

# *In situ* apoptotic and proliferation index in laryngeal squamous cell carcinomas\*

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**Abstract.** The rate of cellular growth is mainly influenced by the balance between cell proliferation and cellular decay. Since to our knowledge, no study so far has analysed the rate of proliferation and apoptosis in the normal laryngeal mucosa and in invasive laryngeal carcinomas, we performed a morphological analysis on both parameters in biopsies from 30 patients with laryngeal carcinoma. We applied the TUNEL end labelling technique for the investigation of apoptosis and immunohistochemistry (Ki-67 antigen) for the determination of the cell proliferation.

In our study we demonstrated that invasive tumour growth of the larynx coincides with an increase of both cellular proliferation and apoptosis. Both parameters, however, affected various tumour areas differently. While there was a preferential expression of the Ki-67 antigen at the tumour–stroma interface, apoptotic figures could be found randomly distributed in the tumour. This indicates that the replication of tumour cells and tumour cell decay are differently distributed and possibly independently regulated. Since we observed a particularly strong increase of cell proliferation at the tumour–stroma interface which outnumbered the corresponding rate of apoptosis by far, the enhanced cell proliferation at the tumour border seems to be a main factor for tumour growth.

A statistical evaluation revealed significant correlation between the apoptotic index and the degree of tumour cell differentiation, indicating that a high rate of apoptosis coincides with a high level of tumour cell differentiation.

There was, however, no statistically significant correlation between prognostic clinical parameters and the rate of apoptosis or that of proliferation.

Keywords: Larynx carcinoma, apoptosis, Ki-67 antigen, prognostic parameters

## 1. Introduction

The maintainance of a regular balance between cell proliferation and cellular decay is a principle for normal tissue function. This balance seems to be significantly disturbed in autonomous neoplastic growth and it is conceivable that neoplastic growth is promoted by factors increasing cell proliferation or inhibiting cell death. Cell death can manifest as necrosis, which is associated with distinct cell injury, for example, induced by ischemia, or as physiological or programmed death, apoptosis. Apoptosis is considered to be an active regulatory response by inducible cells to a specific stimulus.

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In several malignant tumours the biological significance of the apoptosis has already extensively been investigated, especially in colorectal, ovarian and oesophageal cancer [1,3,6]. These studies provide evidence that the rate of apoptosis provides information on the growth kinetics of tumours and thus indirectly on the clinical course of the disease.

Squamous cell carcinomas (SCC) of the larynx are the most common malignancies of the upper aerodigestive tract in the Western hemisphere with an incidence of about 5–7 cases/100,000 each year. In recent years, a number of studies on prognostic factors in head and neck cancer have been published with the aim of improving the prediction of the course of the disease and the probability of tumour recurrence. Well-established prognostic factors are the TNM-classification and the degree of tumour cell differentiation [8].

Until now, the rate of apoptosis has not been analysed in laryngeal SCC and only a few studies exist on the proliferative capacity of the tumour cells in SCC of the larynx [4,10,15]. In these studies a correlation between the proliferation index, measured by use of the Ki-67 antigen or as AgNOR labelled cells and the histological grading of the laryngeal carcinomas was found.

There exists, however, no comprehensive study analysing the possible interaction between proliferation and apoptosis in laryngeal carcinomas.

We therefore designed a study using immunohistochemical and *in situ* end labelling techniques to determine the rate of proliferative and apoptotic cells in laryngeal SCC, in order to find changes in the level of cellular proliferation and apoptosis between invasive carcinomas and normal laryngeal mucosa and to identify the spatial distribution of both processes.

## 2. Patients and methods

The present series was conducted on 30 cases with laryngeal SCC. Four patients were females, 26 patients were males, with a age ranging between 38 and 79 years. As a common risk factor, all patients presented an history of longstanding nicotine abuse.

The analysis of the degree of tumour cell differentiation revealed 9 cases of well-differentiated carcinomas; 10 were classified as moderately differentiated and 11 cases showed poor tumour cell differentiation.

