The Journal of Laryngology and Otology
June 1997, Vol. **111**, pp. 551–555

The evaluation of salivary gland tumours using proliferating cell nuclear antigen

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Abstract

In order to assess its discriminating and prognostic value, we studied the immunoreactivity for proliferating cell nuclear antigen (PCNA) in tissue from 52 human salivary gland tumours using the murine monoclonal antibody PC10.

The PCNA percentage count, namely, the average number of positive nuclei counted per 100 randomly selected tumour cells was recorded for each tumour. Anaplastic carcinoma was used as a positive control and histologically 'normal' salivary gland and tonsil served as a negative control.

A PCNA count of 30 per cent was postulated to predict malignancy within a given salivary gland tumour i.e. a PCNA count of 30 per cent or above would indicate malignant potential. This gave a sensitivity of 96.9 per cent and a specificity of 95.2 per cent and a positive predictive value of determining malignancy of 96.8 per cent.

We conclude that PCNA immunoreactivity is useful in discriminating between benign and malignant salivary gland tumours and that it may have prognostic value in this diverse group of neoplasms.

Key words: Salivary gland neoplasms; Antigens, differentiation; Immunohistochemistry

Introduction

Salivary gland tumours are a histologically heterogeneous group of neoplasms. To date, the morphological appearances of these tumours has been the gold standard in their evaluation (Seifert *et al.*, 1990). However, many of these tumours have a long indolent course with numerous local recurrences and late metastatic spread. Their histological appearances do not always correlate well with clinical behaviour and prognosis (Hickman *et al.*, 1984). To address this problem other characteristics of these tumours have been looked at.

For some time it has been thought that information on cell kinetics might be a useful addition to histologically based tumour classification in the understanding and prediction of tumour behaviour. In a variety of malignant neoplasms correlation has been noted between proliferative activity and metastatic potential, recurrence rate and overall prognosis. These include such tumours as leiomyomas, haemangiopericytomas and astrocytomas (Louis *et al.*, 1991; Yu *et al.*, 1991).

The presence of the nuclear protein PCNA has been found to correlate with the proliferative status of the cell (Battersby and Anderson, 1990; Linden *et al.*, 1992). PCNA is a 36 kilodalton auxiliary protein of DNA polymerase-delta present in cell nuclei in variable amounts throughout the cell cycle (Mathews *et al.*, 1984; Prelich *et al.*, 1987). The antibody PC10 recognizes a stable epitope on PCNA and can be used on routinely fixed paraffin embedded tissue, permitting retrospective studies on archival tissue.

PCNA immunoreactivity has been detected in normal human tissues and human neoplasms (Hall and Levison, 1990). In some tumours such as haemangiopericytomas (Yu *et al.*, 1991), astrocytomas (Louis *et al.*, 1991), malignant mesotheliomas (Ramael *et al.*, 1994) and gastric carcinomas (Jain *et al.*, 1991), increased PCNA immunoreactivity has been correlated with a worse prognosis.

Our goal was to assess the usefulness of PCNA immunoreactivity for discriminating between benign and malignant tumours and evaluating the utility of PCNA in predicting the prognosis of these neoplasms.

Methods

Patient population and tissue

Samples were available from 52 patients with salivary gland tumours collected at the East Birmingham Hospital between 1975–1992. In most cases resection specimens were available for histological evaluation.

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Accepted for publication: 4 April 1997.

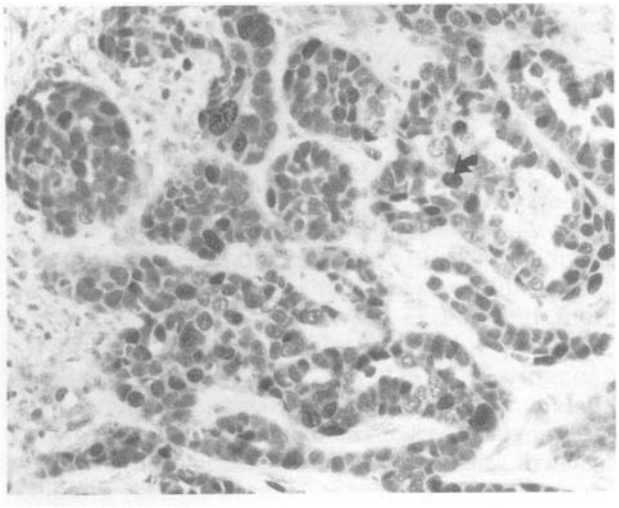

FIG. 1

Photomicrograph showing poorly differentiated adenocarcinoma of salivary gland immunostained with PC10 (PCNA). Small arrow shows uniform dense nuclear staining (H & E × 250)

