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Expression of Ki-67 antigen and proliferative cell nuclear antigen in benign and malignant epithelial lesions of the larynx

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Abstract

In an attempt to analyse the proliferative activity in benign and malignant laryngeal epithelial lesions, and to determine the relationship to their histologic grade, we studied the expression of proliferative cell nuclear antigen (PCNA) and Ki-67 antigen on 20 squamous carcinomas, and on 30 biopsies of epithelial hyperplasia categorized according to the Kambič-Lenart classification into simple, abnormal, and atypical hyperplasias. In simple hyperplasia, both antibodies stained the nuclei of the occasional cells in the basal layer. In abnormal hyperplasia (mild dysplasia), positive cells occupied up to a third, and in atypical hyperplasia (moderate and severe dysplasia) they occupied from two-thirds to the entire epithelial thickness. In squamous carcinoma, we have found a statistically significant correlation between its grade and the percentage of Ki-67–($p<0.01$) and PCNA–($p<0.00001$) positive cells. Our results suggest that the proliferative fraction progressively increases with the degree of epithelial hyperplasia and the grade of carcinoma. We conclude that the patterns of immunoreactivity to PCNA and Ki-67 antigen correspond to the histologic grade of both benign and malignant epithelial lesions of the larynx. This method should be regarded as a useful adjunct to traditional histological techniques allowing more objective grading of benign and malignant epithelial lesions.

Key words: Laryngeal neoplasms; Carcinoma, squamous cell; Epithelial cells, hyperplasia; Immunohistochemistry

Introduction

Epithelial hyperplastic lesions of the larynx with the clinical picture of chronic laryngitis represent a broad spectrum of histomorphological changes ranging from entirely benign simple hyperplasia (squamous cell hyperplasia) to atypical hyperplasia (severe dysplasia) which is a severe condition associated with an increased probability for subsequent cancer. As there is no specific gross appearance, diagnosis is based entirely upon histologic examination (Kambič and Gale, 1986; Kambič *et al.*, 1992; Kambič and Gale, 1995). In squamous cell carcinoma, histological verification is *conditio sine qua non* as well, even when carcinoma has the characteristic gross appearance. In contrast to different groups of epithelial hyperplastic lesions, histologic grade of the carcinoma is of limited value in predicting the clinical course of the disease (Kearsley *et al.*, 1990).

Histologic examination and diagnosis, especially of epithelial hyperplastic lesions, is based on traditional light microscopy. Several supplementary techniques have been tested in order to find a more objective diagnostic and prognostic method (Kearsley *et al.*, 1990; Kambič *et al.*, 1992; Shin *et al.*, 1993; Munck-Wikland *et al.*, 1994; Kambič and Gale, 1995) being mostly of limited value in clinical practice (Kambič and Gale, 1995). Among techniques that enable estimation of proliferative activity, immunohistochemical detection of proteins associated with cell proliferation has gained much attention in the past few years. The most frequently studied proliferative antigens are PCNA and Ki-67. The former is assumed to be needed for DNA synthesis acting as an auxillary protein of DNA polymerase delta, while the exact function of the latter in the cell cycle has not been elucidated yet (Brown and Gatter, 1990; Hall *et al.*, 1990). Both nuclear proteins are believed to be good indicators of proliferative activity and have provided prognostically useful information in many, though not all, malignant tumours (Hall *et al.*, 1990; Linden *et al.*, 1993; Sabattini *et al.*, 1993; Shin *et al.*, 1993; Thomas *et al.*, 1993 Haerslev and Jacobsen, 1995), premalignant (Coltrera *et al.*, 1992; Mittal *et al.*, 1993; Shin *et al.*, 1993; Munck-Wikland *et al*,

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1994), and non-neoplastic conditions (Hall *et al.*, 1990). Few studies have been reported on the head and neck region (Kearsley *et al.*, 1990; Coltrera *et al.*, 1992; Shin *et al.*, 1993; Munck-Wikland *et al.*, 1994).

