

# Prognostic Significance of PCNA Expression in Laryngeal Cancer

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**Objective:** To assess the prognostic value of proliferating cell nuclear antigen (PCNA) in laryngeal carcinoma and its relation with other known prognostic clinicopathologic variables.

**Design:** A retrospective cohort study of 92 patients chosen randomly from patients treated between 1964 and 1993 with the diagnosis of laryngeal cancer. Prognostic factors including PCNA expression, grade, lymphovascular invasion, depth of tumor margins, neck metastasis, and clinical outcome were evaluated.

**Setting:** Hacettepe University Medical Faculty, Ankara, Turkey.

**Patients:** Eighty-five men and 7 women operated on for squamous cell carcinoma of the larynx were studied. Sixty-nine patients had total and 20 patients had partial laryngectomy with neck dissection, and 3 patients had endolaryngeal tumor excision.

**Intervention:** Hematoxylin and eosin-stained sections were reevaluated for grade, lymphovascular invasion, and depth of tumor margins; sections stained with monoclonal antibody against PC10 were examined for PCNA expression.

**Results:** The PCNA index correlated with grade, lymphovascular invasion, depth of tumor margins, neck metastasis, and local-regional recurrence. The PCNA index values of patients with occult metastasis were significantly higher than those of patients without metastasis ( $P=.006$ ).

**Conclusions:** The PCNA index is a more sensitive variable than grade in predicting tumor proliferation, occult lymph node metastasis, and prognosis. These results suggest that the PCNA index can be used in decision making for treatment and assessment of prognosis in laryngeal carcinomas.

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**P**REDICTING THE outcome of laryngeal cancer is of paramount importance for the physician to decide on the treatment modality. Despite the many investigations on cytologic and morphologic properties, currently there are no absolute criteria that can predict the outcome of laryngeal cancer and guide the physician to select the appropriate therapy and to decide on the extent of surgery and the convenience of neck dissection in patients with N0 tumors. One of the most important criteria that shows the aggressive biological behavior is the cellular proliferation rate of a tumor. Consequently, several proliferation markers have been studied for their relation with the recurrence and metastatic rate of the tumor and mortality.

Proliferating cell nuclear antigen (PCNA [cyclin]) is a 36-kd, nuclear, non-histone protein that appears in the nucleus during the late G1 phase, increases during the S phase, and declines during the G2 and M phases.<sup>1</sup> The main function of PCNA is in the regulation of DNA synthesis and cell proliferation as an auxiliary protein of DNA polymerase  $\delta$ .<sup>1</sup> This study was performed to assess the prognostic value of PCNA in laryngeal carcinoma and its relation with

other known prognostic clinical and histopathologic variables.

## RESULTS

Of 92 patients, 85 (92%) were men and 7 (8%) were women. Their ages ranged from 31 to 75 years, and the mean ( $\pm$  SD) age was  $52.9 \pm 9.1$  years.

Tumor staging was done according to the criteria of the American Joint Committee on Cancer.<sup>4</sup> Seven patients (8%) had stage I, 22 patients (24%) had stage II, 41 patients (45%) had stage III, and 22 patients (24%) had stage IV disease.

Six patients (7%) had glottic lesions, 15 patients (16%) had supraglottic lesions, and 71 patients (78%) had transglottic lesions.

According to their lesions, 69 patients (75%) had total laryngectomy, 20 patients (22%) had partial laryngectomy (horizontal supraglottic, vertical, or subtotal), and 3 patients (3%) had endolaryngeal excision. Excluding the 3 patients who had endolaryngeal excision, all patients had unilateral or bilateral modified radical or radical neck dissections.

Histopathologic examination revealed lymph node metastasis in 45 patients (49%). Seventeen patients (19%)

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## PATIENTS, MATERIALS, AND METHODS

For this retrospective study, 100 patients were randomly chosen from 849 patients who underwent surgery for squamous cell carcinoma of the larynx at the Department of Otolaryngology, Head and Neck Surgery, Hacettepe University Medical Faculty, Ankara, Turkey, between 1964 and 1993. Eight patients whose paraffin blocks were missing were excluded from the study. The medical charts of the remaining 92 patients were examined. Sex, age, stage, operative procedure, and disease-free survival periods were recorded. Pathological examinations included conventional histopathologic studies and immunohistochemical identification of PCNA.

