larger in Ca-ex-PA than in PA, in fact there was no statistical difference.

**Immunohistochemical analysis for Ki-67**

In h-APA, the Ki-67 LI ranged from 1.7% to 13.3% (mean, 7.1%), but no significant correlation was seen between the Ki-67 LI and tumor size \( (r = 0.09; P = 0.77) \). In Ca-ex-PA, the Ki-67 LI ranged from 6.7% to 66.7% (mean, 22.5%), but similarly no significant correlation was seen between the Ki-67 LI and tumor size \( (r = 0.36; P = 0.31) \). In PA, the Ki-67 LI ranged from 1.3% to 6.7% (mean, 2.8%), but again no significant correlation was seen between the Ki-67 LI and tumor size \( (r = -0.20; P = 0.42) \). Representative Ki-67 immunohistochemical results are shown in Figs. 1c, 2b and 3b. In comparing the data from each group, the Ki-67 LI was statistically higher in Ca-ex-PA than in other groups, and it was also higher in h-APA than in PA.

**Immunohistochemical analysis for p53**

In h-APA, the p53 LI ranged from 0.3% to 16.7% (mean, 2.5%), but no significant correlation was seen between the p53 LI and the tumor size \( (r = -0.15; P = 0.63) \). In Ca-ex-PA, the p53 LI ranged from 0.3% to 66.7% (mean, 22.6%), but no significant correlation was seen between the p53 LI and tumor size \( (r = 0.27; P = 0.56) \). In PA, the p53 LI ranged from 0.3% to 6.7% (mean, 1.5%), but similarly no significant correlation was seen between the p53 LI and tumor size \( (r = -0.18; P = 0.49) \). Representative p53 immunohistochemical results were shown in Figs. 1d, 2c and 3c. In comparing the data from each group, the p53 LI was statistically higher in Ca-ex-PA than in other groups, but there was no difference in the p53 LI between h-APA and PA.

**Discussion**

In 1996, Auclair and Ellis\(^1\) identified the atypical features seen in PA, and correlated them with malignant transformation. Since then, it has been controversial whether the presence of atypical features in PA is indicative of a precancerous condition and how carcinoma arises in PA. We recently identified, using immunohistochemical staining, that HER2/neu appears in 40% of h-APA. HER2/neu is an important marker for Ca-ex-PA\(^2,3\) and we concluded that h-APA indicates a greater potential for malignant transformation\(^4\). Although there have been many reports of the high incidence of Ki-67 and p53 overexpression that correlates with malignant transformation in Ca-ex-PA cells, there have been no reports focusing on Ki-67 and p53 expression in h-APA cells\(^2-11\).

In previous reports, the Ki-67 LI was from 12% to 58% for Ca-ex-PA\(^2,3\), approximately 3% for PA\(^5\), and the mitotic activity was significantly higher in Ca-ex-PA than in PA. In
this study, Ca-ex-PA cells showed a higher Ki-67 LI than either h-APA or PA, and h-APA also showed a higher incidence than PA. This indicates not only that cancer cells maintain a very high mitotic activity, but also that h-APA should have a higher mitotic activity than PA.

In previous large studies of p53, overexpression was found in 54% to 75% of Ca-ex-PA cases and in 3% to 36% of PA cases. Therefore, the overexpression of p53 is significantly higher in Ca-ex-PA than in PA. In our study, we found a similar result for Ca-ex-PA and PA, but h-APA did not show significantly higher p53 expression than PA. Takeda found that the atypical features of PA did not correlate with higher immunoreactivity for p53. Since there is a close relationship between p53 overexpression and cellular anaplasia, the cells in h-APA that do not show advanced cellular anaplasia are not expected to overexpress the p53 protein.

Overexpression of p53 has been hypothesized to be an early event in the malignant transformation of PA. Since 40% of h-APA cases are positive for HER2/neu expression, we propose that p53 overexpression is a later event than HER2/neu overexpression in the malignant transformation of APA. Further study is required to confirm the roles of p53 and HER2/neu overexpression in this transformation.

The results of this study indicate that carcinogenesis in PA occurs in the following sequence of events. First, an increase of proliferative activity occurs in PA and leads to h-APA. Secondly, an amplification of the HER2/neu oncogene occurs in h-APA. Finally, p53 overexpression occurs in h-APA and leads to Ca-ex-PA. Our hypothesis explains the observed trends in the higher age of patients and greater tumor size in the sequence from PA to h-APA then to Ca-ex-PA.

References


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