The aim of the present study was to analyse the proliferative activity of epithelial cells in benign epithelial hyperplastic lesions and squamous carcinoma of the larynx and to determine the relationship, if any, to the histologic grade of both lesions.

Material and methods

Fifty laryngeal biopsy specimens for which adequate paraffin-embedded tissue was available, were included in this study. The control group consisted of autopsy samples of laryngeal mucous membrane of five non-smokers, aged 20–30 years, who died accidentally and had no clinical or histological evidence of laryngeal disease.

Squamous cell carcinomas were classified according to the standard criteria into well, moderately, and poorly differentiated carcinoma (grades I, II, III) with or without keratinization (Ferlito and Friedmann, 1993). Benign epithelial hyperplastic lesions were categorized according to the criteria of Kambič-Lenart classification into simple, abnormal, and atypical hyperplasias (Kambič and Lenart, 1971; Kambič, 1978; Kambič and Gale, 1986; Kambič *et al.*, 1992; Kambič and Gale, 1995).

Simple hyperplasia is characterized by thickened epithelium because of the augmented prickle cells; the basal layer is regular and consists of one or two rows of basal cells. Simple hyperplasia corresponds to squamous cell hyperplasia of the WHO classification (Shanmugaratnam and Sobin, 1991).

In abnormal hyperplasia, the epithelium is thickened because of basalification. The augmented basal-like cells extend up to the midportion of the epithelium. There are no distinct cellular or nuclear atypias. Abnormal hyperplasia corresponds to mild dysplasia of the WHO classification (Shanmugaratnam and Sobin, 1991).

Atypical hyperplasia, corresponding to moderate and severe dysplasia of the WHO classification (Shanmugaratnam and Sobin, 1991), is characterized by augmentation of immature, basal-like cells which occupy the lower two-thirds and may extend up to the surface of the epithelium. Epithelial cells may show mild to moderate cellular and nuclear atypias.

Immediately after excision, specimens were fixed in 10 per cent neutral formalin, embedded in paraffin, cut at 4–5 μm and stained with haematoxylin and eosin for traditional light microscopy.

For immunohistochemical staining, additional 4 μm thick sections were cut from paraffin blocks. After blockade of endogenous peroxidase with H_2O_2 in methanol for 30 minutes, sections were immersed in citrate buffer (pH 6.0) in a microwave-resistant container. For demonstration of PCNA and Ki-67 antigen, sections were microwaved for 10 and 25 minutes, respectively, at maximum power using a Panasonic microwave oven. The primary antibodies employed were PC-10 (anti-PCNA) and MIB-1 (anti-Ki-67), both from Dakopatts (Glostrup, Denmark). The dilution of PC-10 was 1:300 and of MIB-1 1:450. Immunoperoxidase detection was employed using the ABC method (Dakopatts) and diaminobenzidine substrate. Counter-staining was performed with haematoxylin.

Cell counting was performed by a single observer who did not know the clinical or pathological features of the lesion. Image analysis system IBAS-1000 (Kontron) at 400 total magnification was used. In all cases at least 1000 nuclei were scored and the percentage of tumour nuclei with positive results was determined.

Spearman rank order correlation was used to test the relation between the percentage of PCNA- or Ki-67-positive cells and the grade of carcinoma.

Results

There were 30 cases of hyperplastic epithelial lesions of the larynx (10 cases in each group of the Kambič-Lenart classification), and 20 cases of squamous cell carcinoma. Eight women and 42 men have been analysed, with mean age 53 years, ranging from 33 to 75 years.

