Correlation of proliferating cell nuclear antigen and bcl-2 expression with tumor front grading and metastasis in laryngeal squamous cell carcinoma

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This study was designed to examine the immunohistochemical expression of proliferating cell nuclear antigen (PCNA) and bcl-2 protein in 45 cases with advanced laryngeal squamous cell carcinoma who had undergone total laryngectomy with unilateral modified radical neck dissection, and the relation of this expression to some prognostic factors such as tumor front grading and neck lymph node metastases. Sections were reevaluated for routine histologic grade, tumor front grading and neck lymph node metastases, and were stained with monoclonal antibodies against PCNA and bcl-2. Significant correlation was present between the severity of PCNA expression and incidence of lymph node metastasis ($p<0.05$). No correlation was found between the severity of PCNA expression and tumor front grading. Bcl-2 expression did not associate with either parameters.

In conclusion, PCNA is important in predicting prognosis and no association is present between the bcl-2 protein expression and prognostic factors.

Key words: Laryngeal carcinoma, prognosis, tumor front grading, neck lymph node metastases, bcl-2 protein, PCNA.

Carcinoma of the larynx is the most common head and neck neoplasm in adult patients; it represents about 0.7% of the total cancer mortality [21]. Histopathologic grade has consistently proven unsatisfactory in predicting survival and prognosis [4, 5]. After treatment based on clinical parameters, many patients still are not cured and some patients that are cured may have been treated successfully with less extensive therapy [24]. For predicting the outcome of laryngeal cancer treatment modality decision is of paramount importance for the physician. Thus, the specific biology of the tumor is probably of critical importance in predicting prognosis. In this study, we made a comparison between expression of bcl-2 protein and proliferating cell nuclear antigen (PCNA) with some parameters which had been shown to correlate with tumor progression formerly, and we tried to obtain an idea about specific biology of the tumor.

PCNA, originally known as a cyclin, is a 36-kD nuclear protein that acts as a co-factor for DNA polymerase-δ. PCNA is synthesized in the late G1 and S phase of the cell cycle. It has been suggested that the presence of an increased number of PCNA-positive cancer cells is associated with aggressive malignant behavior [3, 13].

The bcl-2 proto-oncogene has been considered to be a cell death suppressor gene that regulates the programmed cell death – apoptosis. The growth rate of neoplasms depends on the proliferation and death rates of cancer cells. Apoptosis may be related in part to the death rates of cancer cells as a negative regulating system in the growth of neoplasms. Mutations in the bcl-2 gene inhibit apoptosis, which may contribute to the development of tumors and modify their clinical behavior [11, 17, 23].

Regional lymph node metastases at the time of diagnosis were found to carry a poor prognosis in laryngeal carcinoma. Patients who presented with no regional lymph node metastases had significantly better disease-free and overall survival rates than those with involved lymph nodes [5, 8]. Tumor front grading (TFG) was proven to be associated with prognosis in squamous cell carcinomas in larynx and other locations [4, 12, 26].

The purpose of this study was to analyze the expression of PCNA and bcl-2 protein in squamous cell carcinoma of the

larynx and to evaluate the relationship of these markers to prognostic factors such as TFG and regional lymph node metastases.

Material and methods

We conducted a retrospective analysis of 45 man patients with total laryngectomy and unilateral modified radical neck dissection type-1 [2]. All of them had clinically advanced squamous cell carcinoma of stage T3 or T4 before operation, based on the American Joint Committee TNM staging system [1]. All tissues were obtained from the Department of Pathology, Ondokuz Mayis University Medical Faculty, Samsun, Turkey, between 1993–1997. Patients ranged from 40 to 76 years.

The examinations included routine histology, morphologic grading and histological examination of lymph nodes in the neck dissection specimens.

Routine histological paraffin sections were obtained and stained with hematoxylin and eosin (H&E). Tumors were classified according to morphologic differentiation as well, moderately, and poorly differentiated squamous cell carcinoma (grades I, II, III) [9].

TFG was performed on H&E-stained sections according to earlier references [4, 26]. Peripheral and most invasive parts of the tumor (tumor front) were chosen. The tumor front is in direct contact with the host. The following features at the tumor front were analyzed: 1) cytoplasmic differentiation, 2) nuclear differentiation, 3) number of mitoses per high-power field, 4) mode of invasion, 5) stage of invasion, and 6) lymphoid infiltration. These factors were assessed in at least five different tumor regions. Each factor was graded on a scale ranging from 1 to 4, with 1 being mild and 4 being severe. The total TFG score was computed as the sum of the six parameters, with a maximum score of 24 points.

