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Proliferation marker Ki-S11 – a prognostic indicator for squamous cell carcinoma of the hypopharynx

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Abstract As a potential prognostic factor, the proliferative activity of 131 squamous cell carcinomas (SCC) of the hypopharynx and 47 of their cervical lymph-node metastases was analyzed retrospectively by means of monoclonal antibody Ki-S11 immunostaining, which specifically detects the Ki-67 antigen on paraffin-embedded tissue. Median follow-up time was 37.6 months. Ki-S11 revealed distinctive patterns of proliferating cells related to the degree of differentiation. The proliferation fractions in the primaries and their lymph-node metastases did not differ significantly. Patients with high proliferating hypopharynx carcinomas (>45% labeled cells) had a significantly lower 5-year-survival rate (16%) than patients with low proliferating tumors, whose 5-year-survival rate was 30% (P=0.01). A statistically significant positive correlation was also observed between proliferative activity and lymph-node status (P=0.012). In conclusion, the proliferative activity as determined by means of Ki-S11 immunostaining is of prognostic value with respect to both survival and metastatic risk in SCC of the hypopharynx.

Key words Hypopharynx carcinoma · Prognosis · Proliferation · Ki-S11 · Immunohistochemistry

Introduction

Squamous cell carcinomas (SCCs) are the most common malignant neoplasms of the upper aerodigestive tract, accounting for up to 5% of all solid human malignancies [43]. SCCs of the head and neck are fast growing tumors [8], and, despite modern therapeutic approaches combining surgery, radiotherapy, and chemotherapy, the prognosis of these patients is often poor [22]. This is especially true for carcinomas of the hypopharynx [9] because of the absence of symptoms in the early stages [24].

The 5-year survival rate for patients with SCC of the hypopharynx has been reported to range between 14% and 28% [41]. About 75% of the patients present with cervical lymph-node metastases at the time of first surgery [23]. These regional metastases appear to be the most important prognostic factor in head and neck cancer [1]. However, it has been shown that patients with comparable disease receiving the same therapy regimen may show wide variations in their clinical outcomes [25, 35]. As clinical and histopathological parameters appear to be insufficient for predicting the survival of patients with head and neck cancer [35], no generally applicable indicator of prognosis is available as yet. The identification of new prognostic markers is therefore highly desirable. A confident prediction of prognosis would indeed allow oncologists to optimize therapy protocols and follow-up procedures in accordance with individual tumor characteristics, especially with respect to adjuvant or primary chemotherapy.

Overall, there is strong evidence that the proliferative activity of tumors is correlated to biological aggressiveness, unfavorable clinical outcome, and a higher risk of developing metastases [2]. However, SCCs seem to be an exception to this rule, and, accordingly, reports concerning the association of proliferation and prognosis in patients with SCC of the head and neck are inconclusive. This may be attributable to the often limited case numbers studied [6] and to the biological heterogeneity in the group of head and neck cancers [43]. Indeed, tumors from different sites are not comparable in terms of metastasis risk and patient survival. Also, the lack of progress in this field might be related to the limitations of commonly available laboratory techniques [3]. Lastly, the variety of the techniques used for the measurement

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of proliferation may play a role, and the rare analyses of immunohistochemical proliferation markers have been performed on small case series and produced inconsistent results.

With a view toward improving the assessment of proliferation in our material, we employed the recently characterized proliferation-specific monoclonal antibody Ki-S11 [36] reactive with the Ki-67 protein. This antibody yields well reproducible results on formalin-fixed and Bouin-fixed, paraffin-embedded tissue specimens, thus enabling the retrospective analysis of proliferation in vast sample sizes.

Our study was designed to investigate the proliferative activity in a large cohort of hypopharyngeal SCCs in relation to patient survival and lymph-node status. We will show that Ki-S11 immunostaining correlates with the number of lymph-node metastases and provides valuable prognostic information in patients with SCC of the hypopharynx. To our knowledge, this is the first report documenting independent prognostic relevance of an immunohistochemical proliferation marker in head and neck cancer.