Immunoreactivity for PCNA and Ki-67 appeared as diffuse or granular staining limited to the nucleus

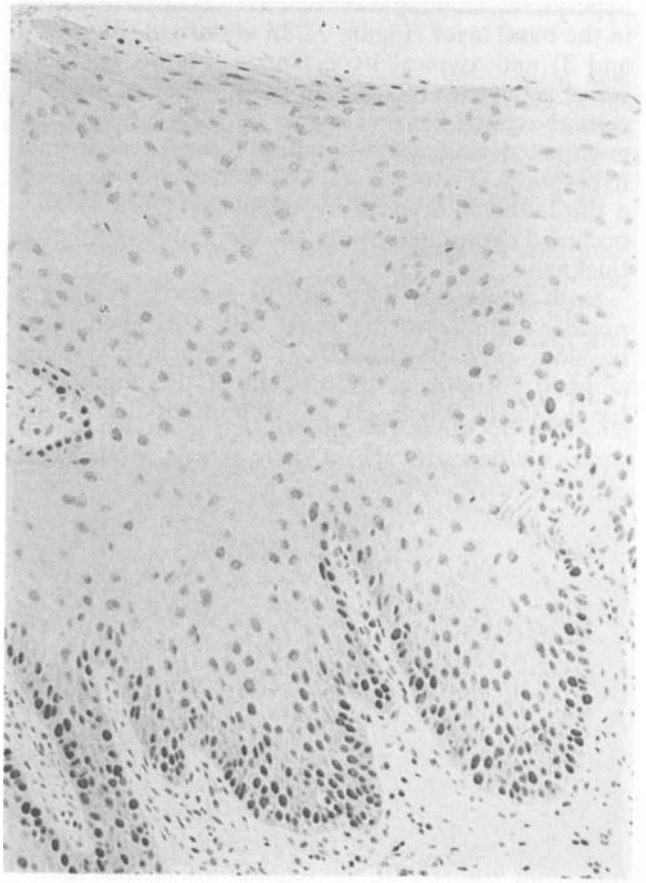

FIG. 1

Simple hyperplasia. PCNA (PC-10)–positive nuclei are seen in the basal and parabasal cells, a faint nuclear staining is found also in the lower third of the spinous layer. (Indirect immunoperoxidase method; original magnification × 40).

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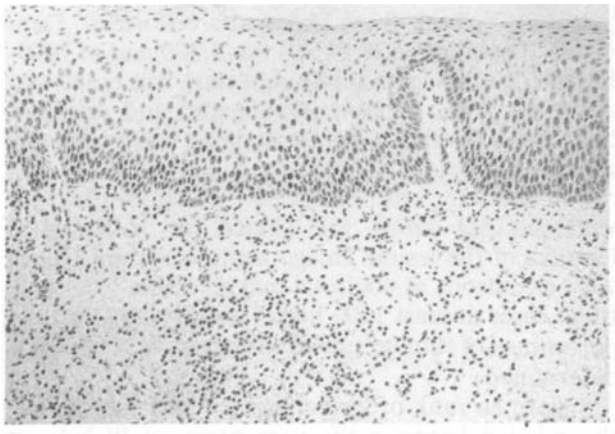

FIG. 2

Abnormal hyperplasia. A heavy PCNA (PC-10) nuclear staining is seen in the augmented basaloid cells in the lower third of the epithelium. A faint nuclear staining is found in the spinous cell layer. (Indirect immunoperoxidase method; original magnification × 16.5).

in all cases tested. No cytoplasmic staining was observed.

Benign hyperplastic epithelial lesions of the larynx

In normal laryngeal epithelium and in simple hyperplasia, staining was confined to occasional cells in the basal layer (Figure 1). In abnormal (Figures 2 and 3) and atypical hyperplasias (Figure 4), there was a significant increase in the number of positive cells above the basal cell layer corresponding to the augmented immature basaloid cells. In abnormal hyperplasia (Figures 2 and 3), positive cells occupied a third, and in atypical hyperplasia (Figure 4) they occupied from two-thirds to the entire epithelial thickness.

