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ARTICLE

Proliferative (MIB1, mdm2) Versus Anti-Proliferative (p53) Markers in Head and Neck Cancer. An Immunohistochemical Study

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Formalin fixed and paraffin embedded samples from 36 squamous cell carcinomas of the larynx and the oral cavity ($pT_2N_0M_0$, R_0) surrounded by non-tumorous mucosa were studied immunohistochemically using a panel of four different anti-p53 antibodies (CM1, PAb1801, D07, PAb240), a monoclonal anti-mdm2 antibody and MIB1, following wet autoclave antigen retrieval. P53 immunoreactivity was detected in 11/14 laryngeal and in 9/22 oral carcinomas. All p53 positive oral, and all but one laryngeal tumors revealed mdm2 positivity as well, whereas in p53 negative tumors 4/12 and 1/3 mdm2 immunopositive cases were demonstrated, respectