LEAD ARTICLE

The Prognostic Significance of Proliferating Cell Nuclear Antigen (PCNA) in Laryngeal Cancer

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Abstract

Squamous cell carcinoma arising from upper aerodigestive tract carries with it a significant morbidity and mortality and, over the last few decades, its incidence has steadily increased. The management of patients requires thorough investigation to determine the local, regional, and distant extent of the disease, and treatment options include surgery, radiotherapy, chemotherapy, or combinations of these.
Despite the large number of therapeutic and hsitopathologic studies in print, there is currently no morphologic or cytologic feature available which consistenly predicts outcome in patients with laryngeal carcinoma. The use of proliferating cell nuclear antigen (PCNA), a newly available marker of a cell's proliferative activity (S-phase fraction), was evaluated in 25 cases of squamous cell carcinoma of the larynx. PCNA scores differed, statistically significantly as far as the localization of the lesion, pathological grade, clinical stage, presence of lymph node metastases and prognosis of the patients were concerned.
These data suggest that PCNA is an indicator of the malignant potential of the larynx. PCNA can be used in decision making for treatment and assessing prognosis in carcinoma of the larynx.

Keywords

Proliferating cell nuclear antigen (PCNA), immunohistochemistry, laryngeal carcinoma, prognosis.

The traditional prognostic factors, including stage of disease and tumour grade, have shown a limited prognostic significance and an inability to predict clinical response to specific treatment in patients with squamous cell carcinoma of the larynx.

Among factors that affect the prognosis of patients with laryngeal carcinoma are clinical stage and the observed degree of differentiation in histologic features. The current method of estimating the tumour malignancy and determining patient prognosis involve measuring the proliferation rate of tumour cells using basic and clinical techniques.

Proliferating cell nuclear antigen (PCNA), which is a nonhistone nuclear protein required for DNA synthesis, acting as the auxiliary protein of DNA polymerase δ , has been described as an indicator of cell proliferation (1). The existence of this protein was first indicated when serum from patients with systemic lupus erythematosus reacted with nuclei of proliferating cells (such as mitogenically stimulated lymphocytes) (2). Previous studies (1) indicate that PCNA is, synthesized during the late G1 to S phase. A series of monoclonal antibodies to PCNA, including PC10, can be used on paraffin sections and is commercially available (3). This marker is much more convenient than other proliferating cell markers, such as Ki- 67, DNA polymerase α, and bromodeoxyuridine (BrdU) because formalin-fixed, paraffin embedded sections can be used for PCNA staining.

This study was performed to assess the prognostic value of PCNA in maryngeal carcinoma and its relation with other known prognostic clinicopathologic parameters.

Material and Methods

For this pilot study, tissue samples from 25 previously untreated patients who underwent surgery for squamous cell carcinoma of the larynx were examined. The records of the patients were examined and the sex, age, localization of lesion, stage, operative procedure and disease free survival

periods were recorded. The pathological examinations included conventional histopathology and immunohisto-chemical identification of PCNA, both of which were performed without knowing the clinical outcome of the patients.

Conventional Histopathology : For conventional histopathology, 5mm paraffin sections stained with hematoxylin and eosin (H &E) were examined by the pathologist. Tumours were graded according to morphologic differentiated as grade 1 (well differentiated), grade 2 (moderately well differentiated) and grade 3 (poorly or undifferentiated) (4).

Immunohistochemical Detection of Proliferation Markers : For Immunohistochemical detection of PCNA, Peroxidase-Anti-Peroxidase (PAP) methods was used. 5mm sections obtained from paraffin block were mounted on poly-i-lysine-coated glass slides and incubated at 60°C overnight. The slides were deparaffinized and dehydrated by xylene and graded alcohol. Endogenous peroxidase activity was blocked by incubating the slides in 30% hydrogen peroxidase for 10 minutes. After pretreatment with antigen retrieval solution and washing in phosphate buffered saline (PBS), monoclonal PCNA antibody (PCNA, AM 206-5M, Biogenex) was overlaid on tissue section for 2 hour at room temperature. Then link was added and incubated for 20 minutes and it was rinsed with PBS. The slides were incubated with label for 20 minutes and again rinsed with PBS. After staining with chromogen substrate (Alkaline phosphatase-conjugated streptavidin) Mayer's hematoxylin was used as counterstain.