Both antibodies gave rise to a strong, clear-cut nuclear staining of the basal cells and augmented basaloid cells. With MIB-1, the nuclear staining stopped abruptly at the margin of the augmented basaloid cells (Figure 3). PC-10 positivity was found

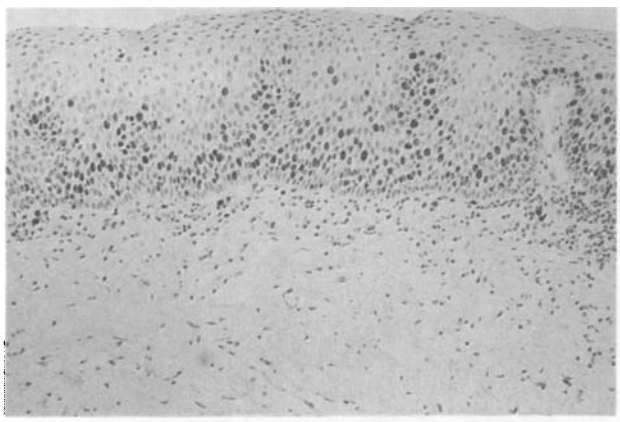

FIG. 3

Abnormal hyperplasia. The same tissue sample as in Figure 2. MIB-1 (Ki-67) immunoreactivity is limited to the augmented basaloid cells in the lower third of the epithelium. (Streptavidin-biotin immunoperoxidase method; original magnification × 16.5).

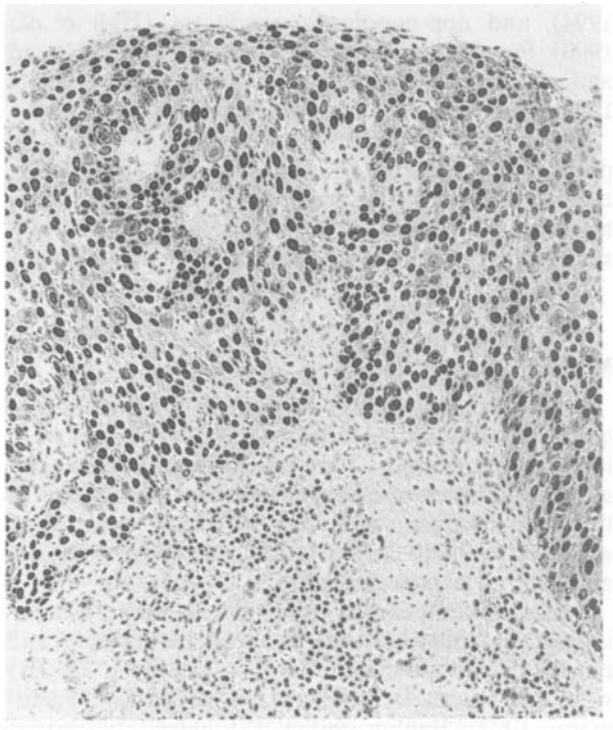

FIG. 4

Atypical hyperplasia. A strong positive nuclear reaction with PC-10 (anti-PCNA) is found in the augmented basaloid cells in almost the whole epithelial thickness. (Indirect immunoperoxidase method; original magnification × 40).

with decreasing intensity also above the basaloid cells - in the spinous cell layer (Figures 1 and 2).

Squamous cell carcinoma of the larynx

In well differentiated, keratinizing carcinomas, MIB-1 and PC-10 tended to stain the basaloid tumour cells at the periphery of the tumour islands, while the more differentiated keratinized cells towards the centre were usually negative (Figure 5). In less differentiated, non-keratinizing carcinomas, the number of positive cells increased and tended to be dispersed throughout the sample (Figure 6).

The percentage of cells, immunostained with MIB-1 and PC-10 in 20 squamous carcinomas with different grades, is shown in Figures 7 and 8, respectively. A statistically significant correlation was found between the grade of the carcinoma and the percentage of the carcinoma cells immunostained with MIB-1 ($r = 0.66$, $p<0.01$) or PC-10 ($r = 0.85$, $p<0.00001$).