Immunohistochemical detection of the PCNA and bcl-2 protein. For immunostaining with the PCNA antibody, sections were deparaffinized in xylene, and then rehydrated through graded ethanol series to water. The slides were then incubated with 3% hydrogen peroxide to block the endogenous peroxidase activity. After washing in phosphate-buffered saline (PBS) and treated with normal horse serum to block the non-specific proteins, the slides were incubated with the PCNA antibody (Ab PC 10, Dako, Glostrup, Denmark) at room temperature for 10 minutes. The slides were then washed again with PBS and stained with the biotin-streptavidin amplified (BioGenex, San Ramon) method. For immunohistochemical detection of the bcl-2 protein, sections were obtained and processed just like in PCNA immunostaining until rehydration was completed. The slides were then incubated with methanol containing 0.3% hydrogen peroxide to block the endogenous peroxidase activity. Thereafter, slides were boiled for 10 minutes in citrate buffer, cooled to room temperature and treated with normal horse serum to block the non-specific proteins. After being incubated with the bcl-2 antibody (Zymed, CA, USA) at room temperature for 50 minutes, they were washed again with PBS and stained with the biotin-streptavidin amplified (BioGenex, San Ramon) method. Counterstaining with Mayer's hematoxylin for 1 minute was final step for both immunoreaction.

Basal cells of squamous epithelium were used as internal positive control tissue for PCNA. For bcl-2 staining, sections from human follicular lymphoma were used as positive control. Internal positive control for bcl-2 staining was performed according to the infiltrating lymphocytes adjacent to the tumor. Negative controls for both immunostainings were carried out by substituting the primary antibody with PBS.

The results of immunostaining were evaluated independently by three pathologists (CB, BK, LY), and any discrepancy was resolved by joint review.

PCNA expression was examined for each tumor at its most invasive zones (tumor front). Evaluation of immunohistochemical staining was performed by counting at least 1000 cells in at least 5 different regions of the tumor front. The final result was expressed as the percentage of positive nuclei per 1000 cells. The percentages of positive cells were classified as follows: more than 50% of the total cells were stained against PCNA in the nucleus (+++ strongly positive); 26–50% of the total cells were stained (++ moderately positive); 1–25% of the total cells were stained (+ slightly positive); no staining present in any of the cancer cells (– negative).

Staining for bcl-2 at the tumor front was semi-quantitatively evaluated: negative (-); slight, which showed less than ‹10% of the cells staining positively (+); moderate, with immunopositivity in 10 to 75% of the cells (++); and strong, more than 75% of the cells staining for the bcl-2 protein (+++).

Statistical analysis. Differences in the degree of bcl-2 and PCNA expression in metastasizing and non-metastasizing cases were analyzed by chi-square test. Front grading scores were compared between metastasizing and non-metastasizing cases using non-parametric test (Mann-Whitney U). The correlation between TFG score and intensity of immunostaining was analyzed using Spearman rank correlation coefficients. Probability values less than 0.05 were considered as statistically significant.

Results

Specimens from 45 patients with laryngeal carcinomas were examined. Squamous cell carcinoma was confirmed histologically in all laryngectomy specimens. Lymph node

Table 1. Results of routine histologic grade, TFG, PCNA and bcl-2 immunoreactivity

Metastasizing					Non-metastasizing				
No	Grade	TFG	PCNA	Bcl-2	No	Grade	TFG	PCNA	Bcl-2
1	I	11	+++	–	1	I	13	+++	+
2	I	13	+++	+	2	I	10	–	++
3	I	16	+++	+	3	I	12	+++	–
4	I	14	+++	+	4	I	16	+++	++
5	I	13	+++	++	5	I	12	+++	++
6	I	18	+++	–	6	I	12	+	++
7	I	12	+++	+	7	I	8	+	–
8	I	15	+++	–	8	II	14	+	+
9	I	16	+++	+	9	II	14	++	+
10	II	14	++	–	10	II	13	++	–
11	II	13	+	–	11	II	12	++	–
12	II	15	+	+	12	II	13	+++	+
13	II	14	+++	++	13	II	12	+++	–
14	II	19	+++	–	14	II	15	++	–
15	II	17	+++	+	15	II	16	+	–
16	II	13	+++	+	16	III	16	+	+
17	II	18	+++	+	17	III	14	–	+
18	II	18	+++	+++	18	III	16	+++	+
19	II	14	+++	–	19	III	16	++	–
20	II	17	+++	–	20	III	18	+++	+
21	III	12	+++	+					
22	III	17	+++	+++					
23	III	21	+++	–					
24	III	19	++ +						
25	III	14	+++	++					

+++ strongly positive, ++ moderately positive, + weakly positive, – negative, TFG: tumor front grading, PCNA: proliferating cell nuclear antigen.

