

Preliminary results of prognostic significance of proliferating cell nuclear antigen expression in advanced primary larynx carcinomas and lymph node metastases

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Abstract

Introduction: The aim of this study was to investigate the prognostic significance of proliferating cell nuclear antigen (PCNA) expression in laryngeal carcinoma in relation to clinicopathological features. Special emphasis was placed on examining the relationship of PCNA expression in the primary tumour and PCNA expression in corresponding lymph node metastases obtained from the same patients.

Material and methods: The study included 60 patients with advanced larynx carcinoma who had received treatment and follow-up for at least 5 years. Sixty laryngeal carcinoma specimens and metastatic lymph nodes from 24 patients were examined for immunohistochemical PCNA expression.

Results: The percentages of PCNA positive cells were significantly higher in the primary tumours which developed lymph node metastases than in those without metastases. The fraction of PCNA immunolabelled cells in metastatic lymph nodes increased significantly when compared with the PCNA positive cell score in their corresponding primary tumours obtained from the same patient. There was a significant difference in PCNA index score in primary tumours between the group of patients who survived a 5-year period and those who died within 5 years after treatment.

Conclusions: Our data demonstrate that a high proliferation index in primary larynx tumours is retained and increased in corresponding lymph node metastases. Measurement of the fraction of cancer cells stained for PCNA in primary larynx carcinomas can be helpful in selecting tumours with high aggressiveness potential that are more likely to develop neck metastases and thereby in identifying patients who need elective lymph node dissection or additional treatment.

Key words: proliferating cell nuclear antigen, larynx cancer, metastases.

Introduction

Predicting the outcome of laryngeal cancer is of utmost importance for the physician to decide on the treatment modality. Detection of a lymph node metastasis is the crucial factor in evaluating the necessity of neck dissection and the extent of surgery in patients with advanced laryngeal

carcinomas. Clinical examination and imaging investigations of lymph node metastases such as computed tomography (CT) and magnetic resonance imaging (MRI) are helpful in the diagnostic process, but they are associated with false-positive or false-negative results [1-3]. Thus, additional parameters of the tumour to predict its more aggressive potential are needed, among them immunohistological analysis. Although different histological tumour biomarkers are not direct factors in predicting the occurrence of lymph node metastases, their evaluation could help categorize primary tumours with clinically more aggressive potential.

One of the most important criteria that show aggressive biological behaviour is the cellular proliferation rate of the tumour. Cell proliferation in a retrospective study involving archival material is mainly measured by means of immunohistochemical expression of Ki-67 protein and proliferating cell nuclear antigen (PCNA) [4, 5]. Proliferating cell nuclear antigen is a DNA polymerase-associated protein needed for DNA replication and repair [6]. Proliferating cell nuclear antigen appears in late G1 phase, increases during S phase, and declines during G2 and M phases of the cell cycle [7], and is a potential biomarker of a variety of tumours [8-10], including laryngeal carcinomas [11-17]. The fraction of PCNA positive

cells progressively increases with the degree of epithelial hyperplasia in the larynx [18, 19], and inversely correlates with the degree of differentiation in larynx cancer [14, 15, 20]. The ratio of PCNA positive cells was also related to the occurrence of nodal metastases [11-14, 17] and PCNA immunolabelling in biopsy specimens was useful in identifying patients with clinically negative lymph nodes who were at considerable risk of occult metastases and may benefit from elective neck dissection [15]. However, these results are in contradiction to other reports showing no association between PCNA expression and presence of neck metastases [20-22]. Although several studies have implicated factors measuring cell proliferation in laryngeal tumours, only one has examined their expression in metastatic deposits [15]. Because therapy is also targeted toward metastatic disease, the assumption is made that expression of the tumour marker in secondary lesions reflects the situation observed in the primary tumour. To investigate this suggestion, the present study has evaluated the expression of the PCNA antigen in primary laryngeal carcinomas and their corresponding lymph node metastases in order to identify tumours which are more likely to metastasize.

Material and methods

Patients

The study included 60 patients, who were diagnosed with laryngeal cancer and underwent surgical treatment. Of these patients, 15 required total laryngectomies (TL), 11 total laryngectomies followed by radiotherapy (TL/RD), 12 total laryngectomies and functional neck dissection (TL/ND), and 22 total laryngectomies and radical neck dissection followed by radiotherapy (TL/ND/RD). Patient data are summarized in Table I.

