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Prognostic significance of apoptosis and associated factors in oral squamous cell carcinoma

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Abstract Tumour progression is characterised by an imbalance between cell proliferation and apoptosis. The aim of our study was to estimate the importance of proliferation and apoptosis associated parameters in primary squamous cell carcinomas (SCCs) of the oral cavity and oropharynx. For determination of apoptosis, the enzymatic labelling of DNA fragmentation with a terminal transferase reaction was used in 156 tissue samples of 107 patients, including corresponding lymph-node metastases in nine cases. P53, bcl-2, and Ki-67 were determined immunohistologically. P53 was detectable in 50.5% of the cases. Positive staining was associated significantly with decreased apoptosis ($P<0.003$). Bcl-2 was upregulated in 31.8% of the cases depending on the tumour grading ($P<0.001$) and correlated negatively with apoptosis ($P<0.001$). Proliferation ($P<0.006$) and apoptosis ($P<0.03$) were enhanced in larger tumours, though a direct correlation between these two parameters was not proven. Nevertheless, in contrast to the conventional tumour staging and grading, neither the expression of *p53* or *bcl-2* nor the apoptosis or Ki-67 measurements were able to predict survival or recurrence-free survival of the patients suffering from a SCC in the oral cavity or oropharynx. Our observations suggest that the function of wild-type *p53* to induce apoptosis is lost in at least half of the SCCs under study and that the physiological function of bcl-2 as potent inhibitor of apoptosis is widely preserved in oral SCC.

Key words Oral · Squamous cell carcinoma · Proliferation · Apoptosis · Tumour suppressor gene · Oncogene · Prognosis

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Introduction

Tissue growth is characterised by an imbalance between cell proliferation and the rate of cell death both under physiological conditions and in tumour tissue [19, 38]. In this context, cell death is defined as a specific morphological change clearly distinct from necrosis, culminating in cell demise called apoptosis or programmed cell death (PCD) [16, 20]. The regulation of apoptosis is not only important for morphogenesis during foetal development and maintenance of tissue homeostasis during adulthood but also for neoplastic transformation in some organs [6, 41]. In cells bearing damage within their genome, the initiation of apoptosis at first depends on the function of wild-type p53 phosphoprotein, which is responsible for an interruption in the late G1-phase of the cell cycle until repair of the genomic damage occurs or the cells are transmitted to apoptosis [7]. As a result, the genomic damage cannot be transferred to daughter cells. Therefore, p53 is called “guardian of the genome” [25]. However, another protein called bcl-2 has been discovered which is involved in the regulation of cell death by inhibiting apoptosis in many cell systems under physiological and neoplastic conditions, probably leading to a growth advantage of tumour cells by blocking the programmed cell death [22, 31, 38]. The role of this oncogene has been revealed primarily in follicular B-cell lymphomas [43, 44]. The *bcl-2* gene located regularly at 18q21 is moved into juxtaposition with powerful enhancer elements in the immunoglobulin heavy-chain locus at 14q32, with the result of overproduction of *bcl-2* mRNAs and their encoded proteins [28]. However, increased *bcl-2* expression has also been reported in epithelial malignancies, such as carcinomas of the lung [34], thyroid [36], breast [9], ovaries [24] and gastric [26] as well as colorectal adenocarcinomas [2]. The aim of this study was to determine the prognostic significance of apoptosis, considering these regulation pathways in primary squamous cell carcinomas (SCCs) of the oral cavity and the oropharynx including their corresponding lymph-node metastases.