Table 2. Comparison of the PCNA expressions in the metastasizing and non

		PCNA Immunoreactivitity			Total
		-/+	++	+++	
Metastasizing cases	n	3	3	19	25
n=25	percent	12	12	76	100
Non-metastasizing cases	n	7	5	8	20
n=20	percent	35	25	40	100
Total	n	10	8	27	45
n=45					

Table 3. Comparison of the bcl-2 protein expressions in the metastasizing and non metastasizing cases

		Bcl-2 Immunoreactivity			Total
		–	+	++/+++	
Metastasizing cases	n	9	9	7	25
n=25	percent	36	36	28	100
Non-metastasizing cases	n	8	8	8	20
n=20	percent	40	40	20	100
Total	n	17	17	11	45
n=45					

metastases were confirmed in 25 of 45 neck dissection specimens. In dissection specimens with lymph node metastasis, at least two or more lymph nodes were involved. Routine histologic grade, TFG, bcl-2 and PCNA immunoreactivity results are shown in Table 1. Comparison of the immunoreactivity results in the metastasizing and non-metastasizing cases are presented in crosstab 2 and 3. PCNA showed a marked increase in staining of metastasizing cases. These results showed that stronger staining for PCNA was associated with a higher incidence of metastasis ($p<0.05$). For bcl-2, the difference between metastasizing and non-metastasizing cases was statistically not significant ($p>0.05$). TFG revealed an average score of 15.32 points in metastasizing cases (ranged from 11 to 21), but an average score of 13.60 points in non-metastasizing cases (ranged from 8 to 18). Difference between these two groups was statistically significant ($p<0.05$).

Statistically no correlation was found between the intensity of PCNA and bcl-2 staining with TFG score (for PCNA: $r=0.192$, $p=0.20$; for bcl-2: $r=–0.026$, $p=0.86$).

Discussion

Proliferating cell nuclear antigen immunoreactivity is found in the proliferating compartment of normal non-neoplastic tissue as a sign of proliferation. For example, staining is seen in germinal centers of lymphoid tissue, basal layer of stratified squamous epithelia, proliferative compartments of gastrointestinal tract epithelia, epithelial and stromal cells of proliferative phase endometrium, and spermatogonia of the testis [7]. There is considerable evidence that assessing cellular proliferation in a variety of tumors provides useful information and may be of major prognostic importance. For example, PCNA immunoreactivity may be of particular value in the identification of hemangiopericytoma and esophageal carcinoma at greatest risk of rapid tumor metastasis and early death [10, 15]. Previous studies suggested that PCNA index can be used in treatment decision making and assessment of prognosis in laryngeal carcinomas [18, 25, 28]. In contrast, PCNA did not associate with the prognosis in another study [16]. We found no or some relation between TFG and PCNA positivity, but the difference between metastasizing and non-metastasizing cases was statistically significant for PCNA immunoreactivity, and for TFG score. We can say that high PCNA positivity and high TFG score may increase the probability of lymph node metastasis.

The bcl-2 oncogene was detected for the first time by cloning the breakpoint of t(14;18) in follicular lymphoma. Initial studies showed that the biological properties of B-cell lymphoma with t(14;18) had an indolent course. Thus the attention was directed to this topic [23]. Expression of the bcl-2 in some kinds of malignant tumors was examined and

association of the clinicopathologic parameters and grade was investigated. Some investigators showed that the bcl-2 protein expression in breast carcinomas was associated with favorable clinicopathological features, on the other hand, expression of the bcl-2 protein in prostate carcinoma was correlated with poor prognosis [14, 20]. The expression of the bcl-2 protein is associated with neck metastases in the carcinoma of the nasopharynx, but no association is present with the histologic grade or neck metastases in the tongue carcinoma [19, 27]. In the laryngeal squamous cell carcinoma, bcl-2 expression was low and not associated with the prognosis and metastasis [6, 22]. In our study, we could neither find association between expression of the bcl-2 protein with both metastasis and TFG in laryngeal carcinoma.

When our results are interpreted together with the literature, it can be concluded that TFG and PCNA is important in understanding and assessing prognosis independent one from the other. On the other hand there are conflicting results in the literature related to the prognostic value of PCNA in laryngeal carcinomas, and further investigations are required for the clarification of this subject. While bcl-2 expression is suggested to have relationship in the prognosis of different types of tumors such as prostate and breast carcinomas, any association seems lacking with the prognostic factors in laryngeal squamous cell carcinoma.

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