Slide preparation and section analysis

Paraffin-embedded 4.5 μ m thin sections were stained with haematoxylin and eosin and assessed histopathologically. Immunohistochemical analysis of proliferating cell nuclear antigen was performed on 60 laryngectomy specimens and on 24 lymph node metastasis specimens using the avidin-biotin-peroxidase technique. In brief, specimens were dewaxed with xylene, dehydrated in ethanol and incubated in 3% hydrogen peroxide for 5 min to exhaust endogenous peroxidase activity. Epitope unmasking was performed using 0.1% trypsin, 0.1% CaCl_2 and 0.05 M Tris solution (pH 7.6) for 20 min at room temperature. After blocking in normal bovine serum, tissue sections were incubated with 1 : 100 diluted monoclonal PC10 antibody (Dako, Glostrup, Denmark). Biotinylated rabbit anti-mouse

Table I. Patient characteristics

Characteristic	Number of patients
Total number	60
Median age (range)	55 (34-77)
Male/female	57/3
Tumour grading:	
• well differentiated (G1)	10
• moderately differentiated (G2)	37
• poorly differentiated (G3)	13
TNM classification:	
• T2	8
• T3	34
• T4	18
• N0	32
• N1	6
• N2	15
• N3	7
Clinical stage:	
• II	7
• III	23
• IV	30

immunoglobulin (Dako, Glostrup, Denmark) was diluted 1 : 300 and used as a secondary antibody.

Proliferating cell nuclear antigen expression was assessed in tumour areas showing the highest density of staining, as determined by an initial scan at a magnification of 40 \times . For PCNA index counting, at 200 \times the field in each of the most stained areas was counted and the average count determined. The growth fraction by PCNA staining was evaluated by counting 500 consecutive cells for each tumour and an index of positive cells to total number of cells was scored. All pathological material was reviewed by two pathologists without knowledge of the clinical outcome and other clinicopathological features. Because of the PCNA scoring discrepancy between pathologists, samples of lymph node metastases from 4 patients were removed from the study.

Statistical analysis

Proliferating cell nuclear antigen immunostaining values (mean \pm SD) were compared with clinicopathological factors by means of ANOVA test, paired Student's *t*-test, and Pearson's correlation test. A value of $p < 0.05$ was considered to be statistically significant.

Results

Proliferating cell nuclear antigen index in primary tumours and lymph node metastases

Positive PCNA immunostaining was found in all 60 larynx tumours and all analyzed lymph node metastases from 24 patients. The PCNA index in laryngeal carcinoma ranged from 15.8 to 73.0% and from 50.4 to 87.4% in lymph node metastases. The percentages of PCNA positive cells were significantly higher in the primary tumours associated with lymph node metastases than in those without metastases (Table II). We also analyzed the degree of cell proliferation directly in metastatic lymph nodes of 24 patients. The fraction of PCNA stained cells in metastatic lymph nodes increased significantly when compared with the PCNA positive cell score in their corresponding primary tumours as calculated by means of paired Student's *t*-test ($p < 0.001$) and Pearson's correlation test ($p = 0.011$). Mean index of PCNA positive cells was 69.97 \pm 10.09% ($n = 24$) in metastases vs. 50.27 \pm 12.89% ($n = 28$) in primary tumours which developed metastases.

PCNA score and TNM/clinical classification

Clinical stage of neoplastic disease is often characterized by TNM classification. In our study the mean PCNA proliferation index was the highest in advanced larynx cancers classified as clinical

TNM stage IV, and decreased in tumours obtained from patients with clinical TNM stages III and II of the disease. ANOVA test analysis revealed that differences between these three values were statistically significant. There was no significant association between mean proliferation rate and T stage.

Proliferating cell nuclear antigen index and histological grade of the tumour

There was also a significant inverse correlation between PCNA mean proliferation index in primary larynx carcinoma and histological grade measured by means of ANOVA test. Mean score of PCNA positive cells was highest in poorly differentiated (G3) carcinomas, decreased in moderately differentiated (G2) carcinomas, and was the lowest in well differentiated (G1) carcinomas (Table II).

