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The influence of p53 and associated factors on the outcome of patients with oral squamous cell carcinoma

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Abstract In several tumour entities the immunohistochemical detection of p53 has proved to be a predictive factor for the survival of the patients. In this study the effector waf1 and the regulator mdm2 responsible for the inactivation of p53 were also determined in 156 tissue samples of primary squamous cell carcinomas in the oral cavity and oropharynx, their lymph node metastases, and the epithelium outside the invasively growing tumour from 107 patients. In this latter epithelium there was a significant correlation between grade of dysplasia and staining for p53 ($P<0.01$). In the dysplastic epithelium a significant correlation between p53, waf1, and mdm2 was shown ($P<0.05$). Differences in the immunohistochemical staining between different blocks of the tumour tissue and also between primary tumours and their lymph node metastases were revealed in 11–44% of cases, but there was no correlation with other variables, such as formation of lymph node metastases. In contrast to the conventional tumour grading and staging, no influence of any of the variables determined on survival or recurrence-free survival could be detected. It seems that p53 and associated factors are important in the early stages of cancerogenesis but not in further tumour progression and metastatic spread.

Key words Oral, squamous cell carcinoma · Tumour suppressor gene · Prognosis · Immunohistochemistry

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Introduction

Knowledge about predictive factors and the biological changes that take place during neoplasia forms the basis for prevention, prognosis, and selection of the adequate therapy. Tumour progression is a process characterized by an imbalance of cell proliferation and apoptosis, both of which are influenced by the shift of proto-oncogenes to oncogenes and deletion, mutation or functional inactivation of tumour suppressor genes or of their products [6].

P53, the product of the tumour suppressor gene *TP53* localized on 17p13.1, has a central role in the control of the cell cycle of cells bearing a sublethal damage within their genome: it arrests the cell cycle in the late G_1-phase until repair of the genomic damage or transference to apoptosis occurs [6]. In this way, p53 maintains the integrity of the genome, and it is called the "guardian of the genome" [17].

The effector molecule of p53 in the cell cycle is the wild-type p53-activated fragment 1 (waf1) [6, 8]. For activation of waf1 the attachment of wild-type p53 homotetramers to specific binding sites in DNA is necessary [35, 36]. Dysfunction of p53 is due to deletion of or mutation within the *TP53* genes or inactivation of the p53 phosphoprotein itself. The product of the murine double minute 2 gene (*mdm2*) localized on 12q13–q14 causes physiological inactivation of p53 in the cell by forming a tight complex not only with wild-type but also with mutant p53 [25]. Originally, *mdm2* was identified as an oncogene, and it is amplified more than 50-fold in the 3T3DM murine fibroblast cell line [9]. Wild-type p53 has a short half-life of 5–40 min in nontransformed cells, and as a result it is not detectable by means of immunohistochemistry. After point mutation within the four hotspots of *TP53* it becomes stable and accumulates in the cell nucleus so that it is discernible immunohistochemically [19].

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]. Squamous cell carcinomas of the oral cavity and the oropharynx make up only 1.5% of all malignant tumours of the human body, but the rate of increase in the last two decades has been immense, namely 10–15% every year [10]. The aim of this study was to determine the influence of p53 on the outcome of patients with oral squamous cell carcinoma, with due consideration for the interaction between p53, waf1, and *mdm2* in tumour tissue, lymph node metastases, and oral epithelium outside the tumour tissue.

Materials and methods

One hundred and seven patients suffering from a primary squamous cell carcinoma of the oral cavity and oropharynx without distant metastases (M0) were included (Table 1) [34]. Twenty-four patients were female (22.4%) and 83, male (77.6%). The age distribution followed a standard distribution (Shapiro-Wilk's W-test, $P<0.02$) with a mean age of 56.5 years and a standard deviation of 10.5 years [32]. For 63 (70.8%) of the 89 patients with a reliable history alcohol and tobacco abuse was known. Four patients (4.5%) declared that they had never smoked but had drunk alcohol; 18 patients (20.2%) had smoked but not drunk. Four (4.5%) patients denied the use of both alcohol and tobacco. All patients had been treated surgically with curative intention supported by an adjuvant radiotherapy or chemotherapy depending on clinical indication [4]. The staging and grading and the highest grade of dysplasia in the epithelium outside the invasively growing tumour are given for all patients in Fig. 1 [29, 34].

