Immunohistochemical Staining of Ki-67 Using the Monoclonal Antibody Ki-S11 Is a Prognostic Indicator for Laryngeal Squamous Cell Carcinoma

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Abstract. *Background: Proliferative activity has been shown to be of prognostic significance for several malignancies. Ki-67, a cell cycle associated antigen, is regarded as a promising proliferation marker. Very few results on the proliferative activity of head and neck cancer and their potential prognostic value are available. Materials and Methods: The proliferative activity of 104 squamous cell carcinomas of the larynx (SCCL) was analyzed retrospectively with the monoclonal antibody Ki-S11 which specifically detects the Ki-67 antigen. Median follow-up time was 47 months. Results: There was a statistically significant correlation ($p<0.05$) between histopathological grading, N-status and proliferative activity. There was also a significant difference for the 5-year survival between low and highly proliferating tumours. The patient group with low proliferating laryngeal cancer had a statistically ($p<0.05$) longer absolute and recurrence-free 5-year-survival time than patients with a highly proliferating cancer. Conclusion: These results show that Ki-67 staining of SCCL with Ki-S11 is a helpful prognostic indicator for squamous cell carcinoma of the larynx with a potential clinical application.*

Squamous cell carcinoma of the larynx (SCCL) is the most common malignancy of the upper aerodigestive tract in the Western hemisphere with an incidence of about 9 cases/100,000 in men and 1.5 cases/100,000 in women each year (1, 2). Prognosis of these patients is not satisfactory (3) and has not improved during the last decades despite progress in therapy (4). Five-year survival rates are described between 52% and 94% depending on tumour site, stage and tumour therapy (2, 5, 6). It is widely known that patients with carcinoma of the upper aerodigestive tract with a comparable tumour staging have a varying clinical course under the same therapy regime (7, 8). The question concerning the patients prognosis is whether there is a correlation between the biological behaviour of the tumour and any tumour biological parameter (9) that may help the clinician to make therapeutical decisions.

Known prognostic factors for survival and prognosis in laryngeal cancer patients are histopathological grading, anatomical localisation of the tumour and tumour stage according to the TNM classification (10). Of these well known prognostic factors, the TNM system is the most sufficient (8). Histopathological grading is only valid with restrictions since grading is not standardized and is regarded as a subjective marker (11, 12).

There has been much progress in the development of proliferation associated antibodies during the past few years. It can be shown that there is a clear correlation between proliferative activity and the biological behaviour of cancer, which might have impact on the patient's prognosis and consequences for the individual therapy concept (13-15).

The aim of this study was to determine the correlation between the proliferative activity of SCCL and the clinical course of the investigated patients. The association of the proliferative activity with other clinical parameters was additionally investigated. For this purpose, the expression of Ki-67, a cell cycle-associated antigen, which is expressed during all phases of the cell cycle (G1-, S-, G2-, M-phase) except the G0-phase (16, 17) was analysed by immunohistochemical staining applying Ki-S11, a monoclonal Ki-67 antibody, on paraffin-embedded laryngeal carcinoma tissues taken from 104 patients.

Materials and Methods

Patients. Tumour specimens of 104 patients with SCCL were investigated. The diagnosis of squamous cell carcinomas was confirmed at the Department of Pathology and Pathologic Anatomy,

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Key Words: Ki-67, Ki-S11, laryngeal cancer, squamous cell carcinoma.

Christian Albrechts University of Kiel. The gender ratio of 89 men to 15 women demonstrated the well-known male predominance in cases of SCCL. The patients' age at time of initial diagnosis ranged from 37 to 87 years with a mean age of 62.4 years. Patients were treated between 1991 and 1998 at the Department of Otorhinolaryngology, Head and Neck Surgery, Christian Albrechts University of Kiel, Germany, by primary surgery and, in most cases, with postoperative irradiation (n=67).