Proliferating cell nuclear antigen score and overall survival rate

We observed that mean PCNA index in primary tumours was lower in patients who survived a 5-year period (Table II). However, having analyzed the PCNA score in connection with different treatment modalities, such a correlation was found only in the group of patients that underwent total

Table II. Correlation between PCNA index and pathological parameters in patients with larynx cancer

Factor	PCNA index (% positive cells) in primary tumours	Number of patients	<i>p</i> Value
Tumour grading:			
• G1	31.63 \pm 11.06	10	0.006
• G2	45.01 \pm 15.64	37	
• G3	51.43 \pm 12.03	13	
T stage:			
• T2	36.88 \pm 23.66	8	0.33
• T3	44.70 \pm 12.99	34	(NS)
• T4	46.44 \pm 15.18	18	
Presence of lymph node metastases:			
• N0	38.84 \pm 15.54	32	0.003
• N1-3	50.27 \pm 12.89	28	
Clinical stage:			
• II	31.84 \pm 20.39	7	0.036
• III	42.95 \pm 13.64	23	
• IV	47.98 \pm 15.37	30	
5-year survival:			
• alive	39.23 \pm 16.06	26	0.025
• died	48.11 \pm 13.77	34	

Table III. Correlation between PCNA index in primary larynx tumour, treatment modality and 5-year survival period

Treatment modality	PCNA index in primary larynx tumour		Statistical significance
	Patients survived 5 years	Patients died within 5 years	
TL (n = 15)	383.7 ±38.73 (n = 11)	521.3 ±60.61 (n = 4)	p = 0.0813, ns
TL + RD (n = 11)	318.6 ±55.58 (n = 5)	571.2 ±65.59 (n = 6)	p = 0.0186*
TL + ND (n = 12)	432.2 ±63.43 (n = 5)	483.9 ±57.21 (n = 7)	p = 0.5565, ns
TL + ND + RD (n = 22)	481.8 ±129.1 (n = 5)	504.3 ±29.17 (n = 17)	p = 0.7930, ns

TL – total laryngectomy, RD – radiotherapy, ND – neck dissection, (Student’s t-test, *p < 0.05, ns – not significant)

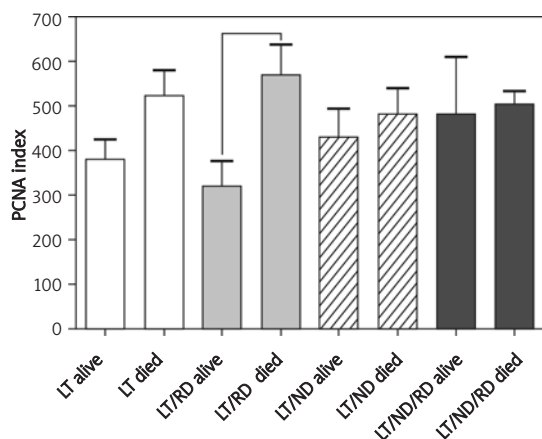


Figure 1. Correlation between PCNA index in primary larynx tumour, treatment modality and 5-year survival period

TL – total laryngectomy, RD – radiotherapy, ND – neck dissection, *p < 0.05, Student’s t-test

laryngectomy followed by radiotherapy (p < 0.05). A similar relationship was observed in patients who received other treatments but it was not statistically significant (Table III, Figure 1).

Discussion

Detection or prediction of the occurrence of lymph node metastases in patients with larynx cancer is a crucial step in the assessment of advancement of the disease state, which influences further therapeutic strategy and clinical outcome.

In general, high proliferative activity in larynx cancer is associated with a worse clinical outcome, including recurrence, shorter survival time and presence of metastases. However, the majority of performed studies analyzed the Ki-67 or PCNA proliferation indices only in primary cancers and then correlated these values with the occurrence of neck metastases [11-13, 16, 17, 20-22]. Very rare are reports where the authors assessed the degree of cell proliferation directly in lymph node metastases. In one of such studies the number of analyzed cases was limited to 10 lymph nodes derived from patients with laryngeal and hypopharyngeal cancers [15]. In our study, we