Discussion

In the present study, we used two commercially available antibodies against proliferative antigens – PC-10 against PCNA and MIB-1 against Ki-67 antigen. The study was performed on 20 squamous cell carcinomas of the larynx, and on 30 biopsies of benign epithelial hyperplastic lesions with the clinical picture of chronic laryngitis, categorized according to the criteria of Kambič-Lenart classification which is

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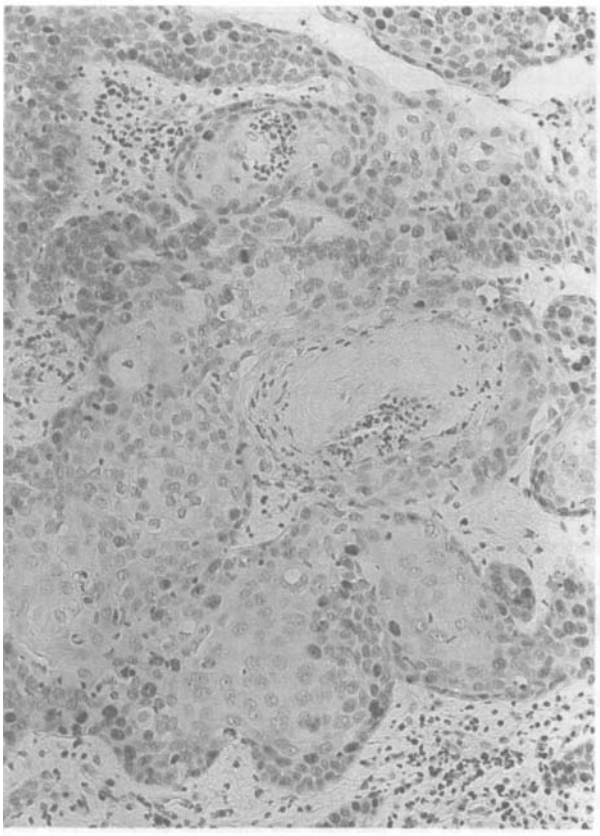

FIG. 5

Squamous cell carcinoma–well differentiated, keratinizing (grade I). Ki-67 (MIB-1) positive cells are seen at the periphery of the tumour islands. More differentiated cells towards the centre are negative. (Streptavidin-biotin immunoperoxidase method; original magnification × 40).

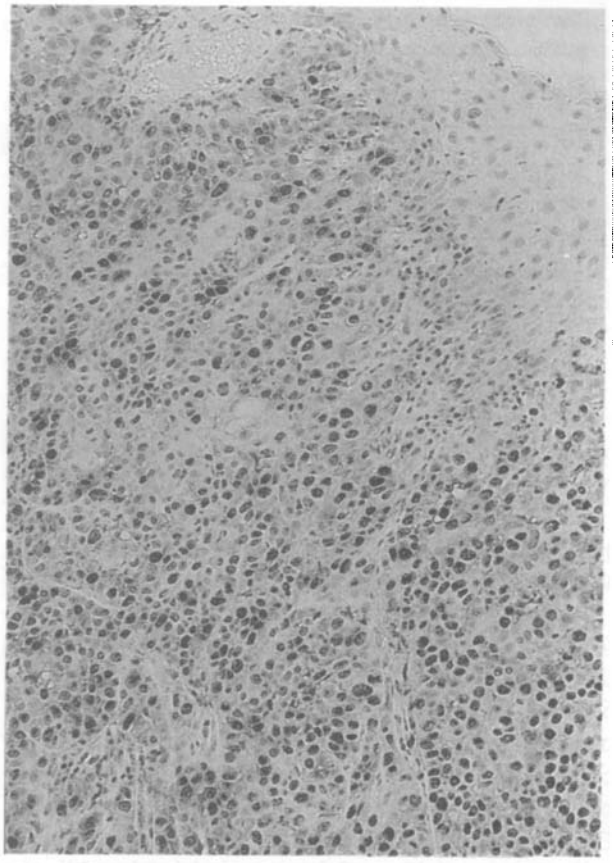

FIG. 6

Squamous cell carcinoma – moderately differentiated, non-keratinizing (grade II). Approximately 70 per cent of the carcinomatous cells are stained by PC-10 (anti-PCNA) and are dispersed throughout the sample. The surface epithelium shows positive reaction only in the basal layer. (Indirect immunoperoxidase method; original magnification × 40).

essentially comparable with the current WHO classification (Shanmugaratnam and Sobin, 1991) despite different terminology (Kambič and Gale, 1995).