During follow-up the subsequent treatment of the patients was not considered; only the recurrence-free survival and the survival of the patients independently of the cause of death were quantified. At the end of the follow-up period data about the death of 56 (52.3%) patients were available. The mean follow-up of the other 51 patients (47.7%), whether alive and still being monitored or lost to follow-up before their death was known: this was 6.4 years, with a minimum of 2.9 years and a maximum of 15.3 years.

The specimens had been routinely fixed in formalin after resection and embedded in paraffin (Table 2). Following dewaxing and rehydrating of the sections of 4 µm thickness drawn on SuperFrost/Plus slides (Menzel, Braunschweig, Germany), a target unmasking step consisting in autoclaving at 120°C for 20 min in 10 mM citric acid at pH 6.0 was performed [28]. The primary antibodies for immunohistochemistry were murine monoclonal antibodies obtained by Oncogene Science (Cambridge, Mass.) [37]. They were incubated in a dilution of 1:10 (p53 [Ab-6], OP43 and mdm2 [Ab-1], OP46) and 1:20 (waf1 [Ab-1], OP64) in phosphate-buffered saline (PBS) at pH 7.6 supplemented with 1% bovine serum albumin for 30 min at room temperature. The same buffer without primary antibody was applied to negative controls. The further process was a biotin–streptavidin standard procedure using the Super Sensitive Multilink Immunodetection System (BioGenex, San Ramon, USA). For visualization of the immunohistochemical reaction Fast Red TR/Naphtol AS-MX Phosphate (Sigma, St. Louis, Mo.) in 0.1 M Tris-buffer was used, and for counterstaining, Mayers haemalum.

The immunohistochemical reaction was evaluated using a microscope at 100-fold magnification. In a circumscribed tumour area the number of cell nuclei showing a positive immunohistochemical reaction in relation to the total number of 500 nuclei counted in each case was determined and divided into three thirds of reaction quantity. The definition of a slight reaction was established as positive staining in less than one third of the nuclei, a moderate reaction as positive staining in more than one third but less than two thirds of the nuclei, and a strong reaction as positive staining in more than two thirds of the nuclei. Absent immunohistochemical staining in all of the nuclei of the tumour tissue was defined as negative. In addition, the immunohistochemical reaction in the epithelium outside the invasively and growing tumour showing the highest grade of dysplasia was noted.

Table 1 Localizations 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 %

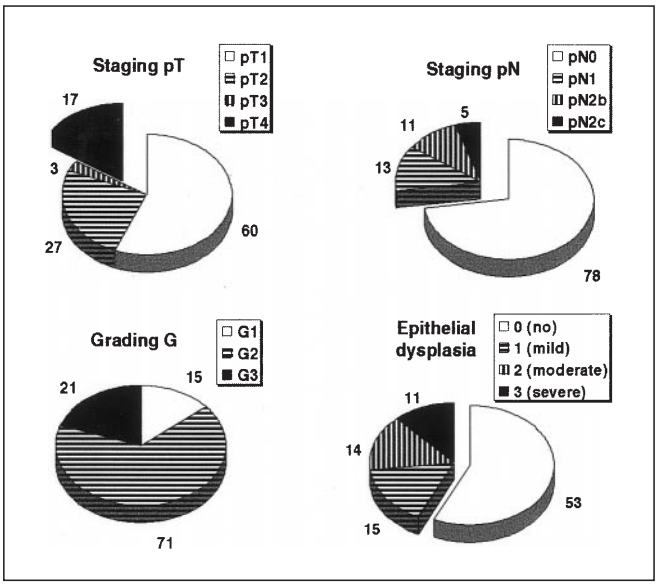

Fig. 1 Staging and grading of the 107 patients suffering from a squamous cell carcinoma in the oral cavity or the oropharynx and the highest grade of dysplasia in the epithelium outside the invasively growing tumour from the 93 cases for which such epithelium was available on the slides