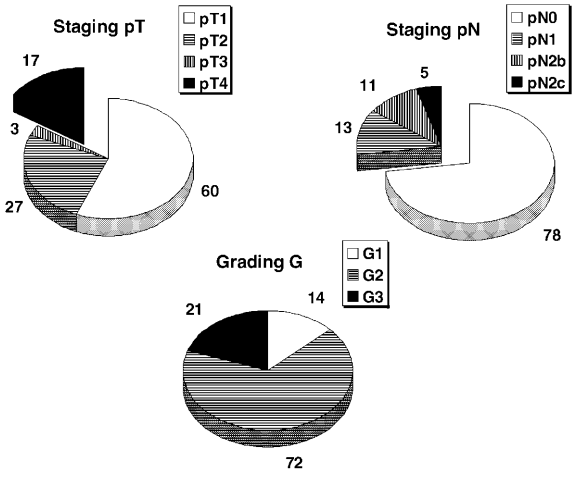

Fig. 1 Staging and grading of the 107 patients suffering from a primary squamous cell carcinoma in the oral cavity or the oropharynx

Table 1 Localisations of the primary squamous cell carcinomas

Floor of the mouth	62	57.9%
Tongue	17	15.9%
Mandibular gingiva or mucosa	12	11.2%
Oropharynx	10	9.3%
Buccal mucosa	3	2.8%
Maxillary gingiva or mucosa	3	2.8%

Table 2 Distribution of the cases yielding one or more different paraffin blocks of the primary squamous cell carcinomas and corresponding lymph node metastases

Paraffin blocks of primary tumours	Paraffin blocks of lymph-node metastases		
	None	One	Two
1	68	7	1
2	23	0	1
3	7	0	0

Materials and methods

One hundred and seven patients suffering from a primary SCC in the oral cavity and oropharynx without distant metastases (M0) were considered (Table 1) [40]. Twenty-four patients were female (22.4%) and 83 male (77.6%). The age at the points of operation ranged from 35.5 years to 86.9 years, with a median of 56.3 years. For 63 of 89 patients, an abuse of alcohol and tobacco was known in their history. Four patients declared to have never smoked but to have drunk alcohol. Eighteen patients were recorded as having consumed tobacco but not alcohol. Four patients denied the use of both alcohol and tobacco. All patients had been treated surgically with curative intention (R0) supported by an adjuvant radiotherapy depending on clinical indication [3]. The staging and grading for all patients are given in Fig. 1 [37, 40]. During follow-up, the subsequent treatment of the patients was not considered in this study. Only the recurrence-free survival and the survival of the patients independently of the cause of death were quantified. Fifty-six patients had died by the end of the follow-up period. In the remaining 51 patients, there was a mean follow-up period of 6.4 years (2.9–15.3 years).

The specimens were routinely fixed in formalin after resection and embedded in paraffin (Table 2). Sections were cut to 4 µm, mounted on SuperFrost/Plus-slides (Menzel, Braunschweig, Germany), dewaxed with xylene and rehydrated in isopropanol, gradually diluted ethanol and distilled water. For immunohistochemistry, a target unmasking step of autoclaving at 120°C for 20 min in 10 mM citric acid at pH 6.0 followed [35]. The primary antibodies against p53 (Ab-6, OP43, Oncogene Science, Cambridge, Mass.) [46] and bcl-2 (clone 124, DAKO, Glostrup, Denmark) [33], and the MIB 1 antibody (dia 505, Dianova, Hamburg, Germany) against the proliferation-associated antigen Ki-67 [12] were murine monoclonal antibodies used in a dilution of 1:10 in phosphate-buffered saline (PBS) at pH 7.6 supplemented with 1% bovine serum albumin. The same buffer without primary antibody was applied to negative controls. After incubation for 30 min in a wet chamber at room temperature, the slides were washed three times with PBS. The further process was a biotin–streptavidin standard procedure using the Super Sensitive Multilink Immunodetection System (BioGenex, San Ramon, Calif.), followed by staining with Fast Red TR/Naphtol AS-MX Phosphate (Sigma, St. Louis, Mo.) in 0.1 M Tris-Buffer and counterstaining with Mayers hemalum.