Four patients (3.8%) presented with a T1 tumour, 29 with a T2 tumour (27.4%), 32 patients (30.2%) with a T3 tumour, and 39 patients had a T4 tumour (36.8%). The tumours were classified as supraglottic in 44 (41.5%) cases, as glottic in 54 (50.9%), and as subglottic in 6 (5.7%). Histopathological grading resulted in only 3 (2.8%) well-differentiated (G1) tumours, 76 (71.7%) moderately differentiated (G2) tumours, and 25 (23.6%) tumours with a low differentiation (G3). Seventy-five patients (70.8%) did not have clinically evident cervical lymph node metastasis, whereas 8 patients (7.5%) suffered from metastasis in one (N1), and 21 patients (19.8%) in more than one (N2) cervical lymph node. Only 2 patients (1.9%) showed distant metastasis at the time of first diagnosis.

Antibodies. Primary SCCL were stained with the monoclonal antibody Ki-S11 which was developed at the Department of Hematopathology, University of Kiel, Germany (17). Antibodies were harvested as described elsewhere (18, 19). Ki-S11 is a monoclonal mouse anti-human antibody that detects the proliferation-associated 395- and 345-kDa heavy DNA-binding nuclear Ki-67 antigen (17). Ki-67 antigen is present in all phases of the cell cycle except at the G0-phase (16).

Immunohistochemistry. Four-μm-thin sections from formalin-fixed, paraffin-embedded tissue samples were routinely deparaffinized and rehydrated. Endogenous peroxidase activity was blocked by incubation with 3% hydrogen peroxide in 97% methanol for 15 minutes. For antigen demasking slides were heated in a microwave oven in a 0.01 M citrate buffer at pH 6.0 for 4 minutes and 45 seconds at maximum power. After cooling in Tris-buffer, slides were incubated with the primary antibody Ki-S11 for 60 minutes. Immunoreaction was enhanced by means of the avidin-biotin complex technique (20) using biotinylated rabbit; anti-mouse antibody (E0354; Dako, Hamburg, Germany), an avidin-biotin-complex (E377; Strept AB Complex/ HRP, Dako, Hamburg, Germany) and diaminobenzidine as a chromogen (DAB; Sigma, Deisenhofen, Germany). The sections were counterstained with Meyer's haematoxylin (Merck, Darmstadt, Germany) (Figure 1).

Tissue of a human tonsil was included in every staining procedure as a positive control. Negative controls were obtained by replacing the primary antibody by buffer to exclude non-specific reactions.

Microscopical evaluation. Evaluation and photo-documentation was performed with a regular microscope (Axioplan, Zeiss, Germany). Tissue sections were evaluated by two independent investigators without knowledge of the clinical data. In areas with intense staining reaction, five fields, were evaluated at 400-fold magnification. In each field 100 cells and the number of proliferating cells were counted. The number of positive cells as a percentage of all counted tumour cells was defined as the proliferative activity index. Only clearly nuclear staining was considered as positive. Nuclear staining of proliferating stromal cells was considered as false positive. In doubtful cases, staining was repeated.

Statistical analysis. For statistical analysis, the statistical packages SPSS PC+3, 0 (Statistical Package for the Social Sciences) and CSS:Statistica (StatSoftTM Inc., Tulsa, USA) were used. Survival curves were generated by Kaplan-Meier analysis. Cause-specific survival was defined as the time elapsed between first tumour diagnosis and tumour-related death. Differences in survival over time were checked by log-rank test and Gehans Wilcoxon test (21). All SCCL were divided into two groups. The borderline between low and highly proliferating tumours was set at 45% of proliferating cells as cut-off point. Only tumour-related deaths were regarded as uncensored observations.

Results

Proliferative activity. The percentage of proliferating cells was between 24% and 95% with a mean of 59.1% (±15.21%) and a median of 58%. The cut-off between highly and low proliferating tumours was set at 45% of proliferating (Ki-67-positive) cells. There were 26 (25%) low and 78 (75%) highly proliferating SCCL.