In benign epithelial hyperplastic lesions, we observed rather constant patterns of immunoreactivity against PCNA and Ki-67, depending on the severity of hyperplasia. In simple hyperplasia (squamous cell hyperplasia), both antibodies stained the nuclei of the occasional cells in the basal layer. An identical staining pattern was also found in normal laryngeal epithelium. These results are comparable to the generally accepted opinions that in normal larygneal epithelium only the cells in the basal layer are capable of proliferating (Hall *et al.*, 1990; Coltrera *et al.*, 1992; Shin *et al*, 1993) and that simple hyperplasia (squamous cell hyperplasia) is only a reactive process (Shanmugaratnam and Sobin, 1991; Kambič and Gale, 1995).

In abnormal and atypical hyperplasias, we observed a significant increase in the number of positive cells, which appeared in the suprabasal portion of the epithelium. In abnormal hyperplasia (mild dysplasia), positive cells occupied up to a third, while in atypical hyperplasia (moderate and severe dysplasia) they occupied from two-thirds to the entire thickness of the epithelium.

The results of our study show that the proliferative fraction of epithelium progressively increases with the severity of hyperplasia (dysplasia). These findings support the hypothesis that abnormal and atypical hyperplasias (moderate and severe dysplasia) are the results of abnormal proliferation and maturation of the epithelial cells (Crissman, 1993; Shurbaji *et al.*, 1993). The PCNA and Ki-67 immunostaining patterns in our study of epithelial hyperplastic lesions of the larynx were exactly as one would expect with a proliferation marker.

Similarly to previous reports (Sabattini *et al.*, 1993), Ki-67 appears to be a more precise proliferative marker of epithelial hyperplastic lesions than PCNA. With MIB-1, the nuclear staining stopped abruptly at the margin of the augmented basaloid cells. In contrast, PC-10 positivity was found with decreasing intensity also above the augmented basaloid cells – in the spinous cell layer. This phenomenon has been reported before (Sabattini *et al.*, 1993), but there is no agreement in the literature as to whether or not the cells with fairly stained nuclei are in the cell cycle (Linden *et al.*, 1993).

Our results are comparable to recently published analyses of proliferative changes in premalignant lesions in the head and neck region, suggesting that the degree of proliferative dysregulation might be used as a prognostic marker for revealing the highest risk of progress to overt carcinoma (Coltrera *et al.*,

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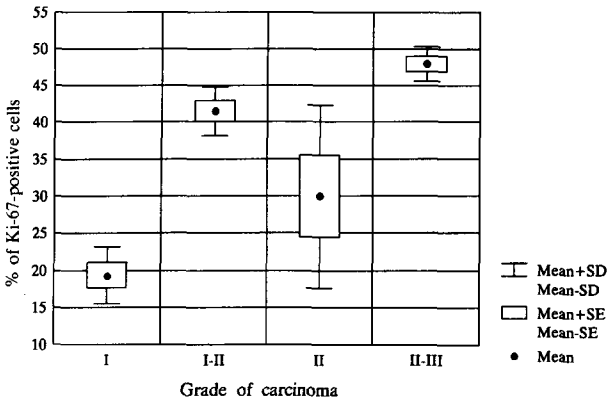

FIG. 7

Box-whiskers diagram showing the percentage of Ki-67 positive cells in 20 cases of squamous cell carcinoma divided on the basis of their grade.