DNA fragments of apoptotic cells were visualised by an enzymatic reaction using the ApopTag Kit obtained by Oncor, Gaithersburg, Md. [1, 10]. In brief, after dewaxing and rehydrating as described above, the slides were incubated for 30 min at room temperature in a 20-mg/l proteinase K solution (Sigma, Deisenhofen, Germany) in PBS at pH 7.6 for protein digestion. After three washing steps with PBS and quenching endogenous peroxidase by incubation in 3% hydrogen peroxide in PBS for 5 min, followed by two further washing steps in PBS, the slides were covered with equilibration buffer for 10 min at room temperature. Afterwards, approximately 25 µl per slide of a mixture of terminal transferase solution and reaction buffer was applied and the slides incubated at 37°C under cover slips. Negative controls only received reaction buffer supplemented with double distilled water instead of terminal transferase solution. The enzymatic reaction was stopped after 60 min by transferring the slides into blocking reagent solution for 30 min at 37°C. Subsequently, the slides were washed three times in PBS again and incubated in a peroxidase-conjugated mouse anti-digoxigenin antibody solution for 30 min at room temperature. After three further washing steps using PBS, 200 µl of a 0.05% diaminobenzidine solution (Merck, Darmstadt, Germany) supplemented with 0.02% hydrogen peroxide was applied to each section. Six minutes later, the slides were transferred to double distilled water, washed three times, counterstained with 0.5% methyl green, covered with Kaisers glycerine gelatine (Merck, Darmstadt, Germany), and mounted with cover slips.

The slides were evaluated using a microscope at 100-fold magnification. Detecting p53, the number of cell nuclei showing a positive immunohistochemical reaction in relation to the total number of 500 nuclei in a circumscribed tumour strand leading off from the invasive front was counted and divided into three gradations assigned to a slight (less than one-third of the cell nuclei were positive), moderate (more than one-third but less than two-thirds of the cell nuclei were positive) and strong (more than two-thirds of the cell nuclei were positive) reaction in addition to a negative result for a completely absent staining in all tumour cells as described before [42]. Similarly, the immunohistochemical detection of bcl-2 was evaluated. The tumour cells were classified as positive if the cytoplasmic red staining showed at least half of the intensity of lymphocytes, present in all tumour samples without exception and used as internal positive controls. With regard to apoptosis and cell proliferation, positively stained nuclei were counted from either 4000 (PCD) or 500 (Ki-67) cells, and the percentage of positive cells gave the PCD-index and Ki-67-index, respectively. Identifying apoptotic cells had to take into account characteristic morphological signs, such as cell shrinkage, increased cell density, karyopyknosis and karyorrhexis up to formation of apoptotic bodies at high magnification in order to avoid false positive results due to artificial staining of necrosis [16, 20].

Statistical analysis was performed using Statistica for Windows 5.1 (StatSoft, Tulsa, Okla.). Correlations between staging,

Table 3 Positive immunohistological staining against bcl-2 was detectable more frequently in less well-differentiated tumours ($P<0.001$)

Bcl-2	Grading					
	G1		G2		G3	
Negative	13	(92.9%)	50	(69.4%)	10	(47.6%)
Slight reaction	1	(7.1%)	9	(12.5%)	1	(4.8%)
Moderate reaction	0	(0.0%)	8	(11.1%)	5	(23.8%)
Strong reaction	0	(0.0%)	5	(6.9%)	5	(23.8%)
Sum	14	(100.0%)	72	(100.0%)	21	(100.0%)

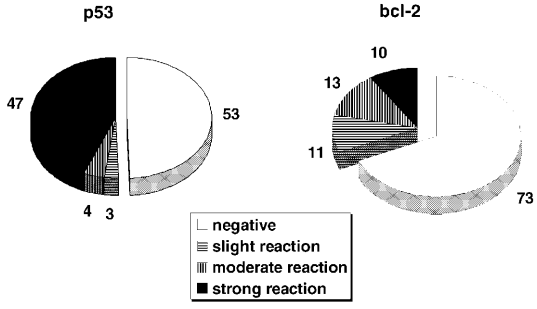

Fig. 2 Immunohistochemical staining was positive in 54 cases (50.5%) of primary tumours for p53 and in 34 cases (31.8%) for bcl-2