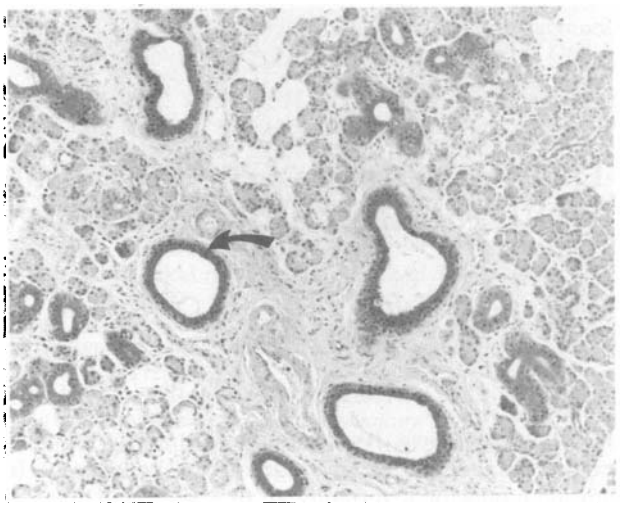

FIG. 2

Photomicrograph of histologically normal salivary gland showing weak uniform cytoplasmic immunostaining of ductal epithelial cells using PC10 (PCNA) indicated by large arrow (H & E × 100).

Histological evaluation

All tumours were classified according to the World Health Organization international histological classification (Siefert *et al.*, 1990) using formalin-fixed, paraffin-embedded tissue sections stained with haematoxylin and eosin. In most cases resection specimens were available and an average of four blocks were taken from each specimen with a range from two to eight. Further histochemical and immunohistochemical studies were performed as necessary to assist complete histological diagnosis. Five μm sections were cut, mounted on poly-L-lysine coated glass slides and air dried overnight at room temperature as oven drying at 60°C for one hour generally reduces the PCNA immunoreactivity (Prelich *et al.*, 1987).

PCNA staining was performed using a Strep-AB kit (Dako, Denmark). Sections were dewaxed in xylene, rehydrated through alcohol, then immersed in three per cent hydrogen peroxide in methanol for 10 minutes to block endogenous peroxidase activity. Sections were subsequently washed in phosphate buffered saline (PBS) and normal goat serum was applied for 10 minutes to reduce non-specific antibody binding.

PC10 (isotype IgG1 Medac, Germany) was used at a concentration of 0.5 g/ml for 10 minutes at room temperature. Biotinylated goat-anti-mouse antibody was used as the linker molecule and was applied at a dilution of 1 in 100 for 10 minutes.

After further washing with PBS, sections were incubated in streptavidin-horseradish peroxidase complex at a 1 in 100 dilution for 10 minutes. Diaminobenzidine-hydrogen peroxide complex was used as a chromagen and a light Mayer's haematoxylin counterstain was applied. Sections were dehydrated in alcohol, cleared in xylene, and mounted in DPX.

To ensure consistency of PCNA staining between batches, a known positive control salivary anaplastic carcinoma was included in each round. The experiment was repeated if there was any measurable fluctuation in the PCNA count of this control. Negative controls were included by performing duplicate assays, on one of which the primary antibody was replaced by PBS.

Assessment of PCNA staining

Sections were visualized at high power (× 400) and five fields of tumour were chosen at random. Nuclei from 500 tumour cells were counted in each case with the aid of an eyepiece graticule. The PCNA percentage count was the mean average number of positive nuclei per 100 tumour cells.

Results

PCNA staining was confined to cell nuclei (Figure 1), except in the case of 'normal' salivary gland duct cells in which fine uniform cytoplasmic staining was noted (Figure 2). Although some cell nuclei stained more strongly than others, some with granular and some with more homogenous staining, all identifiable staining was regarded as positive.

Results have been divided into quantitative and qualitative. The most important staining and counts were those of epithelial elements, however comment is also made on stromal and lymphoid infiltrate staining.

