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In situ assessment of cell proliferation at the invasive front of oral squamous cell carcinomas

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Abstract In oral squamous cell carcinoma (OSCC) the histopathological malignancy grading of the invasive front has been found to offer the most reliable prognostic parameter. In the present study we compared such tumour front grading of 100 OSCCs with the in situ growth fraction demonstrated by MIB1 immunostaining following wet autoclave antigen retrieval. MIB1 labelling indices (LIs) were estimated both at the invasive front and in the central parts of OSCCs using two different evaluation methods (overall and random counting) to investigate whether MIB1 LIs represent a possible biological background for the tumour front grading. Statistically highly significantly increased MIB1 LIs were found at the invasive tumour fronts with both counting methods compared with the centres of the same tumours. For LI estimation the classic overall counting procedure proved to be superior. However, in contrast to tumour front grading, MIB1 LIs revealed no correlation with the clinical outcome of the patients concerned. Our results demonstrate that the invasive tumour front of an OSCC is composed of (a) tumour subpopulation(s) with higher proliferative activity. However, determination of the proliferative activity by MIB1 of this tumour area offers no prognostic information.

Key words Oral cancer · Proliferation · Archival immunohistochemistry · MIB1 · Wet autoclave antigen retrieval

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Introduction

A rapid tumour growth rate is associated with poor prognosis in many human malignancies [16, 27, 33, 34, 39]. With the advent of monoclonal antibodies against proliferation-associated antigens, immunohistochemical estimation of tumour growth fraction has become possible. One of the most commonly used antibodies for this purpose is the anti-Ki-67 antibody [4, 13] which reacts with a non-histone nuclear antigen present in all phases of the cell cycle, except G_0 and early G_1 [14]. A major drawback of its use, however, is the need for fresh material. This particular problem has been overcome by the development of an anti-Ki-67 analogous monoclonal antibody (MIB1) [8] that can be applied on routinely processed tissues after microwave oven antigen retrieval [28]. Both the MIB1 and the Ki-67 antibodies react with the same epitope [20].

Our group has recently described wet autoclave pretreatment as an alternative method for antigen recovery in archival tissues [1]. Wet autoclave pretreatment has been shown to yield an even enhancement of immunostaining reactions with a wide variety of antibodies including MIB1, without the common staining inconsistencies and occasional destruction of tissue morphology caused by microwave irradiation.

Data on the immunohistochemical demonstration of the proliferation rate in oral squamous cell carcinomas (OSCCs) are limited and were obtained using either anti-PCNA on specially fixed tissues [12, 29, 30] or anti-Ki-67 antibodies on frozen material [19]. Most of the studies cited conclude that proliferation activity increases sequentially with the severity of dysplasia in precancerous lesions as well as with increasing histopathological tumour grade in invasive oral carcinomas.

In the present study the distributional and quantitative features of MIB1 immunoreactivity were investigated in a series of 100 routinely formalin-fixed and paraffin-embedded OSCCs following wet autoclave antigen retrieval. The effectiveness of two counting methods (overall and random counting) for estimations of MIB1 labelling

indices (LIs) was compared. Possible correlations of MIB1 LIs with histopathological tumour front grading, clinico-pathological variables and survival probabilities of the patients were statistically analysed.

Materials and methods

Routinely formalin-fixed and paraffin-embedded tissues from 100 consecutive cases of primary OSCC (73 carcinomas of the floor of the mouth, 20 carcinomas of the tongue and 7 carcinomas involving both floor of the mouth and tongue; 84 male, 16 female patients; mean age 54 years) were investigated in this study. Patients had been operated on at the Department of Maxillofacial Surgery, University of Münster, Germany, between 1985 and 1990, either with curative (n=90) or with palliative (n=10) intent; postoperative irradiation (administered to all patients with pT4 tumours, positive lymph nodes and/or positive resection margins [R1, R2]) was performed according to adequate treatment protocols based on the TNM stage of the tumours (Union Internationale Contre le Cancer – UICC). Complete clinical follow-up data of the patients were available from the tumour register of the Department of Maxillofacial Surgery (Table 1). The tissues were retrieved from the files of the Gerhard-Domagk-Institute of Pathology, University of Münster, Germany. The postsurgical staging and grading were performed according to UICC and Broders' criteria, respectively [3, 17]. Histopathological grading of the invasive tumour front was performed as described by Bryne et al. [6] with some modifications concerning the extent of lympho-plasmocytic infiltration (scored as 1 if abundant and 2 if moderate, weak or negligible infiltration was present).