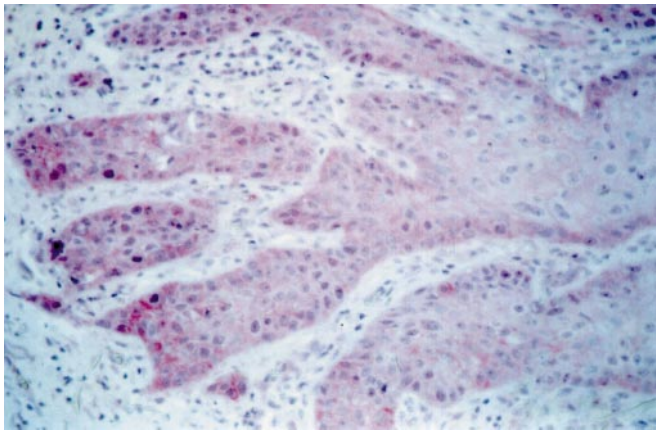

Fig. 3 Immunohistochemical detection of bcl-2 protein in the cytoplasm of an oral squamous cell carcinoma. Original magnification ×100

grading, immunohistochemical results, PCD-index and Ki-67-index were evaluated using Gamma test [13], and possible links of these parameters to different sites within the oral cavity and oropharynx using Kruskal-Wallis analysis of variance (ANOVA) [23]. The survival of the patients was estimated using the product-limit method according to Kaplan and Meier [18]. The multiple sample test to compare different groups of patients was an extension of Gehan's generalised Wilcoxon test [11]. First, a score was assigned to each survival time using Mantel's procedure [27]; next a Chi-square value was computed based on the sums for each group of this score. A probability level of $P<0.05$ was regarded as statistically significant in all tests.

Results

In 50.5% of the cases of SCC, p53 could be detected immunohistologically, irrespectively of site, tumour staging and grading. In most cases, tumours were either uniformly positive or negative, but in seven cases (6.5%) only a proportion of the tumour cells were positive (Fig. 2). When only a part of the cell nuclei was stained positively for p53, it was always the outer, less well-differentiated cell layer of a tumour strand, whereas the inner more mature cells were negative [42]. In 4 of 31 cases yielding various paraffin blocks of the primary tumour, differences of more than one gradation in the reaction between distinct sites within the tumour as a sign of heterogeneity were obvious. Between primary tumours and their corresponding lymph-node metastases in nine cases, a comparison was possible, showing in one case only a negative immunohistological staining in the primary tumour and a moderate reaction for p53 in the lymph-node metastasis. No differences were found comparing separate lymph-node metastases of the same patient.

Bcl-2 could be detected in 31.8% of all cases in the cytoplasm of the tumour cells showing various staining intensities (Fig. 2 and Fig. 3), irrespectively of site and tumour staging. The staining pattern within the tumour was comparable to the distribution of p53 when only a part of the cells was stained positively, being positive in the outer and negative in the inner areas of the tumour strands. In 3 of 31 cases making more than one paraffin block of the primary tumour available, differences of at least two gradations in the reaction between distinct sites within the tumour as a sign of heterogeneity could be found. Various staining between primary tumours and their corresponding lymph-node metastases or among distinct lymph-node metastases of the same tumour never became evident. In addition, there was no correlation between the detection of p53 and bcl-2, but bcl-2 was detectable more frequently in less well-differentiated tumours ($P<0.001$; Table 3).

In 55.0% of the cases, only a few apoptotic cells could be visualised enzymatically (PCD-index <0.5%, Fig. 4 and Fig. 5). The results ranged from a completely undetectable apoptosis up to a maximum PCD-index of 5% in one case, showing a decreasing case frequency towards higher PCD-indices, but independently of the tumour sites. Again, in 3 of 31 cases yielding more than one paraffin block of the primary tumour, differences of at least 1% in the PCD-index between distinct sites within the tumour as a sign of heterogeneity could be determined. Differences between primary tumours and their corresponding lymph-node metastases or among distinct