### CONVENTIONAL HISTOPATHOLOGIC EXAMINATIONS

For conventional histopathologic examination, 6- $\mu$ m paraffin sections stained with hematoxylin and eosin were examined. Tumors were graded according to morphologic differentiation: grade 1 is well differentiated, grade 2 is moderately differentiated, and grade 3 is poorly differentiated or undifferentiated.<sup>2</sup> Tumor margins were classified as  $\alpha$  (pushing borders),  $\beta$  (pushing-infiltrative borders), and  $\gamma$  (infiltrative borders). Lymphovascular invasion was classified as positive or negative according to the presence or absence of tumor cells in lymphovascular spaces.

### IMMUNOHISTOCHEMICAL EXAMINATIONS

For immunohistochemical detection of PCNA, 6- $\mu$ m-thick sections obtained from paraffin blocks were mounted on glass slides and incubated at 56°C overnight. A modi-

fication of the immunoglobulin enzyme bridge technique was used, as described previously.<sup>3</sup> The slides were air-dried, deparaffinized, and dehydrated and rehydrated with phosphate-buffered saline solution. The slides were then incubated with methanol containing 0.3% hydrogen peroxide to block the endogenous peroxidase activity and treated with normal horse serum to block the nonspecific proteins. After washing with phosphate-buffered saline solution, the slides were incubated with monoclonal PCNA antibody against PC10 (DAKO, Glostrup, Denmark) for 60 minutes at room temperature. The slides were washed again with phosphate-buffered saline solution and finally stained with avidin-biotin peroxidase complex (Vectastain, Vector Laboratories, Burlingame, Calif) method. Appropriate positive and negative control tests were done.

Evaluation of immunohistochemical staining was performed by counting at least 1000 cells in at least 5 different regions of the tumor. In patients with cervical lymph node metastasis, the primary tumor and the metastatic node were evaluated separately. The ratio of cells stained for PCNA to the total number of cells was recorded as the proliferative index (PI) (**Figure 1**). The PI in primary tumors is called TPI and the PI in lymph node metastasis is called LPI.

Conventional histopathologic and immunohistochemical examinations were performed by the pathologist (A.A.) without knowing the clinical outcome of the patients.

### STATISTICAL ANALYSIS

Statistical analysis was performed with a computer program (Statistical Package for the Social Sciences, SPSS for Windows v 6.0, SPSS Inc, Chicago, Ill). The data obtained from this study were evaluated by nonparametric tests (Mann-Whitney *U* and Kruskal-Wallis), the Wilcoxon signed rank test, the Fisher exact test, the log-rank test, Cox regression analysis, and Kaplan-Meier survival analysis.

died of local-regional recurrence, and 5 patients (5%) developed distant metastasis. Five patients were lost to follow-up. The other patients have at least 2 years' follow-up, with median follow-up of 118 months.

### CONVENTIONAL HISTOPATHOLOGIC EXAMINATIONS

Results of conventional histopathologic examination revealed that 27 patients (29%) had grade I, 39 patients (42%) had grade II, and 26 patients (28%) had grade III tumors.

Tumor margins were  $\alpha$  in 29 patients (32%),  $\beta$  in 37 patients (40%), and  $\gamma$  in 24 patients (26%). Two patients had endolaryngeal strippings, so tumor margins and lymphovascular invasion could not be evaluated.

Lymphovascular invasion was positive in 61 patients (66%) and negative in 29 patients (32%).

### PCNA IMMUNOHISTOCHEMICAL ANALYSES

Results of PCNA immunohistochemical analysis revealed that TPI values ranged from 3.1 to 94.3 (median, 45.2) and LPI values ranged from 21.3 to 98.0 (median, 67.0).