Proliferative activity and TNM classification. The Ki-S11 proliferative activity varied depending on the size of the tumour. The arithmetic mean of the Ki-67 proliferative activity was 52.3% (±7.27%) for T1 tumours (n=4), 54.7% (±14.33%) for T2 tumours (n=29), 60.9% (±16.2%) for tumours of T3 category (n=31), and 61.5% (±15.13%) for T4 tumours (n=39). An increasing proliferative activity with increasing T category could clearly be shown. These results were not statistically significant (p=0.1).

However, correlating the proliferative activity with the nodal status demonstrated statistical significance, with p=0.001. Patients (n=75) without nodal metastases (N0) had a proliferative activity with an arythmetic mean of 57.2% (±14.69%). Patients (n=8) with N1 status had an average proliferative activity of 56.5% (±18.43%), and patients with an N2 status (n=21) an arythmetic mean of 67.1% (±13.85%). Figure 2 illustrates the correlation of the proliferative activity with the nodal status. Furthermore, a clear difference in the average proliferative activity between M0 and M1 patients could be shown. Due to only two cases with distant metastasis, the correlation of proliferative activity and distant metastasis was without statistical significance. The proliferative activity for the M0 stage (n=102) was 58.7%, and for the M1 stage (n=2) was 76.5%.

Proliferative activity and grading. A strong correlation between proliferative activity and histopathological grading was shown. According to the grading, the mean proliferation index was as follows: 34.67% (±9.24%) for G1 (n=3), 56.32% (±13.58%) for G2 (n=75) and 70.24% (±13.36%) for G3 (n=25). This correlation was statistically significant (p=0.001).

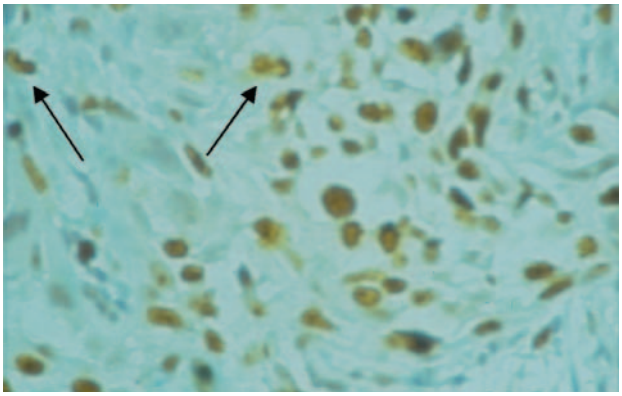

Figure 1. *Immunohistochemical staining of the nuclear Ki-67 antigen by Ki-S11 antibody. Mitosis of two SCCL cells in the late telophase (arrows) (magnification ×200).*