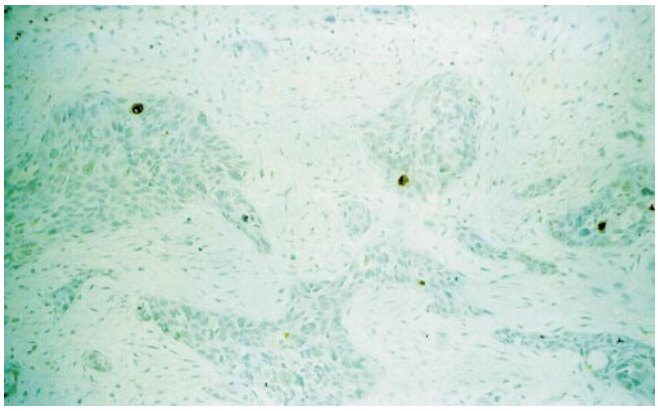

Fig. 4 Oral squamous cell carcinoma showing single apoptotic cell nuclei marked enzymatically by labelling of DNA-fragmentation using a terminal transferase reaction. Original magnification ×100

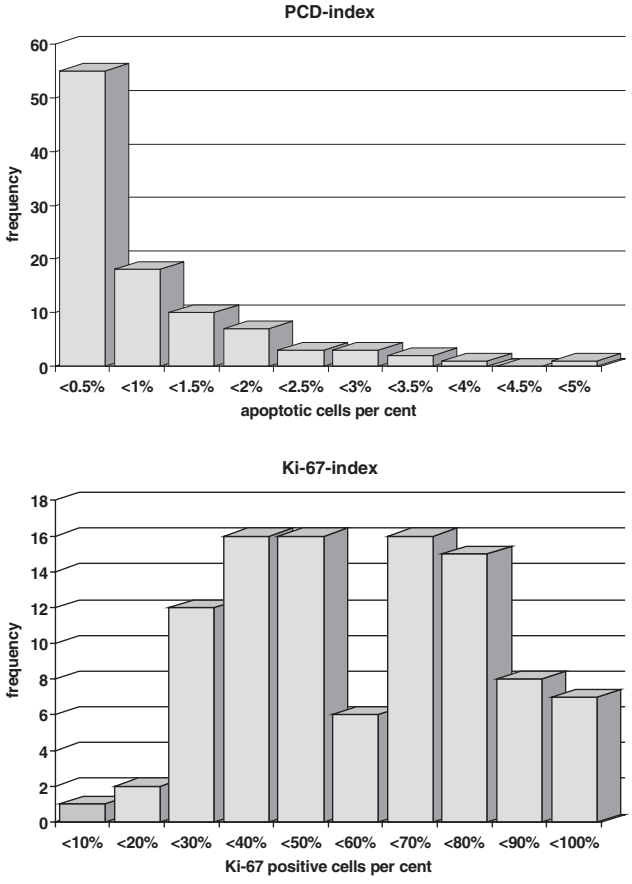

Fig. 5 In about half of the cases, only a few apoptotic cell nuclei were discernible in the primary tumours [programmed cell death (PCD)-index <0.5%]. Ki-67 was detected more frequently by far immunohistochemically, showing a wide range of results

lymph-node metastases of the same tumour could never be revealed. There was a significant correlation between positive immunohistochemical detection of p53 in the tumour cell nuclei and a decreased PCD-index on the one hand (P<0.003), and, on the other, in particular, positive

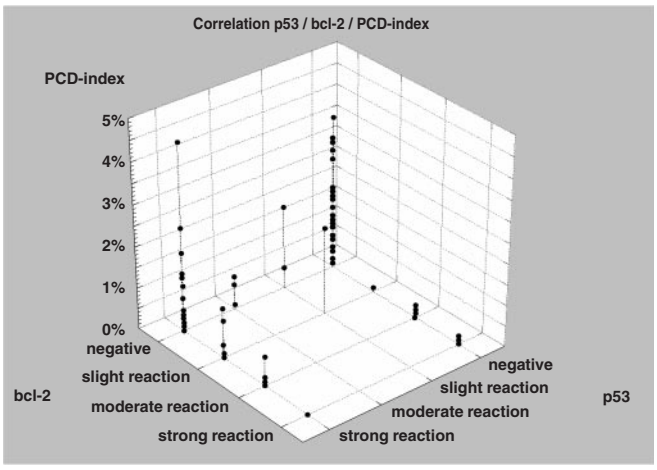

Fig. 6 There was a significant correlation between positive immunohistochemical detection of both p53 (P<0.003) and bcl-2 (P<0.001) in the tumour cells and a decreased programmed cell death (PCD)-index

cytoplasmic bcl-2 detection in the tumour cells was connected with a lower PCD-index (P<0.001; Fig. 6). In addition, the PCD-index was correlated positively with the staging parameter pT (P<0.03).