Scores were determined microscopically by counting at least 1000 cells at the tumour front in at least five different regions. The extent of PCNA positivity was evaluated by determining the amount of the positively stained nuclei present in tissue. A minimum threshold of nuclear staining intensity was used , so that all cells were counted as positive regardless of the staining intensity. When positive cells were observed in the tissue specimen, it was considered to be a positive case.

Statistical Analysis : Statistical analysis was carried out by using the SPSS for MS Windows Release 5.0 software package programme. Fisher's Exact Chi-Square test, Mann-Whitney-U test and Kruskal-Wallis one-way ANOVA test were used in the statistical analysis of the collected data. Only P values of < 0.05 were regarded as signficant.

Results

All specimens were obtained from excisions of the larynx from the Department of Otolaryngology Head and neck Surgery at the PTT Hospital, Turkey, during 1990-96. The patients included 24 men and 1 woman; median age 50, ranging between 30-73, mean age 50.76 with a standard deviation of 10.88.

All patients had glottic, supraglottic or transglottic tumours, with stages I through IV represented. Lymph node metastases in the neck were evident by clinical palpation and / or ultrasonography. All patients underwent surgery. The minimum follow up period was 3 years. The patients had not been given any other treatment .

Tumour staging was done according to the criteria of American Joint Committee on Cancer 1991 (5). 11 patients were in stage 1, 3 patients were in stage II, 11 patients were in stage III and no patients were found in the IV' th stage.

According to their lesions 12 (48%) had total laryngectomy and 13 (52%) had partial laryngectomy (surpraglottic, vertical or subtotal). Invasive squamous cell carcinoma was confirmed histologically in all cases. All margins in each case were free of tumour as determined by frozen section examination. Conventional histopathologic examination revealed that 6 patients had grade 1, 17 had grade II and 2 had grade III tumours. Lymph node metastases were histologically confirmed in 9 patients.

Immunohistochemistry revealed that PCNA values between 16.58 and 81.57 with the median value being 40, mean value being 17.80. As far as the localization of the lesion was concerned 8 cases were found to have lesion at the glottic level ; 3 cases at the supraglottic level and 14 cases at the transglottic level.

Distribution of PCNA scores of all cases according to the localization of the lesion was analyzed using the Kruskal-Wallis one-way ANOVA test and differences were found to be statistically significant (Table 1; $p=0.0078$). When Mann-Whitney -U tests were performed the most significant difference was

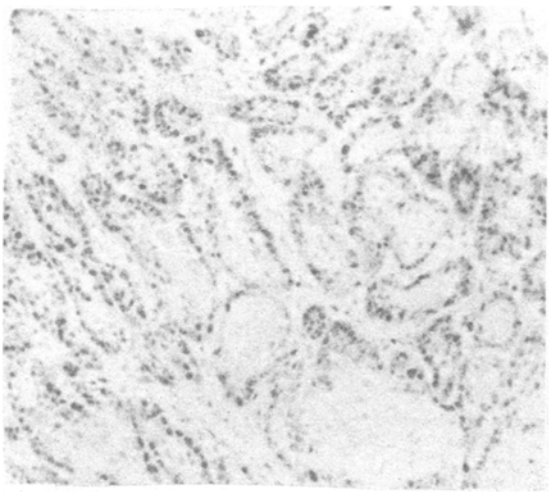

Fig 1 Immunohistochemical staining of moderately differentiated laryngeal squamous cell carcinoma using monoclonal-PCNA antibody (PCNA immunostaining x100).

found to be between glottic versus transglottic lesions (p=0.0027), whereas differences between glottic versus supraglottic lesion and supraglottic versus transglottic lesions were not found to be statistically significant, p-0.22 and p=0.21 respectively.

Similar analysis was performed to analyse the differences between different pathological grades as far as the PCNA scores were concerned and the difference was found to be statistically significant (Table 2 ; Fig 1; p=0.0294)

When further analysis was performed using the mann-Whitney -U test, the difference between grade II and grade III was found not to be statistically signficant (p=0.1840). However, differences between grade I and grade II, and between grade I and grade III were both found to be statistically significant p=0.03 and p=0.45 respectively, again using the Mann-Whitney-U test.