Qualitative

The salivary gland neoplasms showed prominent tumour heterogeneity. This is seen particularly in pleomorphic adenomas (PCNA count range 1 to 36.4 per cent and S.D. 66) and adenoid cystic carcinomas (PCNA count range 31.8 to 84.8 per cent and S.D. 115).

TABLE I
PCNA STAINING (QUANTITATIVE)

Salivary gland tissue	Number of specimens	PCNA percentage range	Mean	S.D.	Overall **5YSR	Metastatic potential**
Normal	4	0–5.4	1			
Ductal cells						
Acinar cells	4	0–1.4	0.4			
Tumour						
Adenolymphoma	9	1.8–13.2	5.8	19.4	100	–
Pleomorphic adenoma	9	1–36.4	17	66	97	+/–
Acinic cell tumour	2	0.2–5.8	3	19.8	82	+
Oncocytoma	1	0.2				
Adenoid cystic carcinoma*	8	31.8–84.8	55	115	62	+
Mucoepidermoid carcinoma*	3	49.8–80.2	63	77.4	70.7	+
Mixed malignant tumour*	3	74.4–92.4	82	46.3	55.7	++
Squamous carcinoma*	4	81.8–86.4	83	10.9	40	++
Adenocarcinoma*	5	84.4–94.4	89	17.9	55	++
Poorly differentiated carcinoma*	8	49.8–83	72	60	50	++

* Tumours regarded as malignant
** 5 YSR = five year survival rate

High PCNA counts were noted in the areas of lymphoid infiltrate where there was lymphoid follicle formation. These areas are observed in the tonsil control.

In all the tissues examined the PCNA staining was confined to the epithelial component with little or no staining in the stroma, except for the lymphoid infiltrate already mentioned.

Quantitative (see Table I)

Of four histologically 'normal' salivary gland specimens examined, three showed no demonstrable PCNA staining whereas one specimen showed a PCNA count of: acinar 1.4 per cent and ductal 5.4 per cent.

The high PCNA count in case of chronic sialoadenitis and benign lymphoepithelial lesions were related to the proliferative centres of the lymphoid follicles present within the lymphoid infiltrate. There were PCNA counts ranging from 1.8 to 13.2 per cent.

A PCNA count of 30 per cent or over was postulated to predict malignancy within a given salivary gland tumour. This gave a sensitivity of 96.9 per cent and a specificity of 95.2 per cent. The positive predictive value of determining malignancy in this group of 52 tumours was 96.8 per cent.

Discussion

Although many studies show a linear correlation of PCNA expression with DNA synthesis and cell proliferation (Jain *et al.*, 1991) recent advances in the understanding of the role of PCNA in cell replication suggest that its use as a proliferation marker is limited.

The fact that PCNA is involved in unscheduled DNA synthesis (i.e. nucleotide excision-repair), following UV irradiation for instance, limits its value as a proliferation marker (Prelich *et al.*, 1987). In addition, it raises the question of its utility in particular clinical settings such as skin tumours in light exposed areas (McCormick and Hall, 1992). Observations of increased, deregulated PCNA expression in non-cycling cells including normal tissue adjacent to tumours (Hall *et al.*, 1990) raises the speculative role of growth factors in regulating PCNA mRNA stability and protein expression.

The half life of PCNA is long at approximately 20 hours and thus it may be present in cells having just left the cycle. This does not present a problem in our study as its speed of degradation should not differ between tumours. However, it does mean that the proliferative compartment is over-estimated in every tumour.

Thus, although recent biological features of PCNA shed doubt on its use as a proliferation marker, there is still evidence that perhaps selective use of PCNA counting may be of diagnostic and prognostic use as an adjunct to conventional histological interpretation serving as an objective independent prognostic variable. Recent reports, supporting this view, studying haemangiopericytomas (Yu *et al.*, 1991), gastrointestinal lymphomas (Woods *et al.*, 1990) and malignant mesotheliomas (Ramael *et al.*, 1994) suggest that PCNA immunoreactivity is of value in histological grading and serves as an independent prognostic variable in these tumours.