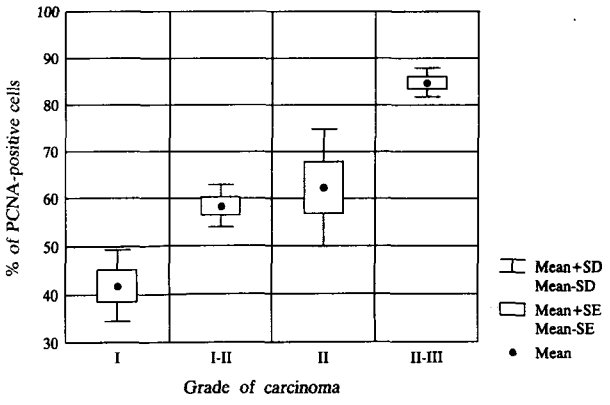

FIG. 8

Box-whiskers diagram showing the percentage of PCNA positive cells in 20 cases of squamous cell carcinoma divided on the basis of their grade.

1992; Nishioka *et al.*, 1993; Shin *et al.*, 1993; Munck-Wikland *et al.*, 1994; Kambič and Gale, 1995).

In squamous cell carcinoma of the larynx, we have found a statistically significant correlation between the grade of the carcinoma and the percentage of Ki-67 ($r = 0.66$, $p<0.01$) and PCNA ($r = 0.85$, $p<0.00001$) positive cells. In well differentiated, keratinizing carcinomas, MIB-1 stained on average 19.36 per cent, and PC-10 41.90 per cent of the tumour cells. Both markers tended to stain the basaloid tumour cells at the periphery of the tumour islands, while the more differentiated keratinized cells towards the centre were usually negative. This staining pattern has been also described by Kearsley *et al.* (1990) and suggests that the tumour periphery is composed of highly primitive proliferative cells, which are the progenitor of the more differentiated cells in the central portion of the tumour. In less differentiated carcinomas, the average percentage of positive cells increased to 48 per cent for MIB-1, and 85 per cent for PC-10. Therefore, PC-10 regularly stained a higher percentage of the tumour cells as compared with MIB-1. This is in concordance with experimental studies which have shown that PCNA grossly overestimates growth fraction, possibly because of the longer half-life of this protein (Hall *et al.*, 1990; Scott *et al.*, 1991). However, a statistically significant correlation was found between the grade of the carcinoma and the percentage of positive cells with both markers. In fact, the correlation was even stronger with PC-10 as compared with MIB-1.

In conclusion, we have demonstrated that PCNA and Ki-67 immunostaining corresponds to the histologic grade of both benign epithelial hyperplastic lesions and squamous cell carcinoma of the larynx. Our findings suggest that the proliferative fraction progressively increases with the severity of epithelial hyperplasia and the grade of the carcinoma. This method should be regarded as a useful adjunct to traditional histological techniques allowing more objective grading of benign and malignant epithelial lesions.

References

Brown, D. C., Gatter, K. C. (1990) Monoclonal antibody Ki-67; its use in histopathology. *Histopathology* **17:** 489–503.

Coltrera, M. D., Zarbo, R. J., Sakr, W. A., Gown, A. M. (1992) Markers of dysplasia of the upper aerodigestive tract. *American Journal of Pathology* **141:** 817–825.

Crissman, J. D. (1993) Upper aerodigestive tract. In *Pathology of Incipient Neoplasia.* 2nd Edition. (Henson, D. E., Albores-Saavedra, J., eds.), Saunders, Philadelphia, pp 44–63.

Ferlito, A., Friedmann, I. (1993) Squamous cell carcinoma. In *Neoplasms of the Larynx.* 1st Edition. (Ferlito, A., ed.), Churchill Livingstone, Edinburgh, pp 113–133.

Haerslev, T., Jacobsen, G. K. (1995) An immunohistochemical study of p53 with correlations to histopathological parameters, c-*erb*B-2, proliferating cell nuclear antigen, and prognosis. *Human Pathology* **26:** 295–301.

Hall, P. A., Levison, D. A., Woods, A. L., Yu, C.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.

Kambič, V., Lenart, I. (1971) Notre classification des hyperplasies de l'épithélium du larynx au point de vue pronostic. *Journal Français d'Oto-Rhino-Laryngologie* **20:** 1145–1150.

