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# Comparison of Proliferating Cell Nuclear Antigen Index in Benign and Malignant Salivary Pleomorphic Adenoma

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**The expression of proliferating cell nuclear antigen (PCNA) was studied in benign and malignant pleomorphic adenomas by using monoclonal antibody to PCNA. Carcinoma in pleomorphic adenoma ($n=8$), cell-rich variant ($n=6$) and typical pleomorphic adenoma ($n=6$) were selected in this study. The PCNA index in carcinoma in pleomorphic adenoma showed a higher index of nuclear staining (mean 22.9%, S.D. 6.2) than in typical pleomorphic adenoma (mean 6.9%, S.D. 3.4) or a cell-rich variant of pleomorphic adenoma (mean 8.8%, S.D. 3.3). A significant difference in PCNA index was found between benign and malignant pleomorphic adenoma ($P<0.05$). The present study suggests that PCNA index significantly differs between pleomorphic adenoma and carcinoma in pleomorphic adenoma, but in the prediction of malignant transformation potential it should be combined with routine histopathological examination.**
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## INTRODUCTION

THE PROBLEMS occasionally confronting clinicians and pathologists concerning pleomorphic adenoma are the frequency of local recurrence and the possibility of malignant transformation. The malignant tumours arising in pleomorphic adenoma develop from the epithelial component of tumour cells as a second neoplasm [1]. Seifert *et al.* have suggested that malignancy in a pre-existing pleomorphic adenoma can be observed in 3–4% of all the pleomorphic adenomas [2]. However, it is critical, but difficult to detect early progression into malignancy in benign pleomorphic adenoma. Recently, there have been many reports on the use of monoclonal antibody to proliferating cell nuclear antigen (PCNA) as a useful marker for proliferating activity of various tumours [3–6]. Tsuji *et al.* have stated that PCNA index represents a growth fraction which correlates with the degree of dysplasia in pre-malignant lesions [7]. In a previous study, we demonstrated the use of monoclonal antibody PCNA P10 in the evaluation of growth patterns of salivary gland tumours [8]. In the present study, aspects of different cellular kinetics in benign and malignant pleomorphic adenomas are described.

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## MATERIALS AND METHODS

8 cases of carcinoma in pleomorphic adenomas of salivary gland origin and 12 cases of benign pleomorphic adenomas (6 cases of cell-rich variant and 6 cases of typical pleomorphic adenoma) were included in this study. All the specimens were obtained by surgical resection at the Department of Oral and Maxillofacial Surgery, the 4th Military Medical University, Xian, China. None of the malignant cases were treated by radiotherapy or chemotherapy. All of the cases were diagnosed according to the WHO International Histological Classification of Salivary Gland Tumours [2]. The specimens were fixed in 10% formalin and embedded in paraffin. Sections were cut at 4 μm, stained with haematoxylin and eosin for routine histopathological assessment and for PCNA immunohistochemical staining.

The sections were deparaffinised and rehydrated. Endogenous peroxidase was blocked by methanol containing 0.3% $H_2O_2$ for 30 min. Sections were washed with distilled water for 5 min. An antigen retrieval method was used according to Shi *et al.* [9]. Slides were placed in a plastic jar containing 1% zinc sulphate. The jar was heated in a microwave oven for 10 min to 90–100°C. The jar was then removed from the oven and allowed to cool for 15 min and washed in distilled water and phosphate buffered saline (PBS) for 5 min.

Monoclonal antibody against PCNA (PC10, Dakopatts, Denmark) was used as primary antibody using the three stage avidin–biotin complex peroxidase method. Details of the immunohistochemical method have been described previously [7].

PCNA positivity was evaluated by determining the positive nuclei present in at least 1000 tumour cells in areas showing

intense nuclear staining but low background staining using a Nikon microscope (20 × objective and 10 × eye-piece). The significance of PCNA positivity and tumour grades were assessed by Student's t-test and the results were considered as significant when $P < 0.05$.