Materials and methods

Materials

Patients

Formalin-fixed, paraffin-embedded tumor samples from 131 patients treated at the Department of Otorhinolaryngology, Head and Neck Surgery, University of Kiel, Germany, between 1984 and 1992 for the primary manifestation of hypopharyngeal SCC were examined. Additionally, tissue of cervical lymph-node metastases from 47 of the 131 patients was available. One to three tumor-infiltrated lymph nodes were examined in each of these cases. All patients were treated with radical primary surgery, followed by locoregional irradiation. The sex ratio of 115 men to 16 women showed a marked male predominance. Patient age at the time of diagnosis ranged from 36 years to 93 years, with a mean age of 56.8 years.

All tumors were staged according to the TNM classification [Union Internationale Contra la Cancrum (UICC) 1987]. Five patients (3.8%) presented with a T_1, 27 (20.6%) a T_2, and 36 (27.5%) a T_3 tumor; the largest number of patients (n=63, 48.1%) presented with a T_4 carcinoma at first diagnosis. By histopathological grading, 96 (73.3%) of the carcinomas were moderately differentiated. Poor differentiation was found in 22 cases (16.8%) and only eight tumors (6.1%) were well differentiated. More than half of the patients (n=75, 57.3%) had an N_2 lymph-node status at first diagnosis. Otherwise, the lymph-node status was N_1 in 15 (11.5%) and N_3 in 23 patients (17.6%). Only 18 (13.7%) patients had no detectable neck lymph-node metastasis. Distant metastases were present at diagnosis in three patients. This signifies that only two (1.5%) patients had stage-I disease. Four (3.1%) patients were stage II and 12 (9.2%) patients stage III. The overwhelming majority of the patients (n=113, 86.3%) suffered from stage-IV disease. The patients were followed for a median time of 37.6 months (1–72 months). No patients were lost to follow-up.

Antibody

The mouse monoclonal antibody Ki-S11 recognizes a fixation-resistant epitope of the Ki-67 antigen and, thus, recognizes the totality of cycling cells but not resting (G_0) or terminally differentiated cells. Ki-S11 was recently generated in the Department of Hematopathology, University of Kiel, Germany [36] by means of somatic hybridization of permanent mouse myeloma cells and splenocytes from mice immunized with nuclear extracts from L428 cells, as described previously [29]. Specificity and sensitivity of the antibody were verified by means of Western-blot analysis [36]. Ki-S11 was further compared with other Ki-67-specific monoclonal antibodies by means of immunohistochemistry on paraffin sections. On most routine samples, the distribution and staining intensity of Ki-S11 does not noticeably differ from that of the immunohistochemical proliferation marker MIB-1. In specimens with reduced immunoreactivity (e.g., due to long storage, poor preservation, or Bouin fixation), however, Ki-S11 displays a markedly higher sensitivity [36].

Methods

Immunohistochemistry

Paraffin sections, 2- to 4-µm thick, were mounted on precoated slides, routinely deparaffinized and rehydrated, then incubated with 3% hydrogen peroxide in methanol for 30 min to block endogenous peroxidase activity. Antigen demasking was performed according to the method of Shi et al. [38], with minor modifications. Briefly, the slides were boiled for 45 min in 0.1 mol/l citrate buffer, pH 6.1, in a microwave oven (Sharp, Hamburg, Germany) at maximum power. The sections were then incubated for 30 min with Ki-S11 (undiluted cell culture supernatant) as a primary antibody in a humidified chamber at room temperature. The immunoreaction was visualized by means of the avidin-biotin-complex technique [21] using biotinylated rabbit anti-mouse antiserum, avidin-biotin-complex (both Dako, Hamburg, Germany) and diaminobenzidine (Sigma, Deisenhofen, Germany). The sections were counterstained with Mayer's hematoxylin (Merck, Darmstadt, Germany).