Paraffin sections 2 µm thick were cut and mounted on poly-L-lysine-coated slides (Sigma, Munich, Germany). Following routine dewaxing, sections were placed into a heat-resistant plastic Coplin jar filled with citrate-buffer (0.01 M Na-citrate monohydrate, pH 6.0) and incubated at 120°C in a Gössner laboratory autoclave (GLA-40–2, Hamburg, Germany) as described previously [1]. Incubation with the mouse monoclonal MIB1 antibody (Dianova, Hamburg, Germany) was carried out overnight (14–16 h) at 4°C in a humidified chamber (dilution: 1:500 in 10% RPMI-1640 solution containing 10% heat-inactivated bovine serum albumin). For secondary immunoreactions a rabbit anti-mouse IgG diluted in 1:30 in RPMI was applied for 30 min at room temperature, followed by a mouse alkaline phosphatase-anti alkaline phosphatase (APAAP) complex (1:100 in RPMI for 60 min at room temperature). Secondary reagents were purchased from Dakopatts (Copenhagen, Denmark). Enzymatic development was performed in a freshly prepared Fast Red solution (Sigma) containing naphthol-As- MX-phosphate (Sigma) for 30 min at room temperature. Finally, sections were thoroughly washed in tap water, slightly counterstained with haematoxylin and mounted with Kaiser's glycerin-gelatin.

Omission of the primary antibody served as a negative control and normal human tonsil, as a positive control.

Tumour cells with distinct red nuclear staining were regarded as MIB1 positive irrespective of the intensity of the reactions. Tumour cell nuclei were independently counted at the invasive margin (the deepest, approximately 1-mm zone of tumour invasion), defined by scanning at low magnification, and in the central parts of carcinomas. Cell counts were made at 400-fold magnification using a 10×10 eyepiece grid on a Zeiss Axioscope microscope (Zeiss, Göttingen, Germany) in at least five neighbouring viewing fields (yielding 500–1000 cells). All tumour cell nuclei within the 10×10 grid were counted individually according to the convention that all nuclei wholly within the boundary of the grid and those intersected by or tangential to the upper and left grid edges were counted (overall counting). The MIB1 labelling index (LI) was determined as the percentage of positive cells among the total number of cells counted in each case. In 75 cases tumour cell nuclei were enumerated in 10% randomly selected squares within the 10×10 eyepiece grid with separate random number tables for each.

Table 1 Clinico-pathological parameters and follow-up data of the 100 oral squamous cell carcinomas (OSCCs) investigated

Parameter	No.(%)
Tumour localisation	
Floor of the mouth	73
Tongue	20
Floor of the mouth and tongue	7
pT stage (UICC)	
pT1	22
pT2	55
pT3	7
pT4	16
pN stage	
pNo	57
pN1	26
pN2	12
pNx	5
Histological grading (Broders')	
Grade 1	26
Grade 2	63
Grade 3	9
Grade 4	2
Age	
< = 54 years	42
> 54 years	58
Gender	
Male	84
Female	16
Clinical course[a]	
5-year survival	67%
10-year survival	32%

[a] Mean follow-up period was 61 months

Using the same criteria as described above, the number of nuclei was estimated as the sum of these counts multiplied by 10 (random counting). Percentage ratios of MIB1 LIs were determined as the estimates of positively stained cells divided by the estimates of all cells counted [15].

