The Journal of Laryngology and Otology
March 1998, Vol. **112,** pp. 310–313

Proliferating cell nuclear antigen in laryngeal cancer

TOMASZ KRĘCICKI, M.D.*, MICHAL JELEŃ, PH.D.†

Abstract

The expression of proliferating cell nuclear antigen in paraffin sections from 154 cases of laryngeal squamous cell carcinoma were examined. There was significant difference in PCNA expression between the control group and cancer patients ($p<0.001$). The mean score of PCNA was higher in patients with poor prognosis than in patients with satisfactory outcome after treatment ($p<0.05$). There was no significant correlation between the PCNA count and the patient's age and sex, T and N stage and site of the tumour.

Univariate analysis revealed that the PCNA score correlated with the patients' survival rates. In multivariate analysis the prognostic value of PCNA was on the statistical borderline ($p = 0.049$). In our study clinical features like N and T status had a more important influence on survival rate. Nevertheless it appears that the immunohistological examination of PCNA in paraffin section could be a complementary prognostic tool for laryngeal carcinoma. PCNA expression may also be a valuable tool for differentiating malignant from benign laryngeal epithelium.

Key words: Laryngeal neoplasms; Proliferating cell nuclear antigen (PCNA)

Introduction

Squamous cell carcinomas of the larynx are the most common malignant neoplasms in the head and neck region. The incidence of laryngeal cancer in Poland is especially high.

The estimation of a patient's prognosis is of great importance as the selection of individual therapy is based on it. In the past few years, cell kinetic information has proved to be useful in completing conventional histopathological grading and clinical staging methods for the prediction of the biological behaviour of many neoplasms. Various cellular markers have been reported to be valuable in this field. One such marker is the proliferating cell nuclear antigen (PCNA).

PCNA is a 36-kd, acidic, nonhistone, nuclear protein whose expression is associated with the late G1 and S phases of the cell cycle. The human PCNA gene is a 261 amino acid polypeptide with a high content of aspartate and glutamic acid (Tsuji *et al.*, 1992). PCNA was first described by Miyachi (Miyachi *et al.*, 1978) as a nuclear antigen found in proliferating cells that reacted with the sera of patients who had systemic lupus erythematosus. This antigen was found to be related to cell proliferation and blast transformation. It has been proven that PCNA acts as the auxillary protein of DNA polymerase (Munck-Wikland *et al.*, 1994). A series of monoclonal antibodies to PCNA have been described, including PC10, which can be used on paraffin sections.

The level of PCNA has been shown to correlate with the proliferative activity and prognosis of cancer patients in several papers (Skopelitou *et al.*, 1993; Störkel *et al.*, 1993; Welkoborsky *et al.*, 1995; Golusinski *et al.*, 1996). Other authors have failed to prove the prognostic value of PCNA (Tomasino *et al.*, 1995).

The aim of our study was to investigate the expression of PCNA in laryngeal squamous cell carcinoma and its relationship to the clinical outcome of the patients.

Materials and methods

One hundred and fifty-four patients who underwent biopsy for carcinoma of the larynx were selected for this study. All patients were diagnosed between 1991 and 1994. There were 26 females and 128 males. The mean age was 61.3 ± 10.1 years. Tumour staging was performed according to TMN criteria. In 36 cases cervical lymph node metastases were detected.

All cases were squamous carcinomas. The histopathological grading of tumours (GI–GIII) done on the haematoxylin-eosin sections was as follows: 39 cases – GI, 61 cases – GII and 54 cases – GIII.

After diagnosis the patients were treated by surgery and/or radiotherapy. Eighty-nine patients underwent complete surgical excision of tumour with free margins. Thirty-two patients were treated by radiotherapy alone and thirty-three patients underwent surgery and subsequent radiotherapy.

A minimum follow-up of three years or to a patient's death was available for all the cases.

The tissue specimens, in the form of paraffin blocks, were available in the Department of Pathology, School of Medicine in Wroclaw. For the control, normal laryngeal epithelium obtained from 25 patients with benign lesions of the larynx was examined.