Sections from a formalin-fixed, paraffin-embedded human tonsil were included in each staining procedure as a positive control. Negative control was also performed on human tonsil sections, the primary antibody being replaced by buffer to exclude nonspecific side reactions.

Evaluation of proliferation indices

Each slide was microscopically read by two investigators without knowledge of the clinical data. Only nuclear staining was accepted as a positive reaction. Three intensely stained areas with low background were selected on each section at low magnification, and in each of these areas 500 tumor cells were counted at high magnification. The number of positive cells was rounded off to the nearest five, expressed as percentage of all counted cells and defined as the proliferative index. In case of multiple lymph-node invasion, the mean proliferation index of all nodes was evaluated. Inconsistently stained cases were stained anew or excluded from the study.

Statistical analysis

All analyses were performed using the statistical packages SPSS/PC+ and CSS (StatSoft Inc., Tulsa, Okla.). Survival curves were generated with use of the Kaplan-Meier method. Survival was defined as the time from the date of diagnosis to the date of tumor-related death. To distinguish a low-risk and a high-risk group, the proliferation index was dichotomized with a cutpoint at 45% proliferating cells determined according to the method described by Sigurdsson et al. [39]. The statistical difference between survival estimates was computed by the Mantel-Haenszel log-rank test. Pearson's rank correlation coefficient served to evaluate the relationship between proliferation of the primary tumor and the lymph-node status. The difference between proliferation

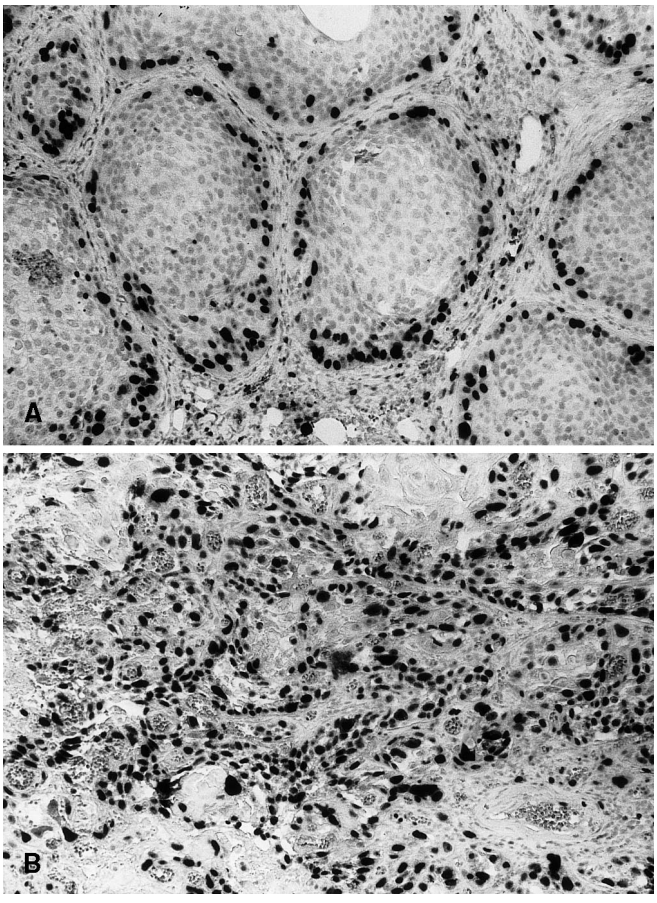

Fig. 1 A Ki-S11 immunostaining of a well-differentiated squamous cell carcinoma (SCC) of the hypopharynx. The proliferative activity is mainly located at the tumor periphery. **B** Moderately well-differentiated SCC of the hypopharynx with a diffuse distribution of proliferating cells. Mitotic figures are highlighted by Ki-S11 immunostaining (ABC technique, weak hematoxylin counterstain, original magnification 350×)

indices in primary tumors and their metastases was evaluated by the Kruskal-Wallis nonparametric analysis of variance. Statistical significance was assumed at $P<0.05$.