Our results show that a postulated PCNA count of 30 per cent or above to predict malignancy in a given salivary gland tumour, gives a sensitivity and specificity of 96.9 per cent and 95.2 per cent respectively. The positive predictive value of determining malignancy is 96.8 per cent. These figures suggest that the PCNA count could be of diagnostic use serving as an additional parameter in histologically difficult tumours. However, it should be born in mind that some tumours show considerable differences in PCNA count both within and between tumours. This is particularly noted in tumours such as pleomorphic adenoma and adenoid cystic carcinoma and is demonstrated by their ranges and standard deviations. For example, pleomorphic adenoma (1 to 36.4 per cent S.D. 66) and adenoid cystic carcinoma (31.8 to 84.8 percent S.D. 115), compared to squamous cell carcinoma (85.8 to 86.4 per cent S.D. 10.9).

https://doi.org/10.1017/S0022215100137880 Published online by Cambridge University Press

A few other studies have looked specifically at the proliferative state of salivary gland tumours in an attempt to relate this to their behaviour. Morgan *et al.*, 1988 stained salivary glands for AgNORs, a silver-staining protein related to nuclear organizing regions and thought to be a marker of proliferation. This showed a statistically significant difference in AgNOR counts between benign and malignant salivary gland tumours. They viewed this as being of diagnostic help in distinguishing between adenocarcinoma, pleomorphic adenoma and adenoid cystic carcinoma especially on small biopsies. Crocker *et al.*, 1985 showed a difference in S100 protein staining in pleomorphic adenoma and adenoid cystic carcinoma, however the reliability of this is now uncertain (Nakazato *et al.*, 1985).

Murakami *et al.*, 1992 showed a statistically significant difference in Ki 67 counts between benign and malignant tumours and 'normal' salivary gland tissue. They also reported a significant difference in Ki67 counts in histological subtypes of adenoid cystic carcinoma with more aggressive lesions having a higher Ki 67 count. Our results for adenoid cystic carcinoma do show marked differences in PCNA counts both within and between tumours but no such association with morphological subtypes.

The cytoplasmic staining observed in a few cases of normal ductal salivary gland epithelium is similar to that reported by other authors in several tumours, but not within histologically 'normal' tissue (Benjamin and Gown, 1991). Although various theories have been postulated, the real significance of this phenomenon remains unclear (Hall *et al.*, 1990).

There are several potential sources of error when assessing PCNA staining in histological sections. Apart from the technical aspects of type of fixative, duration of fixation, and size of tissue blocks, there are other pitfalls which may not be so easily overcome (Wynford-Thomas, 1992). Tissue heterogeneity due to sampling differences is a significant problem. Some groups have attempted to overcome this by employing a semiquantitive method called the PCNA-grade; however, this introduces a greater subjective element. This has been reported to be of use in gastric carcinoma as an independent prognostic variable (Jain *et al.*, 1991).

In summary, our results show that, for a PCNA count of 30 per cent and over, the sensitivity and specificity were 96.9 per cent and 95.2 per cent to predict malignancy within a given salivary gland tumour. The positive predictive value of determining malignancy was 96.8 per cent.

We believe therefore that PCNA immunoreactivity is a useful adjunct to conventional morphological interpretation of salivary gland tumours in predicting benign and malignant behaviour. In addition PCNA immunoreactivity may prove to be a valuable adjunct to fine needle aspiration cytopathology in the early diagnosis of salivary gland neoplasms (Young, 1994).

Prospective studies are required to confirm our results on larger numbers of salivary gland tumours relating PCNA counts to individual five, 10 and 15 year survival rates, metastatic potential and perhaps individual recurrence rates.

References

Battersby, S., Anderson, T. J. (1990) Correlation of proliferative activity in breast tissue using PCNA/cyclin. *Human Pathology* **21:** 781.

Benjamin, D. R., Gown, A. M. (1991) Aberrant cytoplasmic expression of PCNA in Hodgkin's disease. *American Journal of Surgical Pathology* **15;** 764–768.

Crocker, J., Jenkins, R., Campbell, J., Fuggle, W. J., Shah, V. M. (1985) Immunohistochemical determination of S100 protein in salivary gland neoplasms. *Journal of Pathology* **146:** 115–121.

Hall, P. A., Levison, D. A. (1990) Assessment of cell proliferation in histological material. *Journal of Clinical Pathology* **43:** 184–192.