In addition, we analyzed 5 cases of normal laryngeal mucosa which served as a control group.

From all patients tissue specimens were obtained during surgical treatment of SCC of the larynx. For controls, specimens were taken from routine autopsies performed within 6–10 h post-mortem. Control cases had no anamnesis of longstanding nicotine or alcohol abuse and the specimens showed a normal histomorphological pattern. All tumour biopsies as well as the tissue samples of the normal laryngeal mucosa had been fixed in 4–6% buffered formalin and embedded in paraffin wax for routine diagnostic examination.

### 2.1. Immunohistochemistry

Appropriate tissue sections were selected for the immunolocalization of the proliferation marker Ki-67 antigen, using the monoclonal antibody MIB 1, according to established procedures.

Following deparaffinization the immunostaining procedure comprised (i) enzymatic pretreatment for the enhancement of the immunostaining, (ii) reaction with the monospecific primary antibody MIB 1 (Dako, Hamburg, Germany), (iii) reaction with a secondary antibody system coupled with alkaline phosphatase (APAAP method [2]), (iv) visualization of the coupled antigen by a chromogenic substance (fast red.: Sigma Chem., Deisenhofen, Germany).

## 2.2. TUNEL method

For the labelling of apoptotic cells we used the end labelling of DNA (TUNEL method) [5]. After deparaffinization and enzymatic digestion the tissue sections were immersed in 2–8% hydrogen peroxide for 5 min and rinsed twice with PBS for 5 min. Subsequently, the equilibration buffer was placed on the specimens and incubated for 10–15 s at room temperature followed by TdT enzyme with incubation for 1 h at 37°C.

To stop the enzyme activity the specimens were transferred to a washing buffer for 10 min at room temperature. Before applying anti-digoxigenin peroxidase, the slides were washed with three changes of PBS for 5 min, then incubated for 30 min at room temperature. After several washings with PBS, the DAB working solution was added and stained for 3–6 min at room temperature. Counterstaining was performed with haemalaun (10 min at room temperature).

## 2.3. Morphometric evaluation

All slides were subjected to a morphometric evaluation by counting of labeled cells per 1000 tumour cells. For each specimens, at least 3500 tumour cells were evaluated in at least 5 randomly selected tumour areas. The evaluation was performed by two independent observers, the intra- and inter-observer variability was acceptably low (less than 10%).

## 2.4. Statistics

For statistical analysis of the results, the patients were divided into two groups. As a distinction criterium, we choose the overall mean values for the rate of apoptosis and cell proliferation. Therefore, our patients were separated into a group with a high proliferation index of more than 14% labelled cells and one with less than 14%. For the evaluation of apoptosis, the patients were divided into a group with more than 1% apoptotic figures and a group with less than 1%.

In a further step, were correlated the proliferation and apoptosis index with the clinical outcome of the patients as determined by the tumour stage (TNM-classification) and the presence or absence of local or regional recurrences and for metastases.

The statistical evaluation was performed using the *Fisher exact two-tail test*, with a level of significance of  $p < 0.05$  being regarded as statistically significant. For the evaluation of the tumour recurrence interval, *Kaplan–Meier curves* were calculated.

## 3. Results

### 3.1. Rate of cell proliferation and apoptosis in controls

In all 5 biopsies of normal mucosa a positive staining for MIB 1 could be observed in a few cells, however, affecting only the basal layers of the squamous epithelium. The average proliferation index ranged between 0.5 and 2.3% of all cells.

The level of apoptosis in the normal mucosa was also very low, ranging of up to 0.2% of the mucosa cells. Apoptotic figures were seen exclusively at the superficial layer, but not in the basal region.