Correlation of the two different counting methods was tested using the Spearman rank correlation coefficient. Differences between MIB1 LIs at the invasive margins and in the central parts of the carcinomas and their correlation with tumour grade were analysed using the Wilcoxon signed-rank test and the Mann-Whitney U test [38]. The cumulative patient survival was estimated only for the 90 patients whose tumours were radically resected using the Kaplan-Meier method [18]; for comparison of the survival curves the log-rank test (Mantel-Haenszel method) was applied [23]. Multivariate survival analysis was performed by the Cox stepwise regression analysis [10].

Results

MIB1-positive tumour cells accumulated predominantly at the peripheral zone of invasive trabeculae or tumour islets (Fig. 1). Central tumour areas with keratinization showed little or no MIB1 positivity in well and moderately differentiated carcinomas. In contrast, poorly differentiated carcinomas displayed a more diffuse MIB1 reaction pattern. In some OSCCs a complete focal lack of MIB1 immunoreactivity was observed even at the invasive front if those tumour cells showed pronounced maturation and keratinization (Fig. 2). In non-tumorous epithelium located adjacent to the carcinomas, MIB1-

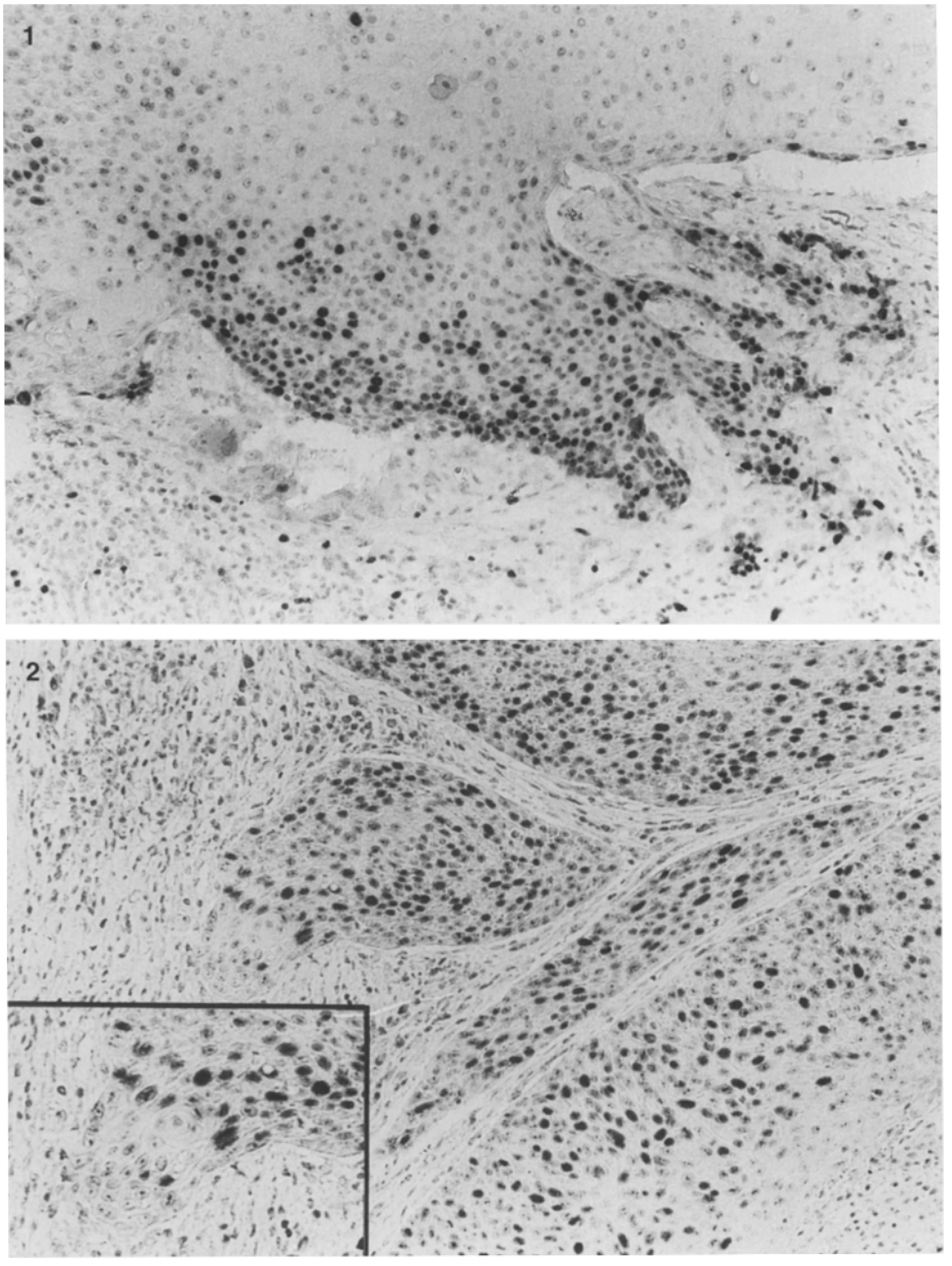

Fig. 1 Peripheral accumulation of MIB1-positive cells at the invasive margin of an oral squamous cell carcinoma. Paraffin section, APAAP technique, ×100

Fig. 2 Focal lack of MIB1 reactivity at the point of invasion. Paraffin section, APAAP technique, ×100 (*insert* ×250)