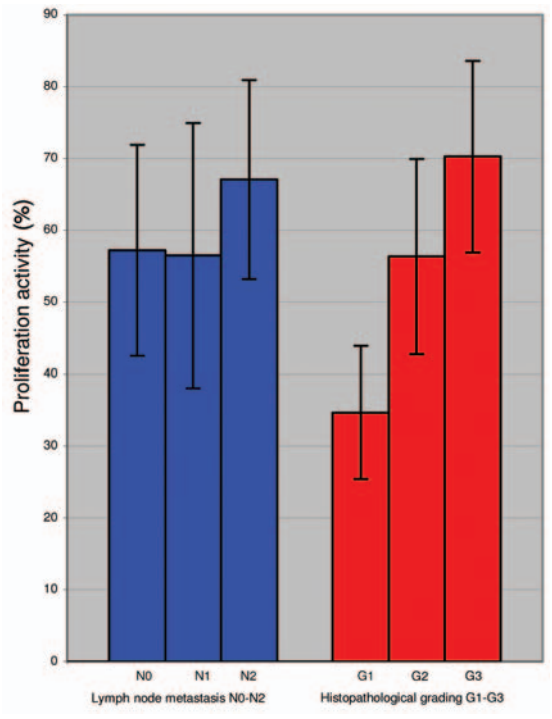

Figure 2. *Graphical illustration of the significant correlation ($p<0.05$) of Ki-67 proliferative activity and N status in primary laryngeal carcinoma (n=104) and graphical illustration of the significant correlation ($p<0.05$) of Ki-67 proliferative activity and grading in primary laryngeal carcinoma (n=104). The arithmetic mean of the proliferative activity increases with the N status. The higher the proliferative activity, the higher the grading of the laryngeal carcinoma.*

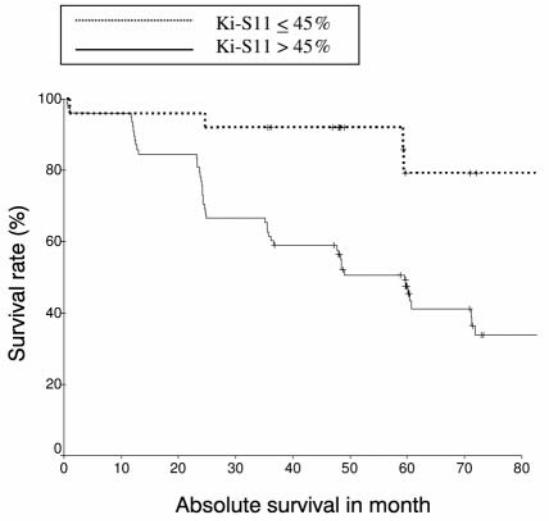

Figure 3. *Significant difference ($p<0.05$) in the absolute survival rate according to Kaplan-Meier analysis of 104 patients with SCCL, divided into low (≤45%) and high (>45%) proliferative activity of tumours.*

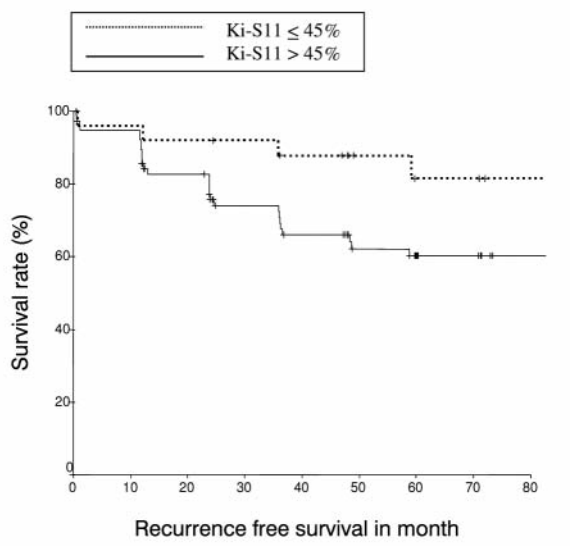

Figure 4. *Significant difference ($p<0.05$) of the recurrence-free survival rate according to Kaplan-Meier analysis of 104 patients with SCCL, divided into low (≤45%) and high (>45%) proliferative activity of tumours.*

Proliferative activity and survival. The overall five-year survival rate of all examined patients suffering from a SCCL (n=104) was 49%, which was a lower rate than expected for a comparable study group. There were significant differences for two distinct groups of tumours ($p<0.05$): Patients with tumours demonstrating low proliferating activity in their carcinomas had a five-year survival rate of 84%, whereas patients with a highly proliferating carcinoma had a five-year survival rate of only 47.18% ($p<0.05$) (Figure 3).

Regarding the relapse-free-survival, there was also a statistically significant difference for the five-year survival rate between the patient groups with low and highly proliferating tumours ($p<0.05$). Patients with a low

proliferating carcinoma had a 5-year survival rate of 84% in contrast to patients with a highly proliferating carcinoma showing a 5-year survival rate of 64.1% (Figure 4).