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

Statistical analysis of the survival of the patients was estimated by the product-limit method according to Kaplan and Meier [16]. The multiple sample test used to compare different groups of patients was an extension of Gehan's generalized Wilcoxon test [11]. First, a score was assigned to each survival time using Mantel's procedure [21]; 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

Staining for p53 in the epithelium outside the invasive growing tumour tissue was always limited to the basal-

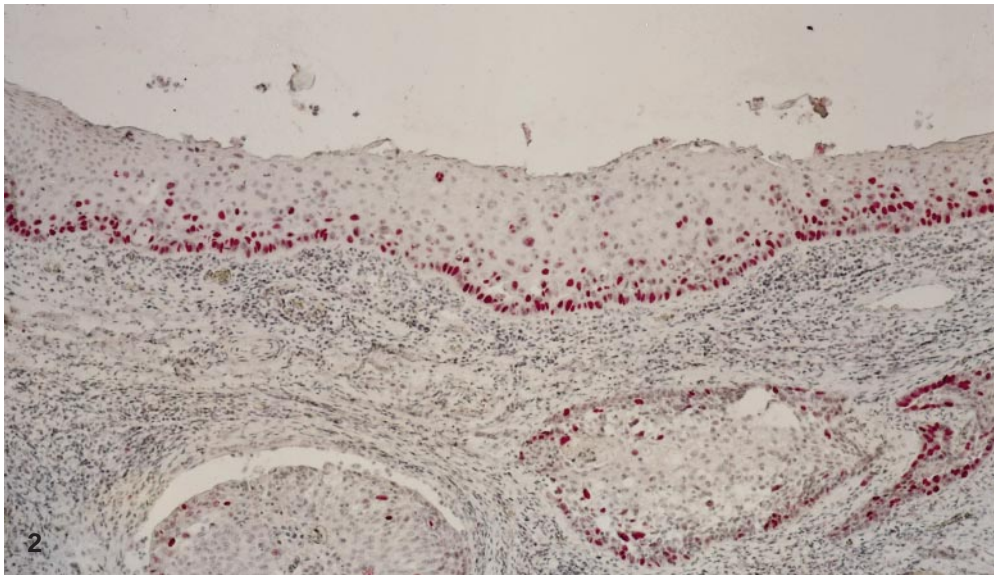

Fig. 2 Staining for p53 in the epithelium outside the invasively growing tumour tissue was limited to the basal cell layer of the epithelium for the most part. When only a part of the cell nucleus was stained positively for p53 in the tumour tissue, it was always the outer, less well-differentiated cell layer of a tumour strand, while the inner, more mature cells were negative. Original magnification, ×100