Table I
Distribution of PCNA Scores by the localization of the lesion

Lesion	PCNA Score				P=0.0078 *
	Median	Mean	Std. Error	95% CI for the Mean	
Glottic (n=8)	32	31.30	3.44	23.17-39.43 (Range, 16.58-44.07)	glottic versus supraglottic ** (p > 0.05)
Supraglottic (n = 3)	35	42.29	7.85	8.51-76.07 (Range, 33.56-57.96)	supraglottic versus transglottic** (p > 0.05)
Transglottic (n=14)	58	55.43	4.42	45.87-64.98 (Range 29.25-81.57)	glottic versus transglottic ** (p= < 0.05)

* Kruskal-Wallis one-way ANOVA
** Mann-Whitney-U tests

Table II
Distribution of PCNA Scores by the Pathological Grade

Grade	PCNA Score				P=0.0294 *
	Median	Mean	Std. Error	95% CI for the Mean	
I (n=6)	33	32.25	3.00	24.52-39.97 (Range, 18.36-40.36)	grade I and grade II** (p > 0.05)
II (n = 17)	44	48.42	4.18	39.55-57.28 (Range,16.158–75.00)	Grade II and grade III** (p > 0.05)
III (n=2)	68	68.37	13.2	99.35-236.09 (Range, 55.17-81.57)	grade I and grade III ** (p= < 0.05)

* Kruskal-Wallis one-way ANOVA
** Mann-Whitney-U tests

Table III
Distribution of PCNA scores by the Clinical Stage

Stage	PCNA SCORE Median	Mean	Std. Error	95% CI for the mean	P=0.0198 *
I (n=11)	34	35.68	4.64	25.34-46.03 (Range, 6.58-75.00)	stage I and stage II** (p > 0.05)
II (n = 3)	44	46.59	5.92	21.13-72.05 (Range, 38.06-57.96)	stage II and stage III** (p > 0.05)
III (n=11)	60	56.46	4.91	45.52-67.39 (Range, 29.25-81.57)	stage I and stage III ** (p= < 0.05)

* Kruskal-Wallis one-way ANOVA

Table IV
Distribution of Lymph node Metastases According to Prognosis of the Patients.

	Prognosis		
Node	Remission (%)	Recurrence (%)	Total (%)
absent	13 (81.3)	3 (18.8)	16 (100.0)
present	0 (0.0)	9 (100.0)	9 (100.0)
Total	13 (52.0)	12 (48.0)	25 (100.0)

Fisher's Exact chi-square Test p =0.00011

Table V
Distribution of PCNA scores according to lymph node metastases and prognosis of patients

	PCNA SCORE Median	Mean	Std. Error	95% CI of the mean	Mann-Whitney-U-test
NODE absent (n=16)	36	36.77	2.72	(-)38.65-(–)13.34	p=0.0013
Present (n=19)	66	62.77	5.18		
PROGNOSIS remission (n=13)	34	34.29	2.75	(-)35.68-(–)13.65	p=0.0005
recurrence (n=12)	61	58.96	4.47		

Dfferences between different PCNA scores and clinical stages of the patients were analysed using the nonparametric test of Kruskal-Wallis one-way ANOVA and the overall difference was found to be statistically significant (P=0.0198). When further statistical analysis was carried out using the Mann-Whitney-U test the actual significant difference was found to be between PCNA scores of stage I and stage III (Table 3, Fig 2 and p=0.0095)

In table 4 classification of patients according to the presence of lymph node metastases in the neck and prognosis of the patients is given. The difference was found to be statistically significant using the Fisher's Exact Chi-Square Test (p=0.00011).

When lymph node metastasis was present recurrence occurred in all of the patients; however when there was no lymph node metastasis detected, recurrence occurred only in 18.8% of the patients (p=0.00011).