The histological diagnosis was made by an experienced pathologist on the basis of haematoxylin-eosin stain. All specimens were fixed in 10 per cent formalin and routinely processed for paraffin embedding. Sections were cut at 3–4 μm and mounted on coated glass slides. The slides were air-dried at room temperature overnight and then

From the Department and Clinic of Otolaryngology* and the Department of Pathology†, Medical University of Wrocław, Poland.
Accepted for publication: 5 January 1998.

TABLE I
PCNA EXPRESSION IN CONTROL GROUP AND CANCER PATIENTS

Groups of patients	Number of cases	PCNA expression	p
Control	25	26.56 ± 24.64	
Cancer	154	56.18 ± 19.05	$p<0.001$

stained immunohistochemically for PCNA. The three-step immunoperoxidase method using the streptovidin-biotin complex and the monoclonal antibody PC-10 was performed, according to the procedure described by Hall (Hall *et al.*, 1990). PC-10 was diluted 1:100 and the incubation time was two hours.

All identifiable nuclear staining in neoplastic cells was recorded as positive regardless of intensity. At least 500 cells were observed under high-power (× 40) microscopy and counted vertically from one corner to the other. The PCNA index was calculated as the quotient of positive neoplastic cells to the total number of neoplastic cells counted, expressed as a percentage.

In a few cases cytoplasmic staining was observed but nuclear staining was always stronger. The intensity of staining was not analysed because this feature may depend on varying staining procedures in different laboratories.

Prognostic factors analysed for their influence on survival were age and sex of patients, site and size of tumour, lymph node metastasis, histopathological grading, and PCNA expression. Any deaths resulting from causes other than laryngeal cancer were excluded from the statistical analysis.

Association between PCNA score, and tumour histological grade and/or clinical stage was estimated by one-way analysis of variance (ANOVA).

Univariate survival analyses were based on the Kaplan-Meier product-limit estimates of survival distribution.

Differences between survival curves were tested statistically using the generalized Wilcoxon test. The relative importance of multiple prognostic factors on survival was estimated using the Cox proportional hazards regression model.

All data were processed with S-PLUS (MathSoft, Seattle, WA) statistical software. A p value less than 0.05 was considered significant. The experimental values are given as a mean ± SD (standard deviation).

TABLE II
CLINICOPATHOLOGICAL FINDINGS AND PCNA EXPRESSION IN LARYNGEAL CANCER

Features	Number of cases	PCNA expression (mean ± SD)	Statistical significance
Sex			
Female	26	53.61 ± 22.79	
Male	128	56.55 ± 18.53	NS
Age (yr)			
<55	41	54.45 ± 16.22	
56–65	47	52.57 ± 20.17	
>65	66	59.71 ± 18.63	NS
Site of tumour			
Epiglottic	56	57.01 ± 21.14	
Glottic	65	55.81 ± 16.89	
Subglottic	8	65.76 ± 5.97	
Transglottic	25	47.54 ± 10.36	NS
T stage			
T1	32	61.57 ± 17.81	
T2	28	56.54 ± 19.74	
T3	35	56.82 ± 18.61	
T4	59	53.88 ± 19.08	NS
Lymph node metastasis			
N_0	118	56.73 ± 20.06	
N_{1-3}	36	54.72 ± 16.23	NS
Histopathological grade			
GI	39	52.29 ± 18.66	
GII	61	53.34 ± 20.12	
GIII	54	62.19 ± 16.78	$p<0.05$

Results

The immunoreactivity of PCNA was identified in all cancer and control tissue specimens. There was a significant difference in PCNA expression between the control group and the cancer patients ($p<0.001$) (Table I). The PCNA expression was the highest in the T1 stage but the difference with other stages of the tumour was statistically non-significant.

Patients with regional lymph node metastases had a similar PCNA score as the patients with disease limited to the larynx. The highest PCNA score was observed in subglottic cancer but the difference with other sites of the tumour was statistically non-significant. There was significant correlation between the PCNA score and histopathological grading of the tumour. The highest PCNA expression was detected in stage GIII. No significant relationship was found between PCNA score and sex and age of the patients (Table II).

The PCNA expression correlated with the patients' clinical course. The mean score of PCNA was higher in cases with recurrence than in cases free of disease after treatment (Table III). The difference was on statistical borderline of significance ($p = 0.042$).