Results of nonparametric tests (Kruskal-Wallis and Mann-Whitney *U*) revealed a significant correlation be-

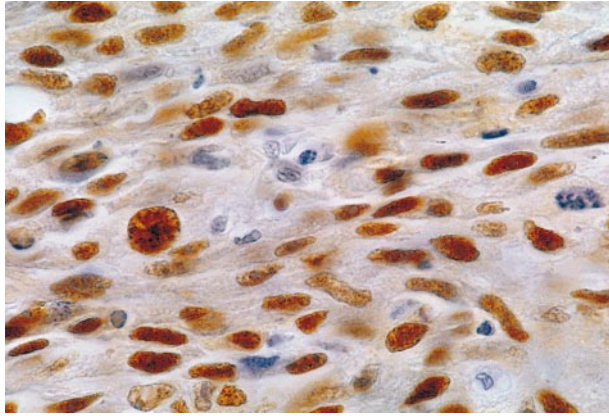
tween TPI and the following variables:

- Grade ( $\chi^2 = 32.4$ ,  $P = .0000$ ;  $P < .05$ ); the correlation was significant between grades I and II ( $z = -4.52$ ,  $P = .0000$ ;  $P < .05$ ) and between grades I and III ( $z = -5.23$ ,  $P = .0000$ ;  $P < .05$ ); no significant correlation was observed between grades II and III ( $z = -1.61$ ,  $P = .11$ ;  $P > .05$ ).
- Lymph node metastasis ( $z = -4.99$ ,  $P = .0000$ ;  $P < .05$ ).
- Lymphovascular invasion ( $z = -2.66$ ,  $P = .007$ ;  $P < .05$ ).
- Tumor margins ( $\chi^2 = 7.1$ ,  $P = .03$ ;  $P < .05$ ); the correlation was significant between  $\alpha$  and  $\gamma$  margins ( $z = -2.61$ ,  $P = .009$ ;  $P < .05$ ); no significant correlation was observed between  $\alpha$  and  $\beta$  ( $z = -1.03$ ,  $P = .30$ ;  $P > .05$ ) and  $\beta$  and  $\gamma$  ( $z = -1.82$ ,  $P = .07$ ;  $P > .05$ ) margins.
- Local-regional recurrence ( $z = -3.41$ ,  $P < .001$ ;  $P < .05$ )

No correlation was found between TPI and stage ( $\chi^2 = 5.7$ ,  $P = .13$ ;  $P > .05$ ) and distant metastasis ( $z = -1.65$ ,  $P = .10$ ;  $P > .05$ ).

Tumor grade was correlated with lymph node metastasis ( $z = -4.22$ ,  $P = .0000$ ;  $P < .05$ ) and lymphovascular invasion ( $z = -4.38$ ,  $P = .0000$ ;  $P < .05$ ).

No correlation was observed between grade and other variables, namely, tumor margins ( $\chi^2 = 4.7$ ,  $P = .19$ ;  $P > .05$ ), stage ( $\chi^2 = 4.5$ ,  $P = .22$ ;  $P > .05$ ), local-regional recurrence ( $z = -1.83$ ,  $P = .07$ ;  $P > .05$ ), and distant metastasis ( $z = -0.64$ ,  $P = .53$ ;  $P > .05$ ).



**Figure 1.** Laryngeal carcinoma showing high proliferating cell nuclear antigen expression, as indicated by brown nuclear staining. Anti-proliferating cell nuclear antigen, immunoperoxidase, original magnification  $\times 725$ .

**Table 1. Comparison of TPI Values in Patients With and Without Occult Metastasis\***

	No. (%)	Mean Rank (TPI)
Metastasis (-)	32 (64)	19.81
Metastasis (+)	18 (36)	35.61
<b>Total</b>	<b>50 (100)</b>	<b>...</b>

\*TPI denotes proliferative index in primary tumors. Mann-Whitney U:  $Z = -3.68$ ;  $P < .05$ .