Discussion

Squamous cell carcinoma of the larynx (SCCL) is the most frequent malignancy in the head and neck region and has shown a growing incidence in women over the last years (1, 2). The combination of surgical or laser-surgical resection and/or adjuvant (chemo-)irradiation is considered as state of the art in the treatment of these tumours (22, 23). Despite these appropriate treatment strategies, the 49% five-year survival rate for all tumour stages, as shown in this study, is disappointing. In literature, a 52%-94% five-year survival rate is described depending on tumour stage and tumour therapy (2, 5, 6). The lower five-year survival in the presented SCCL patient group is explained by a predominance of rather advanced tumour stages (71/104 (68%) patients presented with T3 and T4 tumours).

It still is difficult to give an exact prognosis for the clinical course of SCCL with the established clinical parameters such as the TNM classification and the histopathological grading (7, 8). Moreover, these known parameters even show inhomogeneous results for head and neck cancer (24). Criteria for the histopathological grading are not standardized, making exact classification into a distinct group of differentiation difficult (11). This is one of the reasons why histological differentiation in head and neck carcinoma has only low evidence power (24). Despite its being a most powerful parameter, the TNM classification (10), criticised as an unreliable prognostic indicator for the head and neck region (11, 25) due to unfavourable clinical outcomes especially for small tumours, still remains as the most important prognostic factor (7).

The absence of reliable prognostic parameters may be due to the heterogenic appearance of a variety of different types of head and neck cancer which are often investigated as a homogeneous group of cases. Berrino and Gatta (26) proposed to separately investigate different cancer entities according to their anatomical origin. Therefore, in this study 104 patients exclusively with SCCL were analysed to rule out this particular bias.

An important sign for malignant transformation is the deregulation of the cell cycle as indicated by a large number and high proliferation rate of dividing cells. There are hints that a high proliferation rate of the tumour cells correlates with aggressiveness, unsatisfactory clinical course and tendency to metastasize early (7). Multiple authors reported that an abnormal proliferative activity of tumour tissue can predict the biological behaviour of the tumours (27, 28). This indicates that proliferation of tumours might be an important factor for prognosis and therefore proliferation markers may help to determine patients' survival (7) and may influence therapy indications.

Several basic approaches to determine the proliferative activity of tumour tissues have been achieved, such as the determination of the cell fraction in the S-phase (29) by flow cytometry, determination of 3H-thymidine index, or by histoneH3 mRNA *in situ*-hybridisation (30). Further methods to measure proliferative activity are the determination of the mitotic index, the AgNOR-method, and automatic microscopical picture analysis (29, 31, 32). Most of these studies gave inconsistent or contradictory results on proliferative activity and prognosis. Therefore, cell cycle proteins were used to determine the proliferative activity of normal or neoplastic cell populations. However, only few of these have shown reliable quality as specific proliferation markers, namely Ki-67 and p100 (33).

Ki-67 was first described as a monoclonal anti-human antibody which marks a nuclear antigen that is expressed in all active phases of the cell cycle except the G0-phase. Maximum expression of Ki-67 is seen in the M-phase of the cell cycle, with localisation on the chromosomes themselves (16, 34). Although the role of Ki-67 during the cell cycle is not clear yet (35, 36), Ki-67 is nowadays seen as the most reliable proliferation marker (32, 37, 38).

Gerdes *et al*. described a correlation between Ki-67 positive cells and histological grading and growth fraction of breast cancer (39) and Non-Hodgkin-lymphoma (40). Examination of colorectal carcinoma (41) and lung cancer (42) confirmed this correlation. A review article on the prognostic relevance of Ki-67 in different malignant tumours clearly demonstrated a statistically significant correlation between the prognosis and Ki-67 staining in breast cancer, soft tissue tumours, lung cancer, astrocytoma and meningiomas (43). Several monoclonal antibodies have been described for detecting the Ki-67 antigen. In a large-scale study, Rudolf *et al*. analysed tumour specimens derived from 237 different tumour entities (soft tissue tumours and gastrointestinal stromal tumours) applying Ki-S11 as primary antibody. The authors reported a strong correlation of proliferative activity and prognosis resulting in longer survival times of the patients when the proliferative activity was low. The results presented here are in good accordance to those published by Rudolf and co-workers (44).