## RESULTS

*Carcinoma in pleomorphic adenoma*

Normal salivary gland tissue showed a few cells positive to PCNA (Fig. 1). In the interface between normal salivary gland and pleomorphic adenoma there were many tubular structures which showed a higher frequency of PCNA positivity (Figs 2 and 3). The malignant features of carcinoma in pleomorphic adenoma were, histopathologically demonstrated as epithelial cells with an increased nuclear cytoplasm ratio, prominent nucleoli and hyper chromatic nuclei with numerous mitoses. Infiltrating growth patterns were found in all the specimens; 5 cases showed an adenocarcinomatous proliferation pattern while 2 cases were myoepithelial carcinomas and the last case was undifferentiated carcinoma. Residual benign pleomorphic adenoma was seen in all the cases.

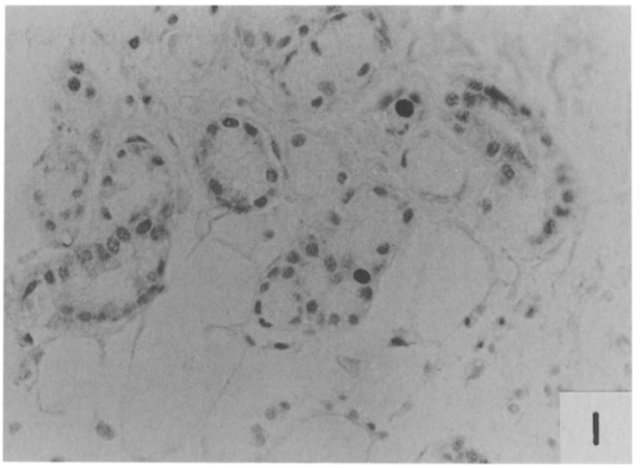
All the cases showed relatively higher rates of positive PCNA staining in nuclei but positive cells were confined to epithelial islands and sheets of the carcinoma components (Figs 4–7). Higher PCNA index rates were seen in modified myoepithelial cells than in luminal or duct-like tumour cells (Figs 8 and 9). In some specimens the positive nuclear staining was predominantly located in the periphery of the tumour mass (Figs 6 and 7). No positive staining was found in the mitotic cells. The mean of PCNA positive nuclei was 22.9% with a standard deviation (S.D.) of 6.9.

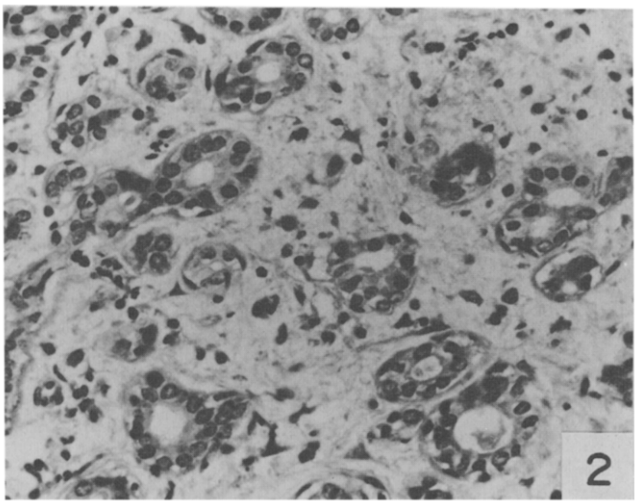
*Pleomorphic adenoma*

Tumour cells with positive nuclear staining were predominantly distributed focally in the epithelial masses and sheets and the outer tumour cells of tubulo-ductal structures but the number of positive nuclei varied widely throughout the specimen. The mean of PCNA positive nuclei in typical pleomorphic adenoma was 6.9% with a standard deviation of 3.4. The epithelial tumour cells of the cell-rich (myo-epithelial participant cells with varying pathological features) had a slightly higher PCNA index (8.8% S.D.:3.3) than the common type of pleomorphic adenoma (Figs 10–13), but there was no statistically significant difference between the two benign types. A statistically significant difference ($P < 0.05$) in PCNA index was found between malignant and benign pleomorphic adenomas. The results of PCNA positive nuclear rates are shown in Table 1.