Ki-67 was detectable in a higher percentage than apoptotic cells by far, yielding a wide range of results (Fig. 5) without association to different tumour sites. There was no relationship between these two parameters, but the Ki-67-index was correlated positively with both of the tumour-staging parameters considered, pT (P<0.006) and pN (P<0.002). In 9 of 31 cases with various paraffin blocks of the primary tumour, differences of at least 10% in the Ki-67-index between distinct sites within the tumour as a sign of heterogeneity could be determined. In 1 of 9 cases comparing primary tumours and their corresponding lymph-node metastases, the Ki-67-index in the primary tumour was 35%, and in the lymph node metastasis, 45%. No differences were found comparing separate lymph-node metastases of the same patient.

Finally, both staging as pT and pN and grading as G proved to be significant predictors of the survival (P<0.01) and recurrence-free survival (P<0.02) of the patients (Fig. 7). In contrast, the immunohistochemical detection of p53 and bcl-2 as well as the PCD-index and Ki-67-index all failed to be predictive for either characteristic (Fig. 8).

Discussion

Tumour progression is characterised by an imbalance between cell proliferation and PCD, finally to the disadvantage of PCD. The aim of our study was to assess the importance of proliferation and apoptosis measurements in primary SCCs of the oral cavity and oropharynx, taking into account their corresponding lymph-node metastases. For determination of apoptosis, the enzymatic la-