Results of histopathologic examination of 50 patients with N0 tumors revealed that 32 patients (64%) had no cervical metastasis, whereas 18 patients (36%) had occult cervical metastasis. The TPI values of patients with occult metastasis were significantly higher than those of patients without metastasis ( $z = -3.68$ ,  $P < .001$ ;  $P < .05$ ) (**Table 1**). When the analysis was performed using grade instead of TPI, no correlation was found (Fisher exact test,  $P = .15$ ;  $P > .05$ ).

The patients with N0 tumors were classified into 2 groups: those having TPI values less than the median TPI value (45.2) and those with TPI values of 45.2 or greater. The occult metastasis rate in the group with TPI less than 45.2 was 17.2%, whereas in the group with TPI of 45.2 or greater, this rate was 61.9%, and this difference was statistically significant ( $t = 3.43$ ,  $P = .006$ ;  $P < .05$ ) (**Table 2**).

When LPI values of 45 patients with cervical metastases were compared with TPI values of the same tumors, it was observed that LPI values were significantly higher than TPI values (Wilcoxon test,  $z = -3.74$ ,  $P = .002$ ;  $P < .05$ ).

Univariate survival analysis using the log-rank test revealed that the factors affecting disease-free survival were TPI, cervical lymph node metastasis, grade, tumor margins, and N stage (**Table 3**).

Multivariate analysis was done by Cox regression analysis with forward selection. The factors affecting the disease-free survival independently were TPI ( $\chi^2 = 10.0$ ,  $P = .002$ ;  $P < .05$ ) and cervical metastasis ( $\chi^2 = 7.9$ ,  $P = .005$ ;  $P < .05$ ), with hazard ratios of 1.04 and 0.45 consecutively for 95% confidence interval. The other clinicopathologic factors that had prognostic significance in univariate analysis did not reveal statistical significance when entered into the multivariate model.

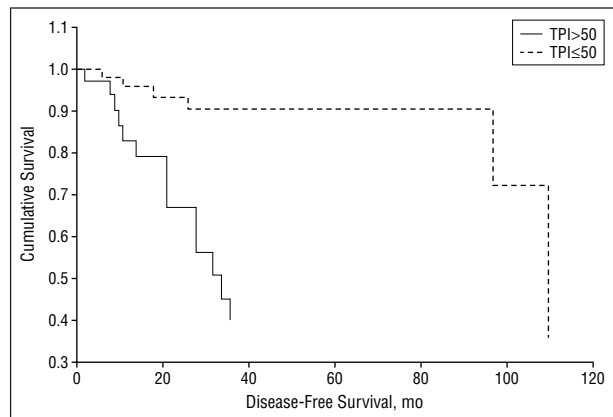
**Table 2. Comparison of Occult Metastasis Rates and TPI Values Below or Above the Median in Patients With N0 Tumors\***

TPI Value	Metastasis (-)	Metastasis (+)	Total	Occult Metastasis Rate, %
<45.2	24	5	29	17.2
$\geq 45.2$	8	13	21	61.9
<b>Total</b>	<b>32</b>	<b>18</b>	<b>50</b>	<b>...</b>

\*TPI denotes proliferative index in primary tumors.  $t = 3.43$ ;  $P < .05$ .

**Table 3. Univariate Survival Analysis for Clinicopathologic Variables**

Variable	Disease-Free Survival	
	$\chi^2$	P
Proliferative index in primary tumor	15.3	<.001
Cervical metastasis	16.7	<.001
T stage	3.8	.28
N stage	8.5	.04
Grade	6.3	.04
Lymphovascular invasion	1.5	.23
Sex	1.2	.28
Tumor margin	6.3	.04
Localization of the lesion	3.7	.29



**Figure 2.** Survival of patients stratified by the proliferative index in primary tumors (TPI) score.

Kaplan-Meier survival analysis was done for TPI by stratifying the TPI score into those with PI values of 50 or less and those with PI values greater than 50. A statistically significant difference in survival was observed between the 2 groups ( $P < .05$ ) (**Figure 2**).