## DISCUSSION

The biological behaviour of pleomorphic adenoma of salivary gland origin is very variable and pleomorphic adenomas with benign histological features may show recurrence, locally aggressive behaviour and occasional malignant transformation. Spiro *et al.* have documented salivary gland carcinomas that have arisen from recurrent, histologically benign pleomorphic adenomas [10]. Attempts have been made to determine possible predictors of aggressive clinical behaviour in otherwise histologically benign pleomorphic adenomas [11]. Myoepithelial cell proliferation has recently been identified as a possible predictor [11]. Cresson *et al.* have suggested



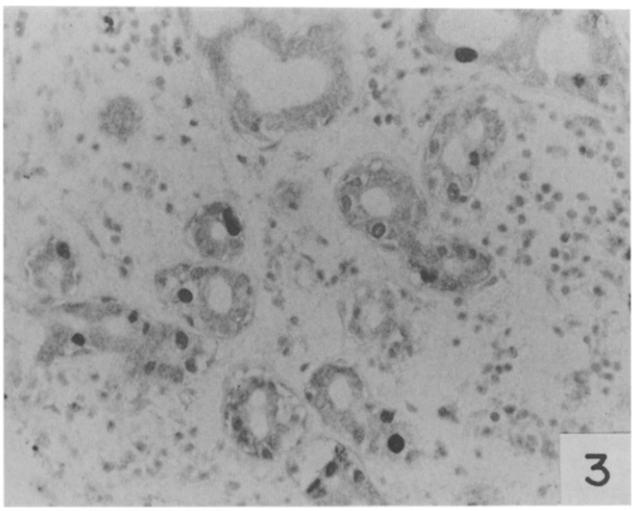




**Fig. 1. PCNA staining in normal oral minor salivary gland in the floor of the mouth. Only two cells labelled with PCNA. × 200.**

**Fig. 2. Haematoxylin and eosin staining shows a number of tubular structures in the neighbourhood of pleomorphic adenoma. × 200.**

**Fig. 3. PCNA staining in the same specimen of Fig. 2. PCNA positive cells are located at tubular structures. × 200.**

that aneuploid populations reflect the malignant potential of pleomorphic adenoma with local recurrence and metastasis [13]. Modified myoepithelial cells in pleomorphic adenoma are characterised by immunohistochemically detectable S-100 protein, vimentin, K8.12 cytokeratin and neuron specific enolase, but, these markers do not label myoepithelial cells in normal salivary glands [14]. It has been suggested that the modified or neoplastic myoepithelial cells in the outer zone of tubulo-ductal structures of pleomorphic adenoma are not the neoplastic counterpart of normal myoepithelial cells in terms of their histogenesis and that these tumour cells are probably ductal basal cell of origin [15]. Sugawara *et al.* have reported that c-*erb*B-2 protein is detected immunohistochemically in adenocarcinomatous elements whereas the remaining pleomorphic adenoma cells are not stained [16]. Shrestha *et al.* have described that c-*erb*B-2 oncoprotein is confined to malignant cells of carcinoma in pleomorphic adenoma [17] and, in breast carcinomas, PCNA index correlates with the positive immunoreaction of c-*erb*B-2 oncoprotein and epidermal growth factor (EGF) receptor status with a significant correlation with survival and disease-free survival [18]. Kernohan *et al.* also suggested that c-*erb*B-2 oncogene plays a role in the malignant development of pleomorphic adenoma [19]. In the present study, tumour specimens with no preoperative radiotherapy or chemotherapy were used in all the selected cases and therefore the specimens represent the natural growth pattern of pleomorphic adenomas, not affected by treatment.

Carcinoma in pleomorphic adenoma had a higher index of PCNA immunoreactivity in comparison with benign pleomorphic adenoma, including the cell-rich variant. It has been reported that the cell-rich variant of pleomorphic adenoma shows a higher risk of malignant transformation [2]. Although no statistically significant difference in PCNA index was observed between the cell-rich variant and typical pleomorphic adenoma, a trend of higher index was seen in the former.