Results

Immunohistochemistry

The antibody Ki-S11 showed distinct nuclear staining with hardly any background. In normal lymphoid tissue unaltered by carcinomatous infiltration, positive staining was essentially restricted to the dark zone (centroblasts) of the germinal centers. Only a few cells in the light zone (centrocytes) and in the mantle zone were positive. The staining intensity of the positive cells ranged from faint to dark brown. When only intensely labeled cells were counted, the interobserver error as well as the intraobserver variability were of no practical significance, and the proliferation indices were independently reproducible. The age of the paraffin-embedded material did not affect the staining quality. In control sections of a human tonsil, nuclear staining was almost mostly restricted to the suprabasal layer of the squamous epithelium and to the centroblast zone of the lymph follicles. No staining reaction was ever detected in the negative controls.

Tumors often displayed heterogeneous staining patterns. In more well differentiated carcinomas, the proliferative activity was accentuated at the periphery of the tumor islands (Fig. 1A), whereas it was diffuse in poorly differentiated tumors (Fig. 1B). Areas with keratinization usually showed a comparatively low proliferative activity.

In the 131 primary tumors, the percentage of positive cells ranged from 25% to 85%, with a mean of 50.1% and a median of 50%. In the 47 cases of lymph-node metastases, histological features such as growth pattern and differentiation closely corresponded to those of the primaries. The percentage of positive cells ranged from 20% to 90%, with a mean of 52.3% and a median of 55%. In comparison, the range of positive cells in the corresponding 47 primary tumors was between 25% and 75%, with a mean proliferation of 49.9% and a median of 50%. The average proliferative activity in the lymph-node metastases appeared to be slightly higher than that of the primary tumors. However, the difference was not statistically significant ($P=0.522$). Also, the correlation between proliferation indices and tumor grade did not achieve statistical significance ($P=0.155$), nor was there any association between the proliferative activity and TNM stage.

Survival analysis

The 5-year survival rate of all 131 patients with SCC of the hypopharynx was 22%. Because of the small number of grade-1 tumors, this group could not be confidently evaluated in the survival analysis. The difference between grade 2 and grade 3 in terms of survival was not statistically significant. Also, the TNM stage was not found to be relevant to prognosis.

Concerning the Ki-S11 index, the series was initially dichotomized by the median value of 50%, which is a simple and statistically legitimate approach. This yielded a statistically significant discrimination. However, with use of this cutpoint, all patients in both the high-risk and the low-risk groups died of the disease. To obtain a plateau documenting survivors, the cutoff level was arbitrarily set down to 45%.

After dichotomization of the Ki-S11 index at 45% proliferating cells, 45 (34.4%) carcinomas were rated as low and 86 (65.6%) as high proliferating. Representative examples of a high- and a low-proliferating hypopharynx carcinoma are shown in Fig. 1A, B. A statistically significant difference in the 5-year survival rate was found between low- and high-proliferating tumors ($P=0.0102$). In the group of patients with low-proliferating carcinomas, the cumulative 5-year survival probability was 30%, in contrast to 16% in patients with high-proliferating tumors (Fig. 2).