In table 5 the differences between lymph node metastases and prognosis according to the PCNA scores are presented and all the differences were found to be statistically significant using the Mann-Whitney-U test (p=0.0013 and p=0.0005 respectively). In other words PCNA scores were shown to be 62.77±5.18 when lymph node metastasis was present; however it was only 36.77±2.72 when there was no lymph node metastasis. In the patients in whom recurrence was observed the PCNA score was 58.96±4.47; whereas in patients in whom there was remission, PCNA scores were lower, being 34.29±2.75; The results can also be observed in Fig 3.

Statistical analysis was carried out to see if there is any difference between age of the patients and the clinical stage and the difference was found to be not significant (p=0.3579; Kruskal-Wallis one-way ANOVA).

Statistical analysis for sex of the patients was not carried out because we had only one female patient.

Discussion

Malignancies arising from the head and neck are of many histopathologic types. The most common malignancy, accounting for 90% of the total, is squamous cell carcinoma (6) Squamous cell carcinoma arising from the upper aerodigestive tract carries with it a significant morbidity and mortality and, over the last few decades, its incidence has steadily increased (7). The management of patients requires thorough investigation to determine the local, regional, and distant extent of the disease, and treatment options include surgery, radiotherapy, chemotherapy, or combinations. Recent advances in treatment have not led to a measurable improvement in overall survival (8). The most significant advances of the last few decades have resulted in improved functional and cosmetic results, but improvement in patient survival has been disappointing.

Despite the large number of therapeutic and histopathologic studies in print, there is currently no morphologic or cytologic feature available that consistently predicts the outcome in patients with laryngeal carcinoma. The main factors influencing the prognosis of laryngeal carcinoma are stage of the tumour, site of the tumour, lymph node metastasis, and therapeutic procedures employed (9-11).

The PCNA immunostaining technique, which is a relatively simple method, estimates the degree of cellular proliferation. Tumour cell proliferation activity is believed to give a fair appraisal of the degree of biological aggression. Rapidly growing cancers generally have a greater mortality than slow growing ones, and in several types of cancer, including head and neck cancer, prognostic significance depends on the cell proliferation rate (12).

PCNA has been studied in many human neoplasms, and has been shown to correlate with the prognosis in non-Hodgkin's lymphoma, chronic lymphoid leukemia, transitional cell carcinoma of bladder, and in gastric, hepatocellular, colorectal, breast, ovarian and pharyngeal cancers (13-21). PCNA has also been used to predict the response to radiotherapy and chemotherapy. Better responses to radiotherapy has been reported in patients with high PCNA has also been used to predict the response to radiotherapy and chemotherapy. Better responses to radiotherapy has been reported in patients with high PCNA scores in cervical and laryngeal cancers (22,23). Tsuji et al (24) observed that, in oral cavity cancers, the mean PCNA score decreases significantly after cancer chemotherapy and concluded that the response of cancer cells to anticancer agents may be estimated by consecutive measurement of PCNA, since the PCNA score dropped after treatment in cases showing a favourable prognosis.

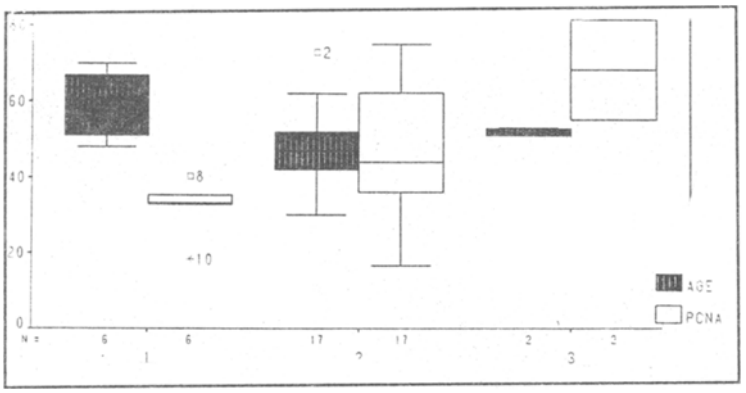

Fig 2. Distribution of PCNA and Age according to pathological grade of the tumour.