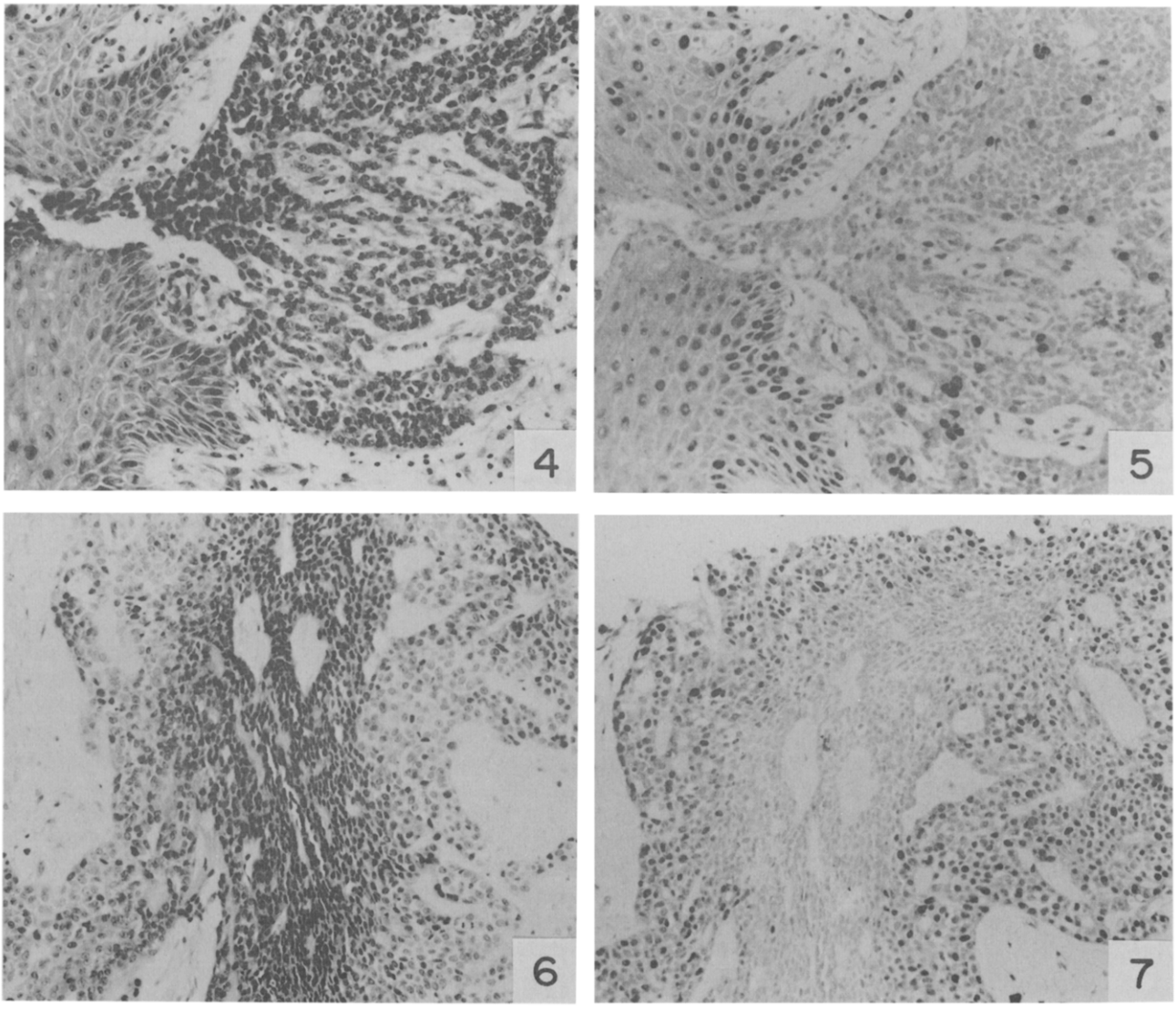



**Fig. 4. Haematoxylin and eosin staining reveals that proliferating cells have invaded into oral mucosa. ×100.**
**Fig. 5. PCNA staining in the same area of Fig. 4 reveals that basal and parabasal zone of oral epithelium and a few tumour cells are positive to PCNA. ×100.**
**Fig. 6. Haematoxylin and eosin staining shows that tumour cells have proliferated into the stromal tissue. ×100.**
**Fig. 7. PCNA staining in the same area of Fig. 6 shows that peripheral tumour cells have a higher PCNA index than central tumour cells. ×100.**