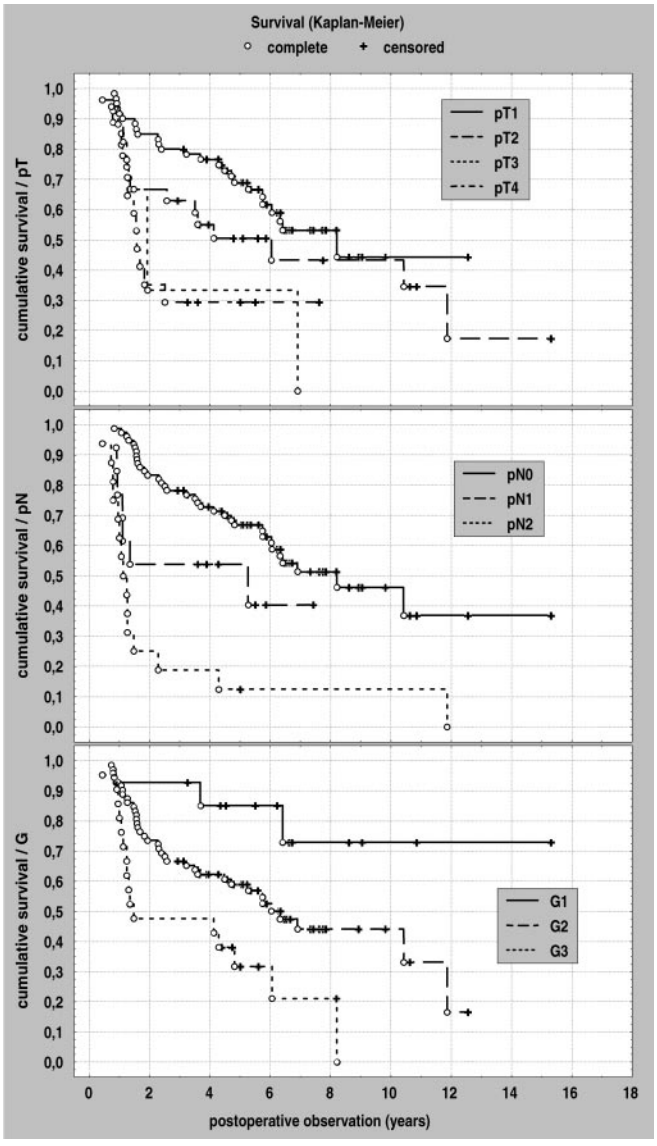

Fig. 7 The staging pT (P<0.01) and pN (P<0.001) as well as the grading G (P<0.005) were significant predictive factors for the survival of the 107 patients with squamous cell carcinomas in the oral cavity or oropharynx

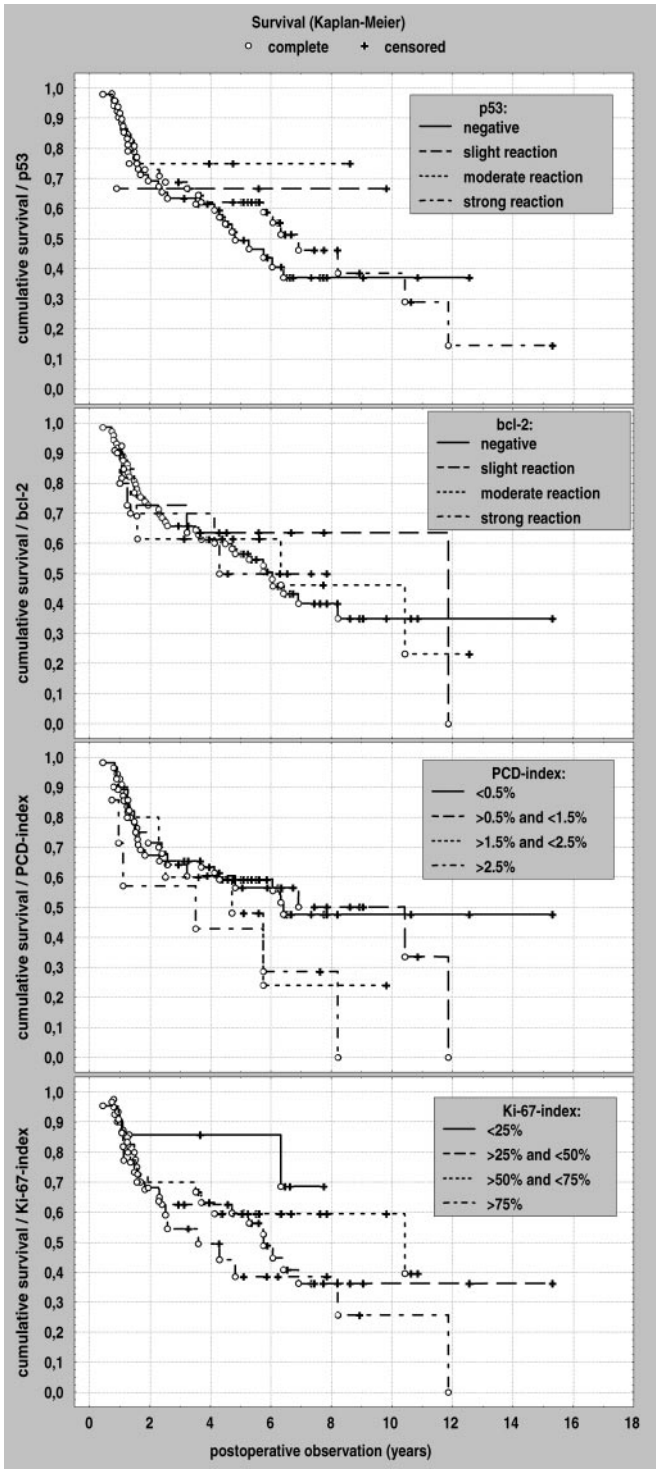

Fig. 8 The immunohistochemical detection of p53 and bcl-2, the enzymatic marking of apoptotic nuclei by labelling of DNA-fragmentation using a terminal transferase reaction, and the determination of the proliferative activity by immunohistochemical staining for Ki-67 failed to predict survival of the patients according to Kaplan and Meier

belling of DNA-fragmentation with a terminal transferase reaction used in our study has been shown to produce reliable results [1, 2, 10, 21, 47]. In addition, morphological aspects of apoptosis were considered [16, 20]. For proliferation assessment, Ki-67 was determined immunohistologically, which seems to be a more reliable marker of cell proliferation in SCC of the oral cavity than PCNA [29].