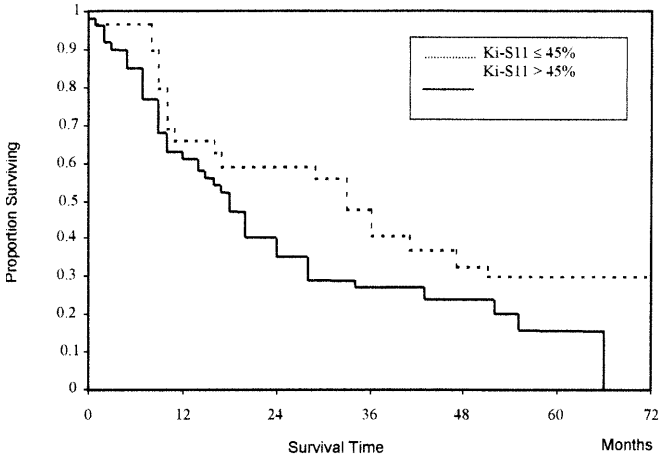

Fig. 2 Kaplan-Meier analysis of survival in 131 patients with hypopharyngeal squamous cell carcinoma (SCC) stratified on the Ki-S11 labeling index with a cutpoint at 45% proliferating cells

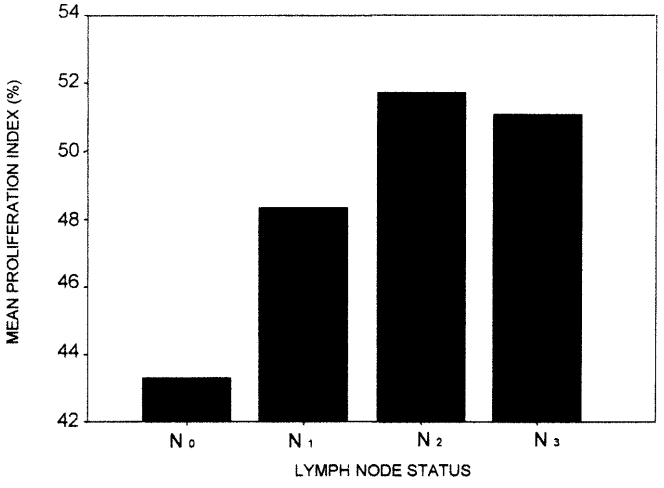

Fig. 3 Histogram showing the association between proliferative activity and nodal status (N_0–N_3) according to the TNM classification (Union Internationale Contra la Cancrum 1987). The correlation is statistically significant

Proliferative activity and lymph-node status

An advanced lymph-node status as defined by the TNM classification went along with increasing mean proliferation indices of the primary hypopharynx tumors (Fig. 3). Pearson's coefficient showed a statistically significant positive correlation between proliferation index and the number of infiltrated lymph nodes (r=0.23, P=0.012). Surprisingly, the tumor infiltration stage (T stage) showed a slightly weaker association with the lymph-node status (r=0.216, P=0.013). Considering the lack of correlation between the Ki-S11 labeling index and TNM stage, the former may be regarded as a stage-independent indicator of metastasis risk.

Discussion

Although SCCs of the hypopharynx are comparatively rare human tumors [24], their incidence has been increasing during the past few years [46]. Prognosis is often poor, due to usually advanced tumor stages at diagnosis and disappointing long-term treatment results [16, 30]. Primary surgery with locoregional irradiation, as performed also on our cohort, is the most widely accepted therapy regimen for this patient group [19]. The experience with primary chemotherapy in SCC of the hypopharynx is limited [45].

There is a strong demand for parameters able to predict the prognosis of the patients prior to adjuvant treatment and to estimate their risk for local lymph-node metastases [12]. Such prognostic markers would be essential for choosing the right balance between therapy aggressiveness and maintenance of an acceptable quality of life, with the final purpose of prolonging the patient's survival. Although the TNM classification and histopathological grading are the most commonly used prognostic parameters for solid tumors, they are of questionable value in head and neck cancer [4, 20, 24, 27, 35, 49]. Biological factors, notably the proliferative activity of the tumor cells, might provide more pertinent prognostic information.