positive cells were generally found in the parabasal compartment; the basal epithelial layer was almost completely negative (Fig. 3).

MIB1 LIs were estimated in 75/100 carcinomas using both overall counts and random tables at the invasive margin and central parts of the tumours, respectively. In 25 cases the tumour was either too small for random counting or a dispersed invasive front hampered sufficient counting of this kind (Fig. 4). In these cases overall estimations only were performed. Statistically significant differences was found between the two counting procedures (exceeding for single cases 20%; Spearman rank correlation coefficient: 0.6, P=0.016). Highly significant differences were demonstrated between MIB1 LIs at the invasive margin and in the central parts of the carcinomas both with random tables (Wilcoxon Signed-Rank test: z=–3.6, P=0.0004) and with overall counting (z=–4.0, P=0.0001). No statistically significant correlation was detected between tumour grade and MIB1 LIs using either the randomized or the overall counting methods. MIB1 LIs at the invasive front of carcinomas (<50%, 51–75%, >76%) overall MIB1 LIs and those obtained in the central parts of the tumour were not associated statistically with survival probabilities.

Some prognostic parameters (pT, pN and R stages) were statistically significantly correlated with survival in the univariate analysis. The age and gender of the patients and histopathological grade of the tumours defined by the Broder's system all lacked significance. The histopathological tumour front score was found to have the