#### COMMENT

The main factors affecting the prognosis of laryngeal carcinoma are cervical lymph node metastasis,<sup>5</sup> grade,<sup>5-7</sup> tumor margins,<sup>6,7</sup> perineural<sup>7</sup> and vascular<sup>8</sup> invasion, depth of tumor,<sup>9</sup> lymphoreticular infiltration, and tumor-associated tissue eosinophilia.<sup>10,11</sup> However, these variables do not suffice for the prediction of prognosis in laryngeal carcinoma. This led us to focus mainly on proliferation markers that reflect the aggressive nature of tumors. The most widely used proliferation markers currently available are thymidine labeling index, bromodeoxyuridine labeling index, argy-

philic nucleolar organizer regions, Ki-67, DNA polymerase  $\alpha$ , DNA flow cytometry, and PCNA.

Proliferating cell nuclear antigen expression has been studied in many human neoplasms. The PI has been shown to correlate with the prognosis in non-Hodgkin lymphoma<sup>12</sup> in gastric,<sup>13</sup> breast,<sup>14</sup> ovarian,<sup>15</sup> and pharyngeal<sup>16</sup> cancers. The risk of regional lymph node metastasis was higher in patients with a high PI in transitional cell carcinoma of bladder<sup>17</sup> and breast cancer.<sup>14</sup> The PI also has been used to predict the response to radiotherapy and chemotherapy. Better responses to radiotherapy have been reported in patients with a high PI in cervix<sup>18</sup> and larynx cancers.<sup>19</sup> Tsuji et al<sup>20</sup> observed that the mean PCNA score decreased significantly after cancer chemotherapy in oral cavity cancers and concluded that the response of cancer cells to anticancer agents may be estimated by consecutive measurement of PCNA because the PCNA score dropped after treatment in patients with a favorable prognosis.

Munck-Wikland et al,<sup>21</sup> in a study of 38 patients, stated that the in situ laryngeal carcinomas that progressed into invasive cancer showed a clear tendency toward more pronounced DNA aberration, a higher percentage of intense PCNA staining, and more frequent p53 positivity. By combining the results of these analyses, the authors classified 82% of the lesions as progressors or nonprogressors. Welkoborsky et al<sup>22</sup> found that the PCNA score was highly associated with prognosis, whereas no correlation was found between PCNA score and recurrent disease.

In the present study, significant correlations were found between TPI and grade, cervical metastasis, lymphovascular invasion, tumor margins, local-regional recurrence, and survival. Although grade was correlated with lymphovascular invasion, depth of tumor margins, and cervical metastasis, it has no value in predicting local-regional recurrence and survival. The TPI score seemed to be a good variable for predicting the occult cervical metastasis as well. The risk of occult cervical metastasis was 17.2% for patients with TPI values less than the median (45.2), whereas the risk increased to 61.9% for those with TPI values of 45.2 or greater.

During immunohistochemical analysis, it was observed that PCNA staining was heterogeneous. This finding, which showed that there are groups of cells with different proliferation rates, also has been emphasized by Shin et al.<sup>23</sup> The theory that cell groups with different proliferation rates may have different metastatic potentials (metastatic heterogeneity) has gained acceptance in recent years.<sup>24,25</sup> According to this theory, the metastatic cells should have higher proliferation rates than the original tumor cells. When we compared LPI values with TPI values, we observed that LPI values are significantly higher than TPI values, thus supporting the metastatic heterogeneity theory.

One of the most important factors affecting prognosis in laryngeal cancer is local-regional recurrence. The TPI score seemed to be strongly correlated with local-regional recurrence. All 17 patients who developed recurrences had TPI values higher than the median (45.2). Multivariate analysis revealed that the only factors affecting survival independently are TPI and cervical metastasis. The role of grade, however, in predicting recurrence and survival was limited and unreliable.

Although there was a strong correlation between tumor grade and PCNA immunostaining, TPI seems to be a more

sensitive variable in predicting tumor proliferation, occult lymph node metastasis, and prognosis. These results suggest that the PCNA index can be used in decision making for treating and assessing prognosis in laryngeal carcinomas.

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