Hall, P. A., Levison, D. A., Woods, A. L., Yu, C. W., Kellock, D. B., Watkins, J. A., Barnes, D. M., Gillett, C. E., Camplejohn, R., Dover, R., Waseem, N. H., Lane, D. P. (1990) Proliferating cell nuclear antigen (PCNA) immunolocalization in paraffin sections: an index of cell proliferation with evidence of deregulated expression in some neoplasms. *Journal of Pathology* **162:** 285–294.

Hickman, R. E., Cawson, R. A., Duffy, S. W. (1984) The prognosis of specific types of salivary gland tumours. *Cancer* **54:** 1620–1624.

Jain, S., Filipe, N. I., Hall, P. A., Waseem, N. H., Lane, D. P., Levison, D. A. (1991) Prognostic value of proliferating cell nuclear antigen in gastric carcinoma. *Journal of Clinical Pathology* **44:** 655–659.

Linden, M. D., Torres, F. X., Kubus, J., Zarbo, R. (1992) Editorial. Clinical implications of morphologic and immunohistochemical assessment of cell proliferation. *American Journal of Clinical Pathology* **97:** (Suppl) S4–S13.

Louis, D. N., Edgerton, S., Thor, A. D., Hedley-Whyte, E. T. (1991) Proliferating cell nuclear antigen and Ki67 immunohistochemistry in brain tumours: a comparative study. *Acta Neuropathologica* **81(6):** 675–679.

Mathews, M. B., Bernstein, R. M., Franza, B. R. J., Garrels, J. I. (1984) Identity of the proliferating cell nuclear antigen and cyclin. Nature **309:** 374–376.

McCormick, D., Hall, P. A. (1992) The complexities of proliferating cell nuclear antigen. *Histopathology* **21:** 591–594.

Morgan, D. W., Crocker, J., Watts, A., Shenoi, P. M. (1988) Salivary gland tumours studied by means of the AgNOR technique. *Histopathology* **13:** 553–594.

Murakami, M., Ohtani, I., Hojo, H., Wakasa, H. (1992) Immunohistochemical evaluation of Ki67: an application to salivary gland tumours. *Journal of Laryngology and Otology* **106:** 35–38.

Nakazato, Y., Ishida, Y., Takahishi, K., Suzuki, K. (1985) Immunohistochemical distribution of S100 protein and GFAP in normal and neoplastic salivary glands. *Virchows Archives* **405:** 299–310.

Prelich, G., Tan, C. K., Kotsura, M., Mathews, M. B., So, A. G., Downey, K. M., Stillman, B. (1987) Functional identity of proliferating cell nuclear antigen and a DNA polymerase-delta auxillary protein. *Nature* **326:** 517–520.

Ramael, M., Jacobs, W., Weyler, J., Van Meerbeeck, J., Bialasiewicz, P., Van Den Bossche, J. (1994) Proliferation in malignant mesothelioma as determined by mitotic counts and immunoreactivity for proliferating cell nuclear antigen. *Journal of Pathology* **172:** 247–253.

Siefert, G., Brocheriou, C., Cardesa, A., Eveson, J. W. (1990) World Health Organization International histological classification of tumours: tentative histological classification of salivary gland tumours. *World Health Organization Publications.*

Woods, A. L., Hanby, A. M., Hall, P. A., Shepherd, N. A., Waseem, N. H., Lane, D. P., Levison, D. A. (1990) The

prognostic value of proliferating cell nuclear antigen immunostaining in gastrointestinal lymphomas (abstract). *Journal of Pathology* **161:** 342A.

Wynford-Thomas, D. (1992) P53 in tumour pathology: can we trust immunohistochemistry? *Journal of Pathology* **166:** 329–330.

Young, J. A. (1994) Diagnostic problems in fine needle aspiration cytology of the salivary gland. *Journal of Clinical Pathology* **47:** 193–198.

Yu, C., Hall, P. A., Fletcher, C. D. M., Camplejohn, R., Waseem, N., Lane, P. D., Levison, D. A. (1991) Immunohistochemical staining with a monoclonal antibody to proliferating cell nuclear antigen may be a good indicator of prognosis in haemangiopericytomas. *Histopathology* **19:** 29–34.

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https://doi.org/10.1017/S0022215100137880 Published online by Cambridge University Press