Univariate analysis revealed a correlation between the PCNA score and survival rates. The three- and five-year survival rates were 62 and 41 per cent for cases with PCNA >45 and 79 and 73 per cent for cases with PCNA ≤ = 45.

Apart from PCNA expression, T stage, N status and size of the tumour correlated significantly with the patients' outcome. T4 stage cases had three- and five-year survival rates of 51 and 28 per cent respectively *vs* 76-85 per cent and 69–82 per cent for other stages. N1-3 patients had three- and five-year survival rates of 49 and 31 per cent *vs* 79 and 61 per cent for N0. Transglottic carcinomas had three- and five-year survival rates of 54 and 24 per cent *vs* 55–65 per cent and 40–54 per cent for epiglottic or subglottic and 91–82 per cent of glottic carcinomas.

The age and sex of the patients and histopathological staging of tumours did not correlate significantly with prognosis.

To determine if PCNA was an independent prognostic variable in laryngeal carcinoma, a multivariate analysis was performed. By testing the association of response with covariates in the Cox model, only three variables showed significant correlation with prognosis: N status ($p = 0.0004$) and, to a lesser degree, T stage ($p = 0.004$) and PCNA score ($p = 0.049$) (Table IV).

Discussion

The importance of cell kinetic parameters for estimation of prognosis for an individual patient is well known and widely accepted. The PCNA-PC10 immunostaining tech-

TABLE III
PCNA EXPRESSION IN PATIENTS WITH DIFFERENT CLINICAL OUTCOME

Clinical status	Number of cases	PCNA expression	p
No recurrence	96	52.31 ± 17.58	
Recurrence	58	59.02 ± 19.51	$p = 0.042$

https://doi.org/10.1017/S0022215100158451 Published online by Cambridge University Press

TABLE IV
MULTIVARIATE ANALYSIS OF COX'S PROPORTIONAL HAZARDS MODEL IN PATIENTS WITH LARYNGEAL CANCER

Variables	*p*
Age	NS*
Sex	NS
Site of tumour	NS
T status	$p<0.05$
N status	$p<0.001$
Grading	NS
PCNA	$p = 0.049$

*NS – not significant.

nique is a relatively simple and reproducible method which indicates the degree of cell proliferation.

Correlation of the PCNA index with poor survival has been shown in gastric carcinoma (Jain *et al.*, 1991), gastrointestinal lymphoma (Woods *et al.*, 1991), breast cancer (Aaltomaa *et al.*, 1992), bladder cancer (Lipponen and Eskelinen, 1992), prostatic cancer (Harper *et al.*, 1992), and carcinoma of the parotid gland (Frankenthaler *et al.*, 1994). In a study by Fontanini *et al.* (1992) of lung cancer, multivariate analyses have shown that PCNA is an independent prognostic factor.

Scopelitou *et al.* (1993) presented a positive correlation of PCNA expression in medullary thyroid carcinoma with the pathological stage and the nuclear grade of the tumour.

Störkel *et al.* (1993) investigated 100 patients with a primary squamous cell carcinoma of the oral cavity and showed that PCNA is useful in estimating the individual prognosis.

Shin *et al.* (1993) analysed dysregulation of PCNA in head and neck squamous cell carcinoma. The tissue samples containing premalignant lesions (hyperplasia and/or dysplasia), cancer tissue and normal epithelium were examined. The results indicate that PCNA could be a useful biomarker for multistep carcinogenesis and may serve as an intermediate end point in chemopreventive trials.

Yang *et al.* (1993) analysed the expression of PCNA in salivary gland tumours and came to the conclusion that PCNA is a useful marker of tumour cell proliferation.

Munk-Wikland *et al.* (1994) examined biopsies from patients with carcinoma *in situ* of the larynx. The lesions which progressed to invasive cancer showed a clear tendency towards a higher percentage of intense PCNA staining.

New methods based on computer analyses of images have recently been applied for PCNA staining estimation (Dawson *et al.*, 1990). Such methods may simplify the laboratory procedures and improve reliability of counting.

The aim of our work was to analyse the correlation between PCNA expression and clinicopathological features of the tumour. We also estimated whether PCNA expression could be a prognostic marker of laryngeal carcinoma.