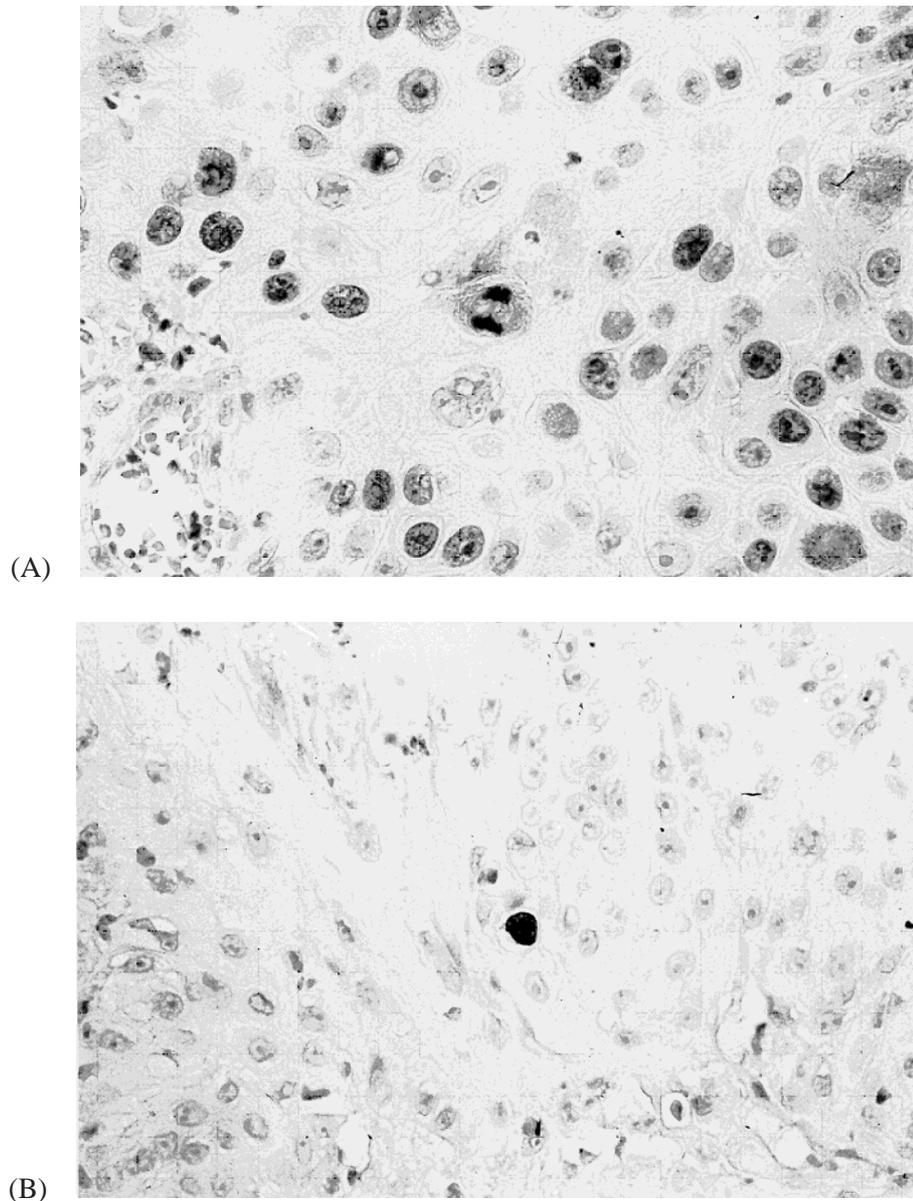


Fig. 1. Cellular proliferation and apoptosis in a poorly differentiated laryngeal carcinoma. (A) In this poorly differentiated carcinoma a high level of proliferation can be observed, measured by positive Ki-67 labelling. (B) The same tumour reveals comparably lower degree of apoptosis. The apoptotic figures are randomly distributed over the tumour. (Magnification  $\times 400$ .)

### 3.2. Rate and localization of apoptosis in laryngeal carcinomas

In general, the number of apoptotic cells detected by the TUNEL method was low in all tumour specimens, ranging between 0.32 and 2.74% of tumour cells. The positively labelled cells showed no preferential localization, they were fairly randomly distributed over the tumour samples (Fig. 1(A)). There was particularly no altered rate of apoptosis to be recorded at the tumour–stroma interface (Fig. 2(A)).