Immunohistochemistry nowadays enables a rapid and easy evaluation of the proliferation fraction in alliance with well-preserved morphology. The Ki-67 antigen [15], which can be detected on archival material by means of specific monoclonal antibodies [7, 18], is a nuclear protein of unknown function that is expressed in proliferating cells throughout the division cycle but absent in resting or terminally differentiated cells. Its expression has been shown to correlate in a significant manner with the clinical course in various types of malignancy [5]. To assess its distribution in hypopharyngeal SCC, we used the newly characterized monoclonal antibody Ki-S11 [36]. Our results characterize Ki-S11 as an outstanding proliferation marker, which is devoid of cross-reactivity and yields neat and well reproducible immunostaining results.

With use of a threshold at 45% labeled cells, we were able to separate the series into two groups with significantly different outcomes, suggesting that the assessment of proliferation may provide useful prognostic information in head and neck cancer. Also, higher immunoreactivity scores were associated with increased numbers of lymph-node metastases. It is worth noting that this association was independent of the tumor invasion stage (T stage), which implies that the proliferation index may be used as an indicator of metastasis risk. By contrast, metastastic spread apparently does not entail a further increase of the proliferative activity.

Analyses of cellular proliferation in head and neck cancer are few as yet, and the methods used vary widely. Some authors found no correlation with the clinical course [33, 35, 40], whereas others observed at least a trend towards prognostic relevance [10, 11, 26, 27, 34,

42, 47, 48]. Amazingly, one study reported a high proliferative activity to be prognostically favorable [44]. These inconsistent results in head and neck cancer studies [9] are probably due to the heterogeneity of this tumor group in addition to the small sample sizes studied. Indeed, studies on patient survival should not be performed without distinction between the different head and neck tumor sites [31], and the series should be large enough to be informative. For this reason, we examined a sizable cohort of SCCs located exclusively in the hypopharynx. Our results show that the proliferative activity as assessed by Ki-67 immunohistochemistry is a prognostic factor in hypopharyngeal SCC and, therefore, might deserve further investigation in well-defined subsets of head and neck cancer.

Several aspects nevertheless have to be considered with respect to the assessment of proliferation in archival tumor material [17]. The fraction of proliferating cells, the cell-cycle duration and the rate of cell loss due to apoptosis, necrosis, or differentiation are the main parameters defining the growth rate of tumors. This growth rate is further influenced by the patient's immune response and therapy modalities [35]. Moreover, it is known that fast growing tumors tend to respond better to radiation and chemotherapy than tumors with low proliferative activity [14]. Therefore, many histological, immunological, biochemical and molecular parameters may have to be investigated to obtain deeper insights into the biological behavior of tumors [13]. One criticism concerning Ki-67 immunohistochemistry is notably that this antibody labels all phases of the cell cycle. However, the biological significance of the cell fraction in G_1 phase is questionable, as these cells may not proceed further through the cell cycle for an indeterminate time or may leave the cell cycle to become quiescent, senescent, or apoptotic [32]. The exclusion of the G_1 fraction might therefore allow a more precise evaluation of the proliferative activity [37], and evaluation of the apoptotic rate might add further information on tumor growth kinetics [17].

In keeping with this idea, in situ hybridization for the S-phase-specific histone H3 mRNA was shown to enable a good estimation of the proliferative activity in head and neck cancer [28]. In our experience, the application of this technique is more delicate, and the evaluation of the results is more time consuming than for Ki-S11 immunostaining. It would nevertheless be of interest to compare the two methods with respect to their prognostic relevance in head and neck cancer.

In conclusion, our results indicate that Ki-S11 immunohistochemistry is a robust and easy method able to provide pertinent prognostic information on patient survival as well as on the risk for the occurrence of local metastases in patients with SCC of the hypopharynx. These results should be confirmed by studies on carcinomas from different head and neck sites. Considering that patients with hypopharyngeal SCC have a bad prognosis in general, the prognostic impact of the proliferative activity as determined by Ki-S11 might be even more powerful in a patient group with wider variations in the clinical outcome, e.g., larynx carcinomas. In combination with clinical, histopathological and biological parameters, Ki-S11 could become an important tool in determining the optimal therapy regimen for these patients.

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