Kambič, V. (1978) Difficulties in management of vocal cord precancerous lesions. *Journal of Laryngology and Otology* **92:** 305–315.

Kambič, V., Gale, N. (1986) Significance of keratosis and dyskeratosis for classifying hyperplastic aberrations of laryngeal mucosa. *American Journal of Otolaryngology* **7:** 323–333.

Kambič, V., Gale, N., Ferluga, D. (1992) Laryngeal hyperplastic lesions, follow-up study and application of lectins and anticytokeratins for their evaluation. *Pathology, Research and Practice* **188:** 1067–1077.

Kambič, V., Gale, N. (1995) *Epithelial Hyperplastic Lesions of the Larynx*, 1st Edition, Elsevier, Amsterdam, pp 39–162.

Kearsley, J. H., Furlong, K. L., Cooke, R. A., Waters, M. J. (1990) An immunohistochemical assessment of cellular proliferation markers in head and neck squamous cell cancers. *British Journal of Cancer* **61:** 821–827.

Linden, M. D., Chan, K., Kubus, J., Brown, R. D., Zarbo, R. J. (1993) Ki-67 and proliferating cell nuclear antigen tumour proliferative indices in DNA diploid colorectal adenocarcinomas. *American Journal of Clinical Pathology* **100:** 206–212.

Mittal, K. R., Demopoulos, R. I., Goswami, S. (1993) Proliferating cell nuclear antigen (cyclin) expression in normal and abnormal cervical squamous epithelia. *American Journal of Surgical Pathology* **17:** 117–122.

Munck-Wikland, E., Edström, S., Jungmark, E., Kuylenstierna, R., Lindholm, J., Auer, G. (1994) Nuclear DNA content, proliferating-cell nuclear antigen (PCNA) and p53 immunostaining in predicting progression of laryngeal cancer in situ lesions. *International Journal of Cancer* **56:** 95–99.

Nishioka, H., Hiasa, Y., Hayashi, I., Kitahori, Y., Konishi, N., Sugimura, M. (1993) Immunohistochemical detection of p53 oncoprotein in human oral squamous cell carcinomas and leukoplakias: comparison with proliferating cell nuclear antigen staining and correlation with clinicopathological findings. *Oncology* **50:** 426–429.

Sabattini, E., Gerdes, J., Gherlinzoni, F., Poggi, S., Zucchini, L., Melilli, G., Grigioni, F., del Vecchio, M. T., Leoncini, L., Falini, B., Pileri, S. A. (1993) Comparison between the monoclonal antibodies Ki-67 and PC10 in 125 malignant lymphomas. *Journal of Pathology* **169:** 397–403.

Scott, R. J., Hall, P. A., Haldane, J. S., Van Noorden, S., Price, Y., Lane, D. P., Wright, N. (1991) A comparison of immunohistochemical markers of cell proliferation with experimentally determined growth fraction. *Journal of Pathology* **165:** 173–178.

Shanmugaratnam, K., Sobin, L. H. (1991) *Histological Typing of Tumours of the Upper Respiratory Tract and Ear*, 2nd Edition, Springer-Verlag, Berlin, pp 26–28.

Shin, D. M., Voravud, N., Ro, J. Y., Lee, J. S., Hong, W. K., Hittelman, W. N. (1993) Sequential increases in proliferating cell nuclear antigen expression in head and neck tumorigenesis: A potential biomarker. *Journal of the National Cancer Institute* **85:** 971–978.

Shurbaji, M. S., Brooks, S. K., Thurmond, T. S. (1993) Proliferating cell nuclear antigen immunoreactivity in cervical intraepithelial neoplasia and benign cervical epithelium. *American Journal of Clinical Pathology* **100:** 22–26.

Thomas, M., Noguchi, M., Kitagawa, H., Kinoshita, K., Miyazaki, I. (1993) Poor prognostic value of proliferating cell nuclear antigen labelling index in breast carcinoma. *Journal of Clinical Pathology* **46:** 525–528.

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