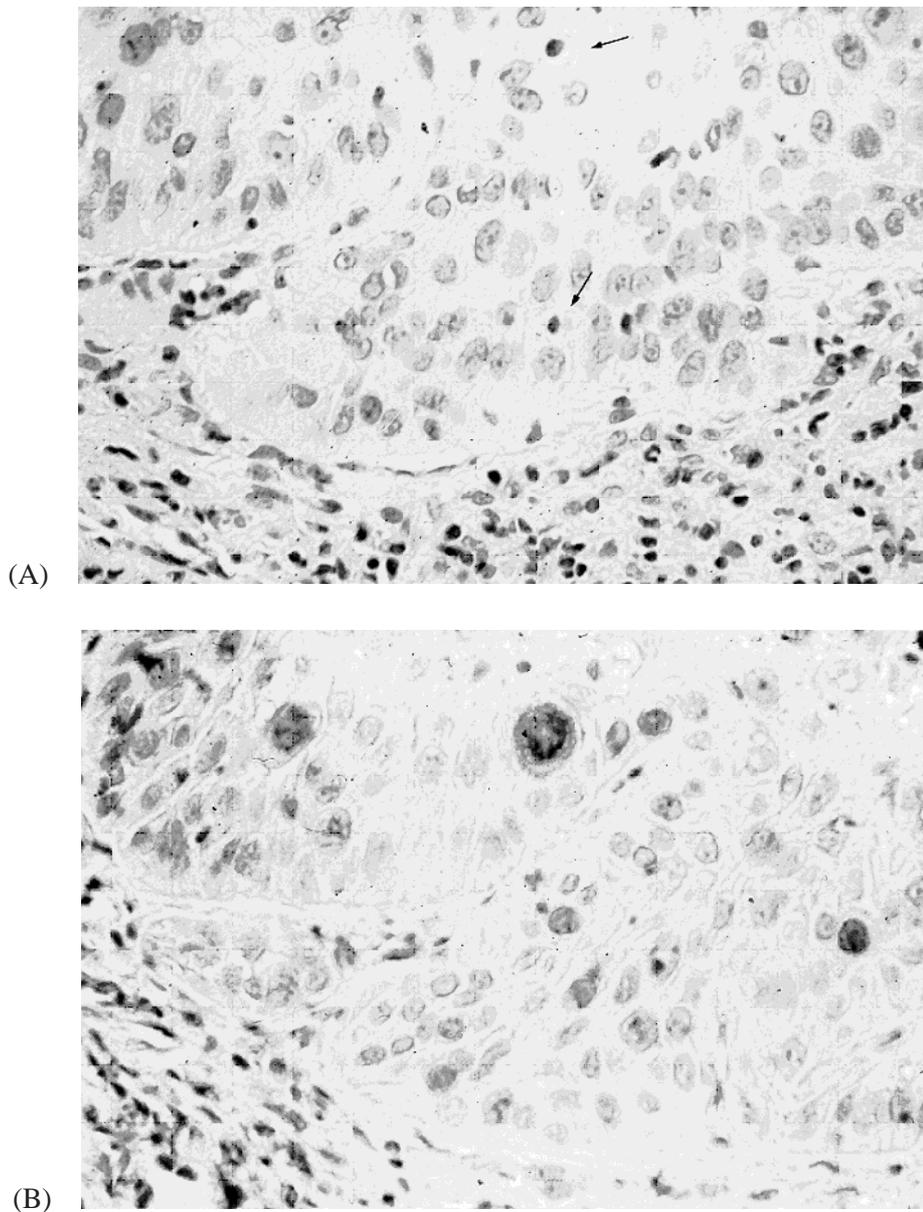


Fig. 2. Immunohistochemical staining of Ki-67 antigen and TUNEL hybridization of cellular apoptosis in a moderately differentiated carcinoma. At the tumour–stroma interface only very few apoptotic cells are present (arrows) (A), while in the same tumour in corresponding areas the Ki-67 index is markedly higher (B). (Magnification  $\times 400$ .)