The regulation of both proliferation and apoptosis is complex and depends on conflicting growth signals [41]. In cells bearing a sublethal damage within their genome, the initiation of apoptosis at first depends on the function of wild-type p53 [7, 25]. In case of a missense mutation within the *TP53* gene or linkage of wild-type p53 to such viral proteins as the large T-antigen from SV40 virus or

the E1B antigen from adenovirus type 5, the loss of function of p53 is associated with a stabilisation of the phosphoprotein [45]. Due to an accumulation in the cell nuclei, it becomes detectable by means of immunohistochemistry. Of course, the immunohistochemical detection of p53, even in conjunction with effector molecules and antagonists, is not sufficient to reveal mutations within the *TP53* gene, especially nonsense or frame shift mutations, but does allow an insight into the regulatory mechanisms [42] and showed, in the case of head and neck SCC, a strong correlation to molecular methods [5]. In many tumour entities, the functional status of p53 has proved to be a valuable predictive factor for the progression of tumour disease and the outcome of patients [15], but, in SCC of the oral cavity and oropharynx, the state of expression is not useful in determining the prognosis [29, 32, 42]. The heterogeneity in p53 expression among different sites within the tumours and between primary tumour and their lymph-node metastases suggests a multifocal cancerogenesis in the oral and oropharyngeal mucosa, though the latter finding could also be caused by a change of the p53 expression during the process of metastatic spread.

Bcl-2 is known to preserve cells from p53-induced apoptosis and has been found increased in many tumour entities [2, 9, 24, 26, 34, 36]. The mechanism leading to bcl-2 over-expression in cells lacking the 14;18-translocation of the bcl-2 gene is still unknown. Bcl-2 forms heterodimers with another protein involved in the regulation of apoptosis, named bax, acting as its antagonist [30]. Thus, a disturbance of the post-translational regulation of bcl-2 might be present in SCC. In SCC of the oral cavity, bcl-2 and bax were associated inversely regarding the number of positive cells as well as the local distribution within the tumours showing a heterogeneous staining pattern [17]. Bcl-2 is not only mainly found in the outer, less well-differentiated cells in SCCs, but is also significantly positive correlated with tumour grading [8, 39]. The preferential finding of bcl-2 in less well-differentiated cells is commonly observed and obviously corresponds to the physiological function of bcl-2 protecting germinal tissue from demise by PCD [14].

Both p53 and particularly bcl-2 proved to be potent regulators of apoptosis in SCC under study. The apoptotic cell rates were quite low compared with other tumour entities [2], but comparable with previous investigations of intra-oral SCC [4, 21, 47]. Ki-67 was detectable more frequently showing a strong association to both tumour staging parameters considered in our study. There was a remarkably wide variation in results not only between tumours but also within individual SCCs, which could be explained, as with the p53 results [42], by multifocal cancerogenesis. It should be noted, however, that such diversity could also arise during progression. Hence, there is a need for a systematic sampling of the tumours.

Our observations suggest that the function of wild-type p53 to induce apoptosis is lost in at least half of the SCCs under study. Bcl-2 is upregulated in about one-third of the cases, depending on the tumour grading, and its physiological function as a potent inhibitor of apoptosis is widely preserved. Proliferation and PCD were enhanced in larger tumours, though a direct correlation between these two parameters was not proven. Nevertheless, in contrast to the conventional tumour staging and grading, neither the expression of p53 or bcl-2 nor the PCD-index or Ki-67-index were able to predict survival or recurrence-free survival of the patients suffering from a SCC in the oral cavity or oropharynx.

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