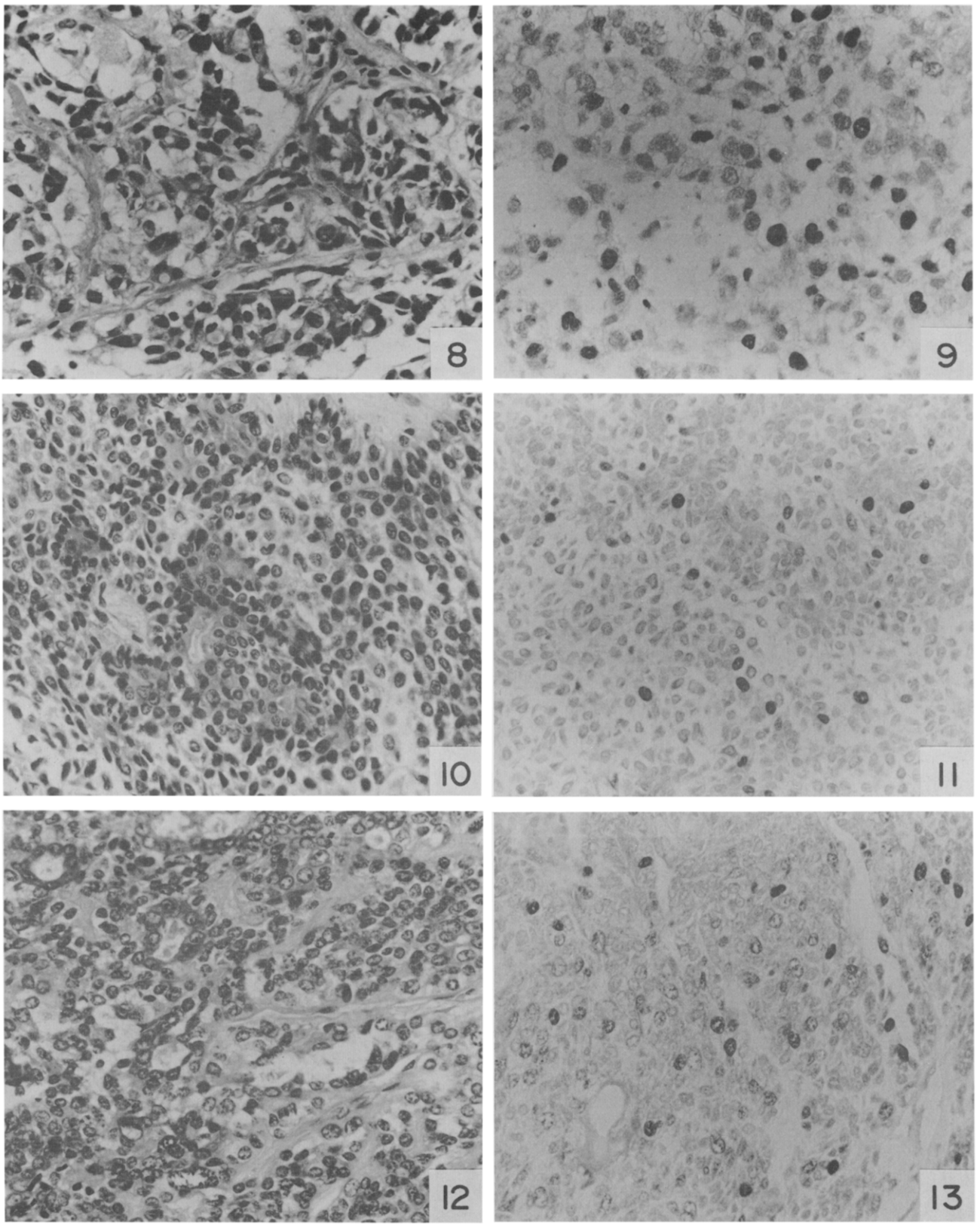



**Fig. 8. Haematoxylin and eosin staining of carcinoma in pleomorphic adenoma. ×200.**
**Fig. 9. PCNA staining in the same area of Fig. 8. PCNA positive nuclei are seen in the tumour focus. ×200.**
**Fig. 10. Haematoxylin and eosin staining of cell rich variant of pleomorphic adenoma. ×200.**
**Fig. 11. PCNA staining in the same area of Figs 10 and 12 shows more PCNA positive cells than typical pleomorphic adenoma. ×200.**
**Fig. 12. Haematoxylin and eosin staining of cell rich variant of pleomorphic adenoma. ×200.**
**Fig. 13. PCNA staining in the same area of Figs 10 and 12 shows more PCNA positive cells than typical pleomorphic adenoma. ×200.**

*Table 1. Percentage of PCNA positive cells in malignant and benign salivary pleomorphic adenomas*

| Tumour types | No. | % of PCNA positive nuclei (Mean ± S.D.) |
|---|---|---|
| Carcinoma in pleomorphic adenoma | 8 | 22.9 ± 6.2 |
| Cell-rich variant of pleomorphic adenoma | 6 | 8.8 ± 3.3 |
| Pleomorphic adenoma | 6 | 6.9 ± 3.4 |

The authors postulate that the cell-rich variant of pleomorphic adenoma may be a transitional type between a rapidly growing tumour of malignant nature and a slow growing, benign tumour. In terms of PCNA immunoreactivity the cell-rich variant of pleomorphic adenoma should not be considered as an independent type of pleomorphic adenoma.

On the other hand, cell types of distinct histological appearances within an individual pleomorphic adenoma can be roughly classified into tumour cells of ductal origin and those of so-called modified myoepithelial cell origin. Cell-rich areas of tumours consist of tumour cells, modified myoepithelial cells, and plasmacytoid or fibroid tumour cells of duct origin. Myxomatous or hyalinised tissue in benign pleomorphic