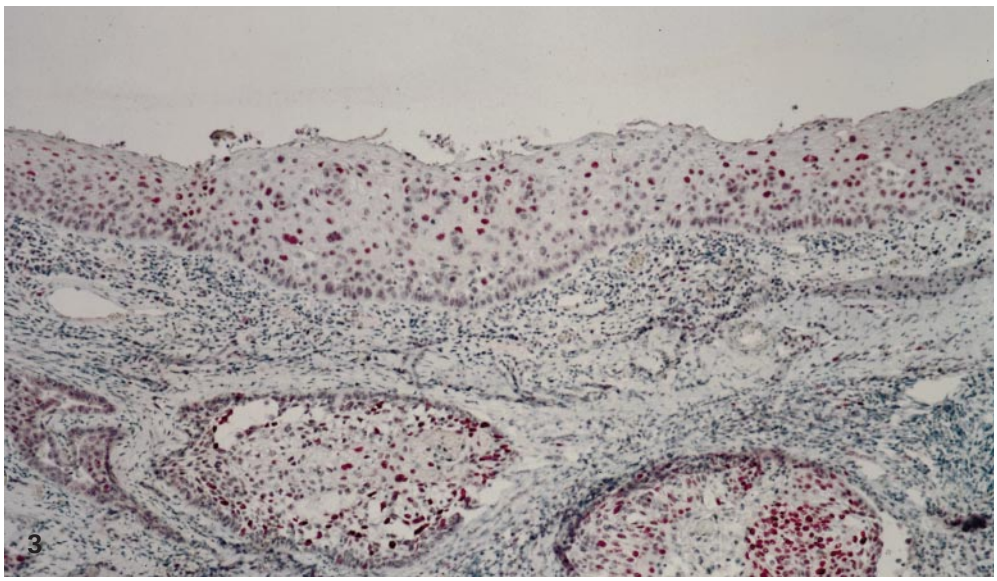

Fig. 3 Immunohistochemical staining for mdm2 (and waf1, not shown) was localized to the prickle-cell and granular layer of the epithelium, leaving the basal-cell layer free. In the tumour tissue from the immunohistochemical staining for mdm2 (and waf1, not shown) showed a contrasting pattern to that for p53. Original magnification, ×100

cell layer of the epithelium (Fig. 2). In contrast, the immunohistochemical staining for waf1 and mdm2 was localized to the prickle-cell and granular layer of the epithelium, leaving the basal-cell layer free (Fig. 3). The epithelium was stained positively for p53 in 30.2% of the cases without dysplasia, in 51.7% of the cases with mild or moderate dysplasia, and in 72.7% of the cases with severe dysplasia at the area of the epithelium showing the highest grade of dysplasia. Thus there is a significant correlation between grade of dysplasia and staining for p53 (Mann-Whitney U test P<0.01; Table 3) [20]. This correlation was missing for waf1, showing positive results in 79.3% of the cases without dysplasia, 75.9% with mild or moderate dysplasia, and 90.9% with severe dysplasia. For mdm2 there were positive results in 67.9% of the cases without dysplasia, 75.9% with mild or moderate dysplasia, and 81.8% with severe dysplasia.

Immunohistochemical staining was positive in 54 cases (50.5%) of primary tumours for p53, in 101 cases (94.4%) for waf1, and in 78 cases (72.9%) for mdm2 (Fig. 4). 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 (Fig. 2). Immunohistochemical staining for waf1 and mdm2 showed a

Table 3 A significant correlation (P<0.01) was found between grade of dysplasia and immunohistochemical staining for p53 in the epithelium showing the highest grade of dysplasia outside the invasively growing tumour

Epithelial dysplasia	p53-negative	p53-positive	Sum
0 (no)	37	16	53
1 (mild)	7	8	15
2 (moderate)	7	7	14
3 (severe)	3	8	11
Sum	54	39	93

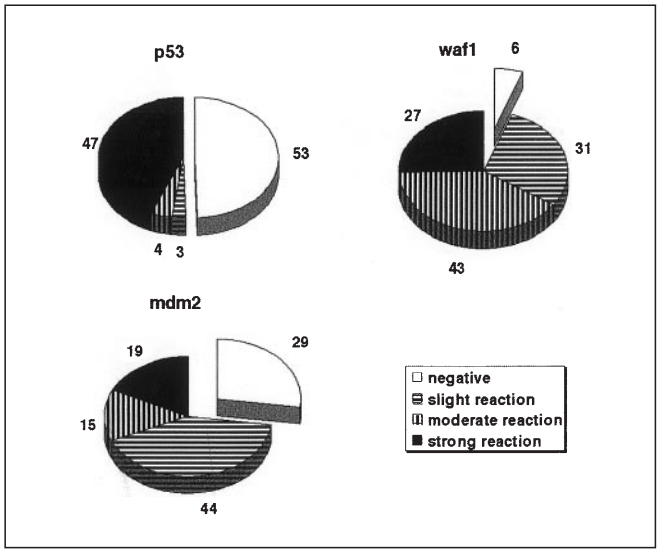

Fig. 4 Immunohistochemical staining was positive in 54 cases (50.5%) of primary tumours for p53, in 101 cases (94.4%) for waf1, and in 78 cases (72.9%) for mdm2

contrasting pattern compared with p53 (Fig. 3). Of 31 cases with more than one paraffin block available, differences in the reaction intensity between distinct sites of a primary tumour as a sign of heterogeneity were detectable in 4 cases (12.9%) for p53 and 6 cases (19.4%) for each of waf1 and mdm2. A reaction quantity for p53 differing between the primary tumour and a lymph node metastasis from it was seen in only 1 (11.1%) of 9 cases usable for this comparison. Here, the primary tumour was negative while in the lymph node metastasis a moderate immunohistochemical reaction for p53 was visible. For waf1 there were 2 cases (22.2%) and for mdm2 4 cases (44.4%) that showed differing reaction intensity. The shift of reaction intensity from the primary tumour to the corresponding lymph node metastasis was equally distributed in both directions concerning both variables. No differences were found between separate lymph node metastases of the same patient.