The PCNA expression was significantly less for benign lesions than for malignant epithelium, in accordance with the findings of Shin *et al.* (1993) and Wen *et al.* (1995). This suggests that PCNA expression may be of value for differentiating benign from malignant squamous epithelial lesions.

In normal epithelium PCNA was confined to isolated cells adjacent to the basal laminae. In invasive, poorly differentiated carcinoma positive PCNA stained cells were present in all tumour infiltrations. When the tumour cells matured into squamous pearls, the PCNA staining disappeared. This observation is in accord with results of other authors (Shin *et al.*, 1993; Störkel *et al.*, 1993; Golusiński *et al.*, 1996).

There was no positive association between PCNA expression and T stage, in accordance with the findings of Kram *et al.* (1996) but in contrast with Welkoborsky *et al.* (1995) and Golusiński *et al.* (1996).

It was difficult to assess the correlation between lymph node status and expression of markers of cell proliferation. Some authors suggested such a correlation (Golusiński *et al.*, 1996) but other authors, including this study, could not confirm this (Roland *et al.*, 1994).

We have demonstrated a significant correlation between PCNA expression and tumour grading which confirms the results of other authors (Lorz *et al.*, 1994; Lam *et al.*, 1996). The explanation of a relationship between histopathological grading and PCNA count may be based on the fact that cell proliferation is one of the factors to consider in the estimation of the grade of histopathological malignancy (Woods *et al.*, 1991).

Our results demonstrate that the PCNA score correlated with the patient's clinical outcome and survival in univariate analysis although this correlation was rather weak. These findings are in line with the results obtained by Pich *et al.* (1992) and Stöerkel *et al.* (1993).

In multivariate analysis the prognostic value of PCNA was on the statistical borderline of significance ($p = 0.049$). Clinical features like N and T status had a more important influence on survival rates. Nevertheless it appears that the immunohistological examination of PCNA on paraffin section could be a complementary prognostic tool in laryngeal carcinoma. PCNA expression may also be a valuable tool in differentiating malignant from benign laryngeal epithelium.

References

Aaltomaa, S., Lipponen, P. M., Syrjanen, K. (1992) Prognostic value of cell proliferation in breast cancer as determined by proliferating cell nuclear antigen (PCNA) immunostaining. *Anticancer Research* **12:** 1281–1286.

Dawson, A. E., Norton, J. A., Weinberg, D. S. (1990) Comparative assessment of proliferation and DNA content in breast carcinoma by image analysis and flow cytometry. *American Journal of Pathology* **136:** 1115–1124.

Fontanini, G., Macchiarini, P., Pepe, S., Ruggiero, A., Hardin, M., Bigini, D., Vignati, S., Pingitare, R., Angeletti, C. A. (1992) The expression of proliferating cell nuclear antigen in paraffin sections of peripheral, node-negative non-small cell lung cancer. *Cancer* **70:** 1520–1527.

Frankenthaler, R. A., El-Naggar, A. K., Ordonez, N. G., Miller, T. S., Batsakis, J. G. (1994) High correlation with survival of proliferating cell nuclear antigen expression in mucoepidermoid carcinoma of the parotid gland. *Otolaryngology – Head and Neck Surgery* **111:** 460–466.

Golusiński, W., Szmeja, Z., Olofsson, J., Biczysko, W., Krygier-Stojalowska, A., Majewski, P. (1996) Wartość diagnostyczna i prognostyczna onkogenu p53 wybranych markerów nowotworowych (Ki67, PCNA, DNA ploidii) ultrastruktury u chorych na raka krtani. *Otolaryngologia Polska* **50:** 607–617.

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

Harper, M. E., Glynne-Jones, E., Goddard, L., Wilson, D. W., Metenhelia, S. S., Conn, I. G., Peeling, W. B., Griffiths, K. (1992) Relationship of proliferating cell nuclear antigen (PCNA) in prostatic carcinomas to various clinical parameters. *Prostate* **20:** 243–253.

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

Kram, A., Domagala, W. (1996) Proliferating cell nuclear antigen (PCNA) expression in laryngeal squamous carcinomas. An immunohistochemical study. *Polish Journal of Pathology* **47:** 183–187.