performed an analysis of PCNA expression in lymph node metastases obtained from 24 patients who developed exclusively laryngeal carcinoma. We have noted a very high percentage of PCNA positive cells in all single metastasis specimens – the lowest score was over 50% of total cells and mean PCNA index was near 70% in metastatic lymph node samples. Paired t-test and Pearson’s correlation test showed a positive correlation between increased PCNA index in metastases and lower PCNA index in corresponding primary carcinoma obtained from the same patient. Liu *et al.* [15] reported similar results, demonstrating higher PCNA index in metastases than in primary carcinomas. Additionally, we have shown that laryngeal tumours associated with neck metastases were characterized by increased PCNA index when compared with tumours without lymph node metastases, which is consistent with a great number of studies [11-17] and in contradiction to only a few reports [20-22]. It is plausible that metastasizing laryngeal carcinomas could develop a cell population with highly proliferative potential, and this potential is retained in arising metastases. Although PCNA is not directly involved in the metastatic behaviour of cancer cells, it could be a good prognosticator for the selection of tumours which are prone to metastasis.

Increased cell proliferation is regarded as one of the most important biological mechanisms in oncogenesis. Proliferating cell nuclear antigen expression is often associated with other proteins involved in cell cycle progression. A positive correlation between PCNA expression and over-expression of cyclin-dependent kinase 2 (CDK2) [23], cyclin A [24], cyclin E [25], cyclin-dependent kinase 4 (CDK4) and cyclin D1 [26] has been found in laryngeal carcinoma, which indicates a close association between PCNA expression and proliferation activity of tumour cells. The property of cell proliferation is related to the progression of various malignancies and influences disease-free and overall survival [4, 10, 14]. In our study we demonstrated that mean PCNA index is negatively correlated with 5-year survival rate in the group of patients who underwent total laryngectomy

followed by radiotherapy. This tendency was also shown in the group of patients that received other treatment modalities, although the differences were not significant and decreased according to more aggressive therapy applied (LT/RD vs. LT/ND vs. LT/ND/RD), which was also connected with increasing clinical stage of the disease. Since patients were qualified for different treatment modalities according to clinical examinations, histological type and grading of the primary tumour, and imaging investigations (CT, occasionally also lymphoscintigraphy), the type of treatment was dependent on clinical stage of the neoplastic disease. Therefore differences between PCNA scores measured in the group of patients who survived and those who died within a 5-year period and received the same therapy could to some extent reflect the proliferation potency of larynx tumours; however, they are greatly influenced by the local progression of the neoplastic disease. The PCNA index assessment was more prognostic in the group of patients with less advanced disease who also received less aggressive therapy. The high PCNA score found in this group of patients should draw our attention to considering more aggressive therapy.

Several studies have reported increased proliferative indices associated with tumour grade [14, 16, 20, 21]. The grade of laryngeal carcinoma was an important factor affecting recurrence [27], and thereby together with biological markers reflecting tumour proliferation potential could be useful for the detection of tumours that require more aggressive treatment [28]. We observed an inverse correlation between PCNA index and tumour grading. These findings are consistent with some studies [14, 16, 20, 21] and in contradiction to others [12, 22]. More detailed histological examinations showed that PCNA immunostaining corresponds to the histological grade of benign epithelial hyperplastic lesions and squamous cell carcinoma of the larynx [18, 19]. In well differentiated, keratinizing carcinomas, PCNA staining was observed in layers adjacent to the basal membrane at the periphery of the tumour islands, while in less differentiated, non-keratinizing carcinomas, the number of PCNA positive cells increased and they tended to be dispersed throughout the sample [19]. It was also reported that immunostaining of PCNA was heterogeneous within each laryngeal carcinoma and varied widely between tumours [14], which could be responsible for the observed discrepancy between different reports.

Despite these practical limitations, the pre-therapeutic assessment of PCNA expression may become of increasing importance in the evaluation of tumour aggressiveness and of prognostic value

in predicting neck metastases in patients with laryngeal carcinoma, especially when analyzed in preoperative biopsy specimens [15].

In conclusion, our data demonstrate that a high proliferation index in primary larynx tumours is retained and increased in corresponding lymph node metastases. The measurement of the fraction of cancer cells stained for PCNA in the primary larynx carcinomas can be helpful in selecting tumours with high aggressiveness potential that are more likely to develop neck metastases, and thereby in identifying patients who need elective lymph node dissection or additional treatment.

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