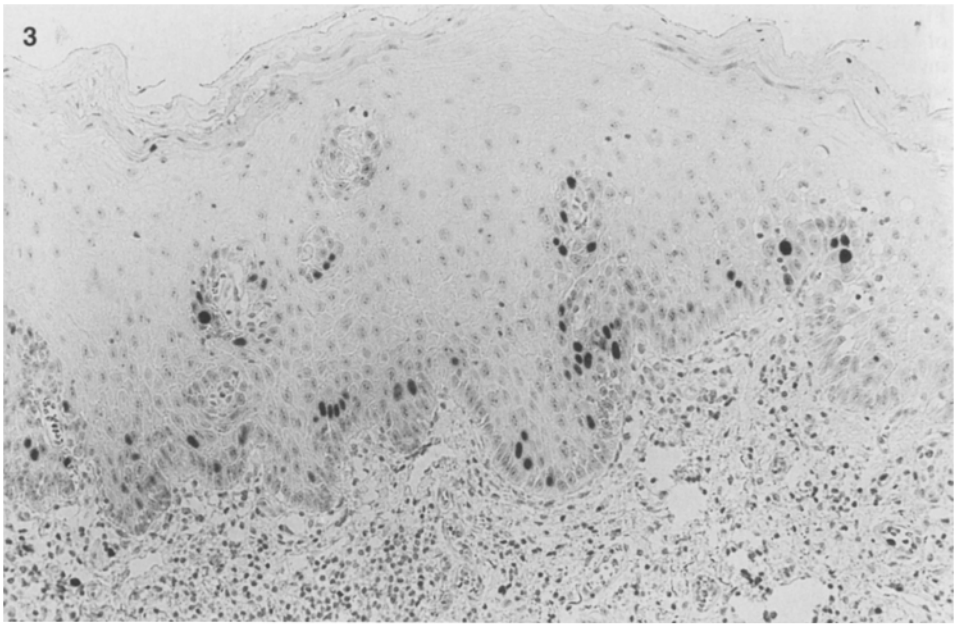

Fig. 3 Parabasal localization of MIB1-reactive cells in the normal oral mucosa adjacent to carcinoma. Paraffin section, APAAP technique, ×100

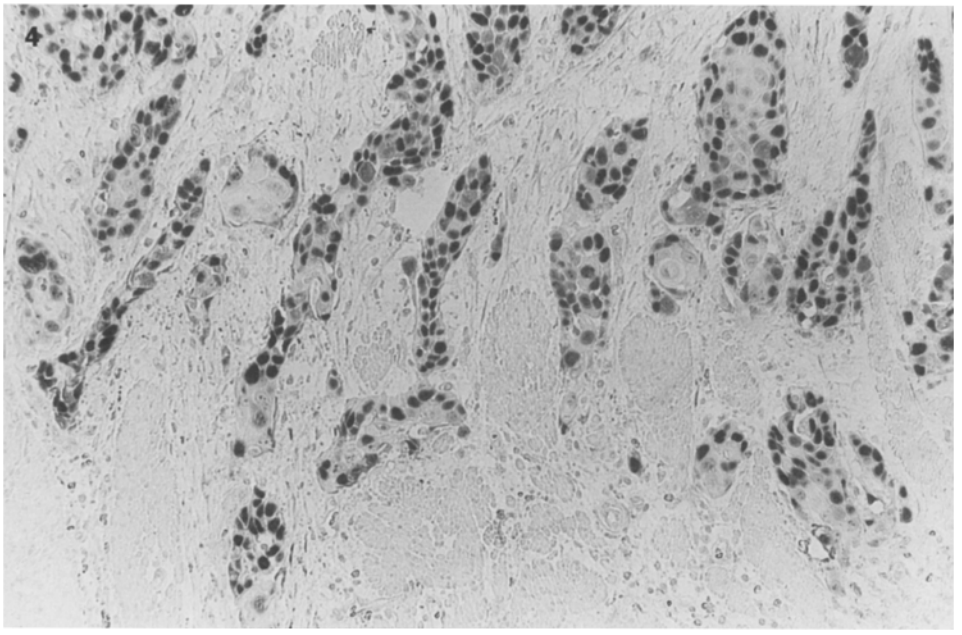

Fig. 4 Dispersed growth pattern of a squamous cell carcinoma at the invasive zone. Paraffin section, MIB1 reaction, APAAP technique, ×100

Table 2 Univariate survival analysis of clinico-pathological factors and MIB1 labelling indices in 100 OSCCs (*NS* not significant)

Parameter	Univariate chi-square for the log-Rank test (Mantel)	*DF*	*P*
Age (<54y vs >54y)	0.1	1	NS
Gender	0.001	1	NS
pT stage	12.4	3	0.006
pN stage	10.4	2	0.006
R stage	9.7	2	0.008
Tumour front score	28.7	2	0.000
Tumour histopathological grade (Broders')	6.1	3	NS
MIB1 LI (overall counting)	0.5	2	NS
MIB1 LI (random counting)	0.01	2	NS

highest prognostic significance. In contrast, MIB1 LIs at the invasive tumour front were not statistically associated with prognosis. The results of the univariate survival analysis are shown in Table 2. According to the multivariate Cox regression analysis the histopathological tumour front score was the only parameter with independent prognostic value (chi-square: 2.9, P=0.001).