Lam, K., Law, S., So, M., Ma, L., Wong, J. (1996) Prognostic implication of proliferative markers MIB-1 and PC10 in esophageal squamous cell carcinoma. *Cancer* **77:** 7–13.

Lipponen, P. K., Eskelinen, M. J. (1992) Cell proliferation of transitional cell bladder tumours determined by PCNA/cyclin immunostaining and its prognostic value. *British Journal of Cancer* **66:** 171–176.

Lorz, M., Meyer-Breiting, E., Bettinger, R. (1994) Proliferating cell nuclear antigen counts as markers of cell proliferation in head and neck cancer. *European Archives of Otorhinolaryngology* **251:** 91–94.

Miyachi, K., Fritzler, M. J., Tan, E. M. (1978) Autoantibody to a nuclear antigen in proliferating cells. *Journal of Immunology* **121:** 2228–2234.

Munck-Wikland, E., Edstroem, 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.

Pich, A., Chiusa, L., Pisani, P., Krengli, M., Pia, F., Novone, R. (1992) Argyrophilic nucleolar organizer region counts and proliferating cell nuclear antigen scores are two reliable indicators of survival in pharyngeal carcinoma. *Journal of Cancer Research and Clinical Oncology* **119:** 106–110.

Roland, N. J., Caslin, A. W., Bowie, G. L., Jones, A. S. (1994) Has the cellular proliferation marker Ki67 any clinical relevance in squamous cell carcinoma of the head and neck? *Clinical Otolaryngology* **19:** 13–18.

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.

Skopelitou, A., Korkolopoulou, P., Papanikolau, A., Hadjiyannakis, M. (1993) Proliferating cell nuclear antigen (PCNA) in medullary thyroid carcinoma. *Journal of Cancer Research and Clinical Oncology* **119:** 379–381.

Störkel, S., Reichert, T., Reiffen, K. A., Wagner, W. (1993) EGFR and PCNA expression in oral squamous cell carcinomas – a valuable tool in estimating the patient's prognosis. *European Journal of Cancer* **29B:** 273–277.

Tomasino, R. M., Daniele, E., Bazan, V., Morello, V., Tralongo, V., Nuara, R., Nagar, C., Salvato, M., Ingria, F., Restivo, S. (1995) Prognostic significance of cell kinetics in laryngeal squamous cell carcinoma: clinicopathological associations. *Cancer Research* **55:** 6103–6108.

Tsuji, T., Shrestha, P., Yamada, K., Takagi, H., Shinozaki, F., Sasaki, K., Maeda, K., Mori, M. (1992) Proliferating cell nuclear antigen in malignant and pre-malignant lesions of epithelial origin in the oral cavity and the skin: an immunohistochemical study. *Virchows Archives A, Pathological Anatomy and Histopathology* **420:** 377–383.

Welkoborsky, H. J., Hinni, M., Dienes, H. P., Mann, W. J. (1995) Predicting recurrence and survival in patients with laryngeal cancer by means of DNA cytometry, tumour front grading, and proliferation markers. *Annals of Otology, Rhinology and Laryngology* **104:** 503–510.

Wen, Q. H., Nishimura, T., Miwa, T., Nagayama, I., Furukawa, M. (1995) Expression of EGF, EGFR and PCNA in laryngeal lesions. *Journal of Laryngology and Otology* **109:** 630–636.

Woods, A. L., Hall, P. A., Shepherd, N. A., Hanby, A. M., Waseem, N. H., Lane, D. P., Levison, D. A. (1991) The assessment of proliferating cell nuclear antigen (PCNA) immunostaining in primary gastrointestinal lymphomas and its relationship to histological grade, S + G2 + M phase fraction (flow cytometric analysis) and prognosis. *Histopathology* **18:** 21–27.

Yang, L., Hashimura, K., Qin, C., Shrestha, P., Sumitomo, S., Mori, M. (1993) Immunoreactivity of proliferating cell nuclear antigen in salivary gland tumours: an assessment of growth potential. *Virchows Archives A, Pathological Anatomy and Histopathology* **422:** 481–486.

Address for correspondence:
Tomasz Kręcicki, M.D.,
ul.Smoleńskiego 4,
51-607 Wrocław,
Poland.

Fax: 0-48713285415