However, for head and neck cancer, contradictory results have been published. Several studies have shown no correlation of proliferative activity with Ki-67 and prognosis. In one of these studies, tumour tissues derived from different anatomical sites (oral cavity, larynx, pharynx and lymph node metastases) were analysed, which might explain the lack of impact of the Ki-67 staining on survival times of the patients (8). The parameter of anatomical sites of the investigated tumour taken alone gives diverging survival

curves in Kaplan-Meier calculations. In another study, only 77 tumours were examined and the authors mentioned problems with the staining procedure for Ki-67 because of an unsatisfactory primary antibody (45). Finally, a third study on 30 cases with SCCL showed no statistical correlation of Ki-67 proliferative activity and prognosis, however an increase of antigen expression was seen in combination with decreased differentiation (46), which is in accordance with the results presented here.

Only tendencies on the correlation of proliferative activity and grading were obtained in two other studies (11, 29). In both reports, the authors found a positive trend of Ki-67 staining with histopathological grading, but results were not statistically significant due to a small patient contingent. Cappiello *et al*. (29) evaluated 30 unselected consecutive primary squamous cell carcinomas of the larynx. Results of Ki-67 staining were compared and subsequently related to histological grading, lymph node status and pT category. Although there was a positive trend when Ki-67 staining was correlated with histological grading, findings were not statistically significant. Kearsley *et al*. (11) investigated 42 fresh tumour samples of patients with squamous cell carcinomas (SCCs) of the upper aerodigestive tract. In that study, the significance of the cellular expression of the Ki-67 antigen was analysed among other tumour biological parameters in a prospective analysis of patients with SCCs of the head and neck. The percentage of cancer cells that reacted with the Ki-67 monoclonal antibody was assessed as low (less than 10%) in 15 samples (35.8%), intermediate (10-30%) in 19 samples (45.2%) and high (more than 30%) in eight samples (19.0%). There was no statistically significant correlation with prognosis or other clinical parameters and proliferative activity, and – in clear evident contrast to the presented study – the percentage of Ki-67-positive cells was much lower.

However, there are studies published showing a definite and statistically significant correlation of proliferative activity of Ki-67 and prognosis in head and neck cancer. Welkoborsky *et al*. (47) investigated an admittedly small group patients (n=40), however the examined, tumours were precisely homogeneous (exclusively glottic SCC). A further study by Kuropkat *et al*. (15) showed a statistical correlation of Ki-67 proliferative activity and prognosis in a study investigating an larger number of tumours (n=131). In that study, only hypopharyngeal SCC were examined also applying the Ki-S11 primary antibody for immunohistochemical staining. The results presented therein are fully in accordance with the results of the present study.

In conclusion, the presented findings on Ki-67 proliferative activity using the Ki-S11 antibody on SCCL seem to be in accordance with other published data for head and neck tumours as well as with results of tumours derived from other tumour sites or even from other tissue entities. The results were comparable mostly to the larger and more homogeneous patient groups (32, 48) and in those cases where the antibody Ki-S11 was employed (15, 17). High proliferative activity seems to correlate with a worse clinical course and stronger metastatic potential. Thus, it is concluded that Ki-67 proliferative activity stained with the Ki-S11 antibody is able to predict prognosis of SCCL and could be a helpful tumour biological marker in clinical oncology for SCCL.

Acknowledgements

We deeply regret the death of our co-author Pierre Rudolph in August 2008.

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Received October 29, 2008
Revised November 27, 2008
Accepted January 14, 2009