In normal epithelium without dysplasia a significant correlation between the reaction intensity for p53 compared with mdm2 ($P<0.05$) and for waf1 compared with mdm2 ($P<0.02$) was obvious. There was no association between the reactions for p53 and waf1. In the dysplastic epithelium outside the invasively growing tumour a positive correlation between all three variables was found ($P<0.05$). In squamous cell carcinoma tissue an association between the reaction intensity for p53 compared with mdm2 ($P=0.02$) and for waf1 compared with mdm2 ($P<0.01$) was also found. A relationship between the reactions for p53 and waf1 was missing, as in normal epithelium. There was no association of these variables with staging and grading of the tumours.

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. 5). In contrast, the immunohistochemical reactions for p53, waf1, and mdm2 all failed to be predictive for either characteristic (Fig. 6).

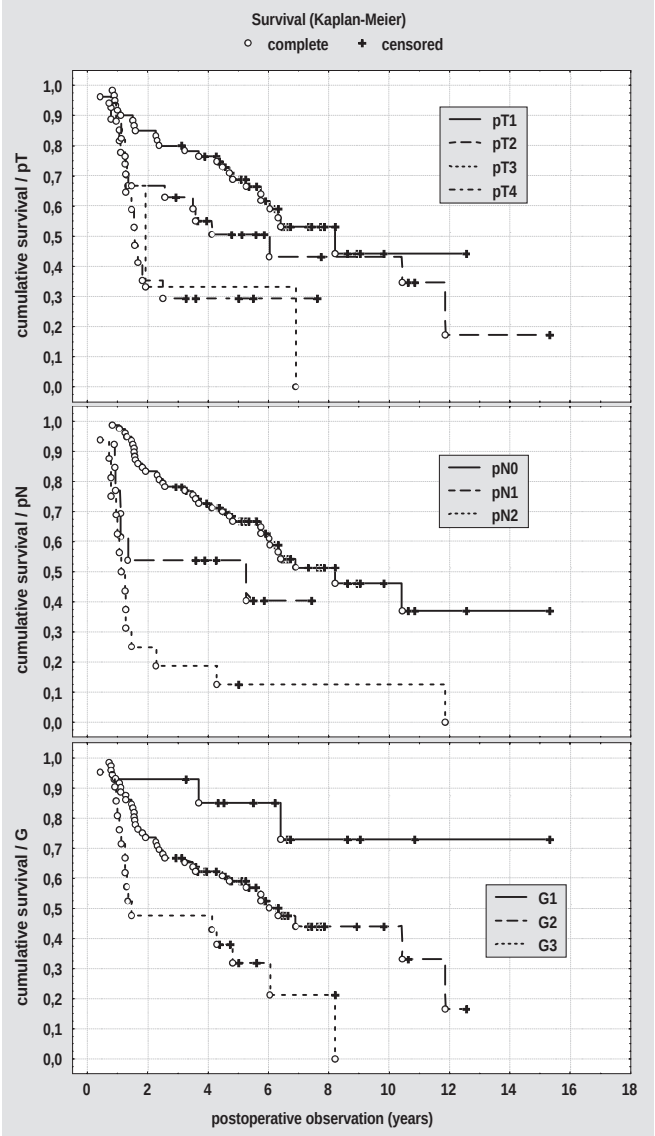

Fig. 5 Staging as pT and pN and grading as G are significant predictive factors for survival of the 107 patients with squamous cell carcinomas in the oral cavity or the oropharynx

Discussion

The numerous studies on the detection of p53 in tumour entities are based on the findings that p53 has a central role in the regulation of the cell cycle of cells bearing a sublethal damage within their genome and that it becomes detectable by means of immunohistochemistry if it accumulates in the cell nucleus [3, 13, 19, 37].