With respect to the tumour cell differentiation, the following particular findings could be made. When compared to less differentiated tumours the highest values were found in the 9 well-differentiated carcinomas, ranging between 0.9 and 2.29% (median = 1.63%; mean = 1.73%). In the 10 moderately differentiated SCC the apoptotic index varied between 0.32 and 2.74 (median = 1.19%; mean = 0.84%) and was therefore lower than the well-differentiated carcinomas. A comparably reduced apoptotic index could be further observed in poorly differentiated SCC. Here the level of

Table 1  
Statistical analysis of the correlation between apoptosis and cell proliferation and prognostic parameters in laryngeal carcinomas

	Apoptosis	Ki-67 antigen
Grading	$p = 0.02$	$p = 0.08$
TNM-classification		
T-stage	$p = 0.11$	$p = 0.57$
N-stage	$p = 0.57$	$p = 0.57$
Tumour recurrence		
12 months	$p = 0.71$	$p = 0.1$
24 months	$p = 0.69$	$p = 0.33$
36 months	$p = 1.0$	$p = 0.3$

apoptosis ranged between 0.4 and 1.3% of tumour cells (median = 0.82%; mean = 0.77%). The statistical evaluation showed a significant correlation between the histological degree of tumour cell differentiation and the level of apoptosis in laryngeal carcinomas using the *Fisher exact two-tail test* ( $p = 0.02$ ) (Table 1).

When we compared the apoptosis index with the prognostic clinical parameter of the tumour stage, divided into two groups with small tumours (T1 and T2) and large tumours (T3 and T4), no further significant correlation was seen with regard to tumour cell apoptosis ( $p = 0.11$ ). The existence of lymph node metastases did also not correlate with the degree of tumour cell apoptosis ( $p = 0.57$ ) (Table 1).

We further compared the apoptotic rate with the local tumour recurrence in the post-operative period (12, 24 and 36 months). Over a period of 36 months, 8 patients developed a local tumour recurrence. However, we could not find any statistically significant correlation between the level of apoptosis of the patients with or without of local recurrence ( $p = 0.71$  for 12 months;  $p = 0.69$  for 24 months;  $p = 1.00$  for 36 months) (Table 1).

### 3.3. Rate and localization of proliferating cell in laryngeal carcinomas

All 30 SCC of the larynx contained positively labelled cells by immunostaining for the proliferation antigen Ki-67 (Fig. 1(B)). These cells were preferentially expressed at the tumour–stroma interface (Fig. 2(B)).

The percentage of positive nuclei varied between 2.27 and 29.9 for all carcinomas. In well-differentiated SCC the proliferation index was between 5.26 and 12.3% (median = 8.4%; mean = 7.4%). Patients with a moderately differentiated carcinoma had on average an almost doubled labelling index for Ki-67, when compared to well-differentiated carcinomas. The value ranged between 8.5 and 26% of labelled tumour cells (median = 16%; mean = 14.3%). We found in moderately differentiated SCC the highest level of proliferation. In poorly differentiated SCC the degree of proliferation was again lower, with a median value of 12% of positively labelled tumour cells (range 2.3–29.9%; mean = 10.3%).

The statistical evaluation revealed no significant correlation between the expression of the Ki-67 antigen and the degree of tumour cell differentiation, but a higher grading of the tumour seems to be associated with a higher expression of Ki-67 antigen ( $p = 0.08$ ) (Table 1).

For the statistical analyses of the relationship between the tumour stage (TNM-classification) and the proliferation index, a  $p$ -value of 0.57 was calculated for both the local tumour size (T-stage) and the lymphatic tumour spread (N-stage) (Table 1). Therefore the proliferation index did not correlate

significantly with the tumour stage. Furthermore, no significant correlation could be found between the local tumour recurrence and the level of tumour cell proliferation ( $p = 0.1$  for 12 months;  $p = 0.33$  for 24 months and  $p = 0.3$  for 36 months) (Table 1).