Discussion

The few studies investigating the proliferation rate by means of antibodies against PCNA or Ki-67 in OSCCs have found an association with the histopathological tumour grade but not with prognosis [12, 19, 29, 30]. This may be because, in the majority of those studies, the entire bulk of the tumours was assessed. In the present study we evaluated the MIB1 immunoreactivity at the invasive tumour front and the tumour centre independently.

Our results, showing an accumulation of MIB1 immunoreactive proliferating tumour cells at the invasive front of oral squamous cell carcinomas, provide further support for the putative biological significance of this particular area in epithelial malignancies [6, 7, 19, 31, 37]. However, MIB1 quantitation of this tumour fraction was also not correlated with the clinical course of the respective carcinomas.

Counting is regarded as the Achilles' heel of cell or nuclear fraction evaluation in histological sections. Appropriate field selection has been shown to be as important as sound counting methods. On the basis of our results, in agreement with the findings of others [19], the invasive margin of squamous cell carcinomas may be the best field for growth fraction estimations in oral cancer. However, as in other studies assessing Ki-67 expression in epithelial malignancies [9, 22], our data showed no statistically significant correlation between MIB1 counts and clinico-pathological features. The proliferative activity characterized by MIB1 LIs at the invasive tumour front does not apparently represent a parameter with influence on the survival of oral cancer patients.

Our findings with regard to the statistically highly significant and independent prognostic value of the histopathological tumour front grading support the results of others [6, 7, 26]. However, it is intriguing to see that the "proliferative activity" detected by MIB1 at the invasive tumour front does not offer a biological explanation for the tumour front grading. These disappointing findings point to the problematic nature of proliferation estimations by rates or ratios of so-called proliferation markers.

Labelling indices – of any kind – of proliferation are static, quantitative measures of the actual amount of cells in cell cycle and do not reflect the real dynamism of proliferation. Neither time-related dimensions of cell division, nor the rate of cell loss can be properly estimated by currently used methods evaluating proliferation rates in histological material. Other techniques, like morphometric analysis of silver-stained nucleolar organizer-associated proteins (AgNORs) have been shown to yield more relevant information concerning both the dynamism of the cell cycle and the clinical outcome of cancer patients [24, 25, 32].

In situ study of cycling cells addresses a few questions concerning the association of proliferation with other processes, such as tumour cell invasion. The focally observed complete loss of MIB1 positivity in apparently invasively growing tumour cells displaying a more mature phenotype may either be due to a slow onset of Ki-67 (or MIB1) expression being undetectably low in the early S-phase [35, 36] or to its rapid degradation at the end of mitosis [5]. This histopathological feature is well known and a hallmark for the diagnosis of microinvasive squamous cell carcinomas of the uterine cervix and the skin [11, 21], suggesting that invading carcinoma cells might display activities other than proliferation. The parabasal localization of MIB1-positive cells observed in the adjacent normal oral epithelium is suggestive of a reserve cell character for the basal cell layer [2].

In conclusion, tumour growth rate determined by calculating MIB1 LIs is not a prognosis-related factor in OSCCs, regardless of whether overall or random counting methods are applied. Nevertheless, the accumulation of MIB1-positive proliferating cells at the invasive tumour front highlights the significance of the tumour–host interface in determining the biological behaviour of OSCCs, whose high prognostic value is reflected in the histopathological tumour front grading. Further research is necessary to investigate whether MIB1 immunolabelling in combination with other proliferation-associated markers, such as AgNORs, may provide additional information.

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