Unfortunately, the association between loss of function of p53 and accumulation in the cell nuclei may be disrupted in some conditions [3, 14, 39]. Apart from technical faults, such as inappropriate tissue handling,

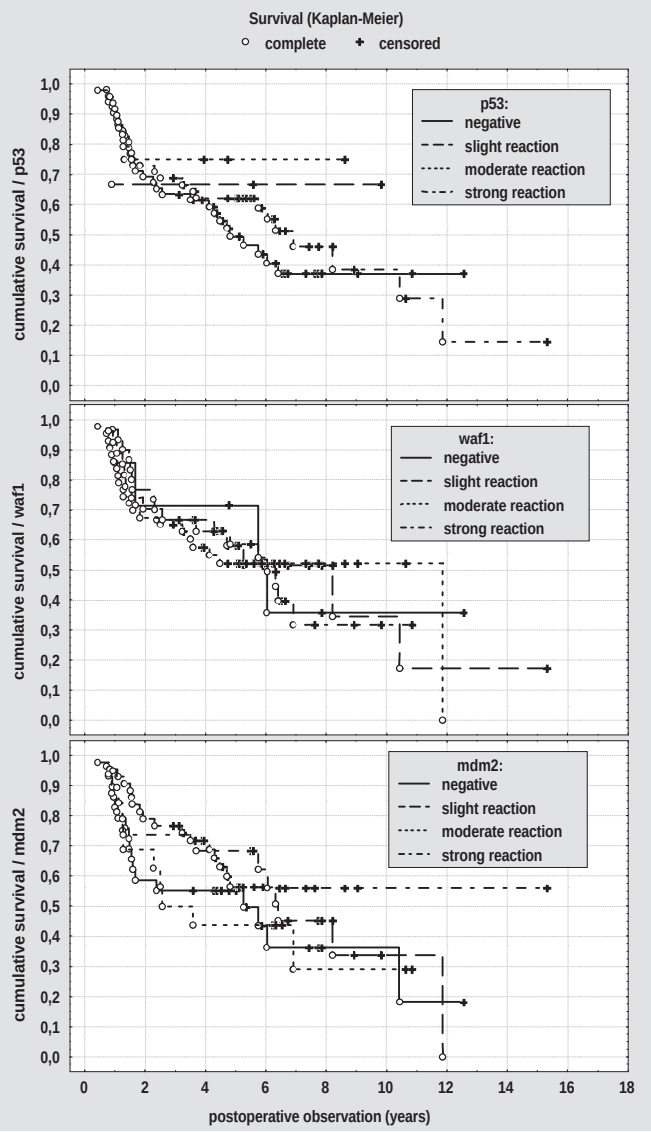

Fig. 6 The immunohistochemical reaction for p53, waf1, and mdm2 failed to predict survival of the patients according to Kaplan and Meier

false-negative results obtained with the immunohistochemical method may be caused by a deletion of the *TP53* alleles or a nonsense mutation leading to an abridged, nonstabilized phosphoprotein [3, 39]. False-positive results can result from an increase of the sensitivity of the immunohistochemistry, so that physiological amounts of p53 will be detected or from an up-regulation of p53 caused by the influence of noxae to the cellular genome [3, 14], corresponding to the physiological regulation stabilizing the phosphoprotein p53 by changing the degree of phosphorylation. Similarly, a linkage to such viral proteins as the large T-antigen from SV40 virus and the E1B antigen from adenovirus type 5 may lead to stabilization and accumulation of p53 [14, 36]. Nevertheless, in this case the loss of function of p53 is associated with a positive immunohistochemical result equivalent to a missense mutation.

The immunohistochemical detection of p53, even in conjunction with the physiological effector molecule waf1 and the antagonist mdm2, is not, on its own, sufficient to reveal mutations within the TP53 gene, but does allow an insight into the regulatory mechanisms [22]. The method is rapid and cheap, and as a further advantage it offers the option of assigning the results to definite areas in the tissue. The immunohistochemical procedure in our study achieved reliable results comparable with those of earlier investigations [2, 5, 18, 22, 26, 30, 33, 38].