#### 4. Discussion

Cell proliferation and cellular decay are essential factors in controlling proper function of the tissues. This control seems to be lost in tumoural growth. At present, the mechanisms are still unknown and there lack data on the extent of both factors in a multitude of different tumours. Only a few reports regarding cell proliferation and SCC of the head and neck have been published [4,9,10,12,13,15]. To the best of our knowledge, no data have been presented for the rate of programmed cell death in this tumour type.

We therefore designed a study to determine the quantitative and localization expression of the Ki-67 antigen, as a proliferation marker, and by use of the TUNEL method, of apoptotic cells in laryngeal SCC. In our study we were able to show that invasive tumour growth of the larynx coincides with an increase in cellular proliferation and also with an increase of cellular apoptosis. In normal epithelium Ki-67 positive cells were confined to isolated basal cells, as described previously [12]. The number of detected apoptotic cells was generally much lower than the level of cellular proliferation, for the normal mucosa as well as for the invasive SCC. This agrees well with the known dynamics of these processes [7]. Therefore, a direct comparison between the rate of cell proliferation and apoptosis is not feasible. However, the estimation of the proportions and their changes seems to be possible.

In carcinomas, the expression of both parameters, increased, however, affecting distinct tumour areas differently. There was a local preferential expression of the Ki-67 antigen at the tumour–stroma interface, similar to the results of [12] whereas apoptotic figures could be found fairly randomly distributed over the tumour. This indicates that the replication of tumour cells and tumour cell decay are differently and possibly independently regulated. Since we observed a particularly strong increase of cell proliferation at the tumour–stroma interface which outnumbered the corresponding rate of apoptosis by far, the enhanced cell proliferation at the tumour border seems to be the main factor for the net growth of laryngeal carcinomas.

A significant correlation could be found between the apoptotic index and the degree of tumour cell differentiation. A low apoptotic index coincides with a poor degree of tumour cell differentiation. This is in line with the findings of Hamada et al. [6] in oesophageal cancer, where they also observed a significant correlation between a high level of apoptosis and a high degree of tumour cell differentiation ( $p < 0.05$ ). In contrast to our observations, in ovarian and colorectal carcinomas a correlation between low apoptotic index and a lower malignant potential of the tumour has been described [3]. Obviously, the regulation of apoptosis in neoplastic tissue seems to be a complex process that is probably regulated differently depending on the respective tumour type [14].

However, we could not find a statistically significant correlation between the proliferation index and the tumour cell differentiation, although we observed an increase of Ki-67 antigen expression with a decrease of SCC differentiation. This may be due to the still limited number of observations in our series requiring clarification by the analysis of further cases. This increase of proliferation in poorly differentiated SCC was also found in other previous studies. Eckel et al. [4] and Olofsson and Golusiunski [9] found a significant correlation between a high level of Ki-67 positive tumour cells and a low degree of tumour cell differentiation. In contrast to these studies, we and Spafford et al. [13] were not able to find a correlation either between the proliferation marker Ki-67 and the TNM-classification,

or between the proliferation and the individual prognosis of the patient, determined by the local tumour recurrence. Reasons for these differences in the statistical evaluation of the Ki-67 antigen expression may lie in the areas of investigation. Our observation indicates an inhomogeneous expression of the Ki-67 antigen within the tumour showing a selectively high rate of proliferation at the tumour–stroma interface. A selection of this area for analysis may provide completely different observations than those made over the whole tumour area. Therefore, we suggest that the selection of the tumour area is of exceptional importance for the evaluation of the proliferation rate. We propose that especially the tumour–stroma interface should be investigated in further studies on the proliferation rate of SCC.

In summary, our observations provide data for a better understanding of invasive laryngeal tumour growth. The observed unbalance between cell proliferation and apoptosis seems to play an important role in the rate and probably in the speed of malignant tumour growth.

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