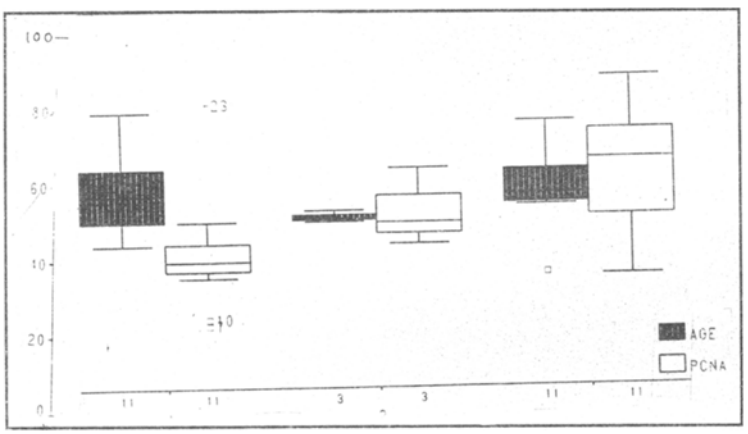

Fig 3. Distribution of PCNA and Age according to clinical stage of the tumour

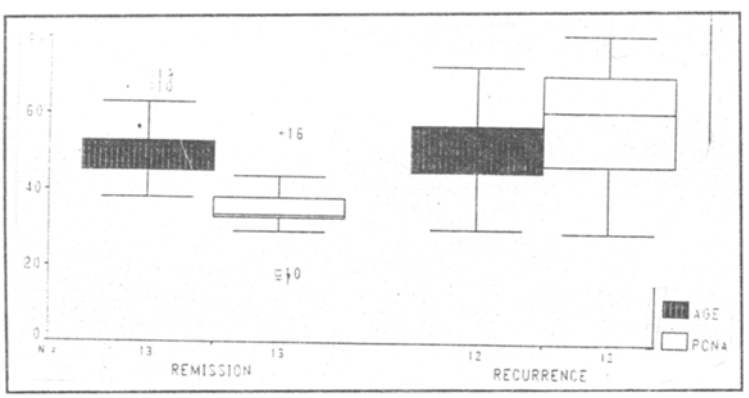

Fig 4. Distribution of PCNA and Age according to prognosis of the tumour.

Lampe (25) studied squamous cell carcinoma of the head and neck and found that DNA aneuploidy and high PCNA scores correlating with advancing pathological nodal stage can be evidence and a measure of biological aggressiveness as related to metastatic potential. The author concluded that the combination of DNA ploidy with PCNA positivity may be the biological marker needed to predict tumour behavior and biological aggressiveness.

Sarac et al. (26) reported 92 patients with squamous cell carcinoma of the larynx. They found significant corelation between PCNA scores and grade to the tumour, cervical metastasis, lymphovascular invasion, tumour margins, local recurrence and survival. The authors concluded that the PCNA score seems to be a more sensitive parameter in predicting tumour proliferation, occult lymph node metastasis and prognosis. Welkoborsky et al. (27) studied 40 patients with laryngeal tumours, No significant correlation was found in their study between the PCNA score and stage of the tumour; however, he reported all recurrent tumours to show high PCNA scores. The authors concluded that PCNA is a better indicator of tumour aggressiveness than tumour stage. Munck-Wikland et al. (28) reported 38 patients with laryngeal carcinoma having in situ lesions which progressed to invasive cancer together with a clear tendency towards more pronounced DNA aberration, a higher percentage of intense PCNA staining and more frequent p53 positivity.

Analysis measuring correlation with PCNA scores was also performed in precancerous lesion and normal tissues of the larynx. PCNA scores showed a statistically significant

difference between precancerous lesion and normal tissues of the larynx (19). PCNA scores of 47 patients with squamous cell carcinoma of the larynx were measured after partial laser resection. A much weaker relationship was found between PCNA scores and tumour recurrence rate (30).

In the present pilot study we found statistically significant differences when the distribution of PCNA scores was analysed according to pathological grades and clinical stages. The differences between lymph node metastasis and prognosis according to the PCNA scores are presented and the difference was found to be statistically significant. When lymph node metastasis was present, recurrence occurred in only 18.8% of the patients.

Several studies as discussed above, show a close relationship between PCNA scores and squamous cell carcinoma of the larynx. These data suggest that PCNA scores can be correlated with the malignant potential of the larynx and might be used as an additional prognostic factor when planning the appropriate treatment.

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