In the epithelium outside the invasively growing tumour, less than one third of the analysable cases without dysplasia were stained positively for p53 in the cell nuclei. Mdm2 and waf1 were discernible more often. The arrangement of the staining for p53 in the basal-cell layer containing the less well-differentiated cells and for waf1 and mdm2 in the prickle-cell and granular layer of the epithelium containing the more mature cells was striking. For waf1 the increased expression in senescent cells compared with young cells is commonly established, leading to the alternative designation of senescent cell-derived inhibitor 1 [6, 23, 27]. Whereas the preferential accumulation of p53 in the basal and parabasal cell layers of squamous cell epithelium, indicating an involvement in the regulation of cell differentiation, is also well known [22, 23], restriction of the detection of mdm2 to suprabasal cell layers of the oral epithelium is different from the situation seen in the normal human skin [7]. Apart from this intraepithelial pattern, there was a significant positive correlation between the expression of p53 and its antagonist mdm2. As p53 is only one of the regulators of waf1, the lack of relationship between p53 and waf1 is probably due to the influence of cytokines linking to the platelet-derived growth factor receptor, the fibroblast growth factor receptor, and the epidermal growth factor receptor, inducing waf1 [6, 23, 24].

In the dysplastic epithelium outside the invasively growing tumour tissue, p53 was significantly more frequently discernible, depending on the grade of dysplasia. One explanation for this could be more frequent damage to the genome in the dysplastic than in the normal epithelium [3, 14] and up-regulation of p53 corresponding to the physiological role of p53 as guardian of the genome [17]. In addition, in the dysplastic epithelium there was a significant correlation between all three variables: p53, waf1, and mdm2, a sign not only that the relationship between p53 and mdm2 functions physiologically but also that the induction of waf1 by p53 predominates over other regulatory mechanisms.

About half of the cases of squamous cell carcinomas were stained positively or negatively for p53, respectively. When only a part of the cell nuclei was stained positively for p53 in a circumscribed tumour area, it was always the outer, less differentiated cell layer of a tumour strand, whereas the inner, more mature cells were negative. These findings are equivalent to the situation in the epithelium. Similarly, immunohistochemical staining for waf1 and mdm2 was always localized to the inner, more

mature cells. Between the immunohistochemical detection of p53 and mdm2 a positive correlation was show; a correlation was lacking between p53 and waf1. One possible explanation is that immunohistochemically detectable p53 in squamous cell carcinomas is enriched owing to a mutation in TP53, so that the function of inducing waf1 is lost [31, 36]. In comparison, even in the case of a missense mutation within the four hotspots p53 should be able to bind and, at least in the case of a mutation within exon 8 of *TP53*, to induce *mdm2* [1, 25].

In 12.9% of the cases suitable for the assessment of heterogeneity within the tumour tissue, differences between various blocks of the primary tumour were found for p53. The proportion of cases with differences was greater for waf1 and mdm2. At least in parts, these findings suggest multifocal cancerogenesis in the oral and oropharyngeal mucosa, though there were also differences between primary tumours and corresponding lymph node metastases. On the one hand, these could be caused by a change in the expression of p53, waf1, and mdm2 during tumour disease, especially in the process of metastatic spread; on the other, the possiblity cannot be ruled out that here primary different cell clones were available, which could not have been detected in the primary tumours by means of these immunohistochemical techniques.

In summary, correlation between the immunohistochemical detection of p53 in the epithelium outside the invasively growing tumour and the corresponding grade of dysplasia is a sign that changes in the regulation of p53 are early events in the cancerogenesis of oral squamous cell carcinomas [33]. The state of expression of p53 is important in premalignant lesions of the oral and oropharyngeal mucosa, but is not useful in determining the prognosis of patients suffering from an invasively growing carcinoma. Investigations concerning other tumour entities gave contrasting results, suggesting that mutations within *TP53* are late in their oncogenesis [12, 15]. Each tumour entity must be considered separately.

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