adenoma consists of large amounts of matrix substance and varying amounts of plasmacytoid and fibroid cells. We suggest that these different types of tumour cell may have different growth activity and that the tumour growth rate depends on the amounts of higher proliferating cells with higher PCNA index. In the present study, PCNA index was relatively higher in modified myoepithelial foci than in luminal tumour cells. Cell-rich variants of pleomorphic adenoma showed relatively higher index rates of PCNA labelling (mean 8.8%, S.D. 3.4) than typical pleomorphic adenomas (mean 6.9%, S.D. 3.4), and this difference of PCNA index was related to the numerous modified myoepithelial cells in the foci.

An antigen retrieval technique for the rescue of PCNA from formalin-fixed paraffin-embedded tissue was applied to enhance the immunostaining of PCNA. In the present study, some sections showed weak or negative PCNA staining without the use of the antigen retrieval technique but, after the microwave heating treatment, the weakly positive sections showed intensive staining in tumour cell nuclei. Thus we suggest that this antigen retrieval method should be used in PCNA staining in specimens fixed for long periods.

In conclusion, our findings suggest that immunohistochemical staining for PCNA may be helpful in supplementing the evaluation combined with the pathological features but not necessarily in the prediction of malignant transformation of benign pleomorphic adenomas. If PCNA displays an increase of 7–8% index in the lesion it may be that this tumour has a trend to rapid proliferation or malignant transformation. However, its practical application should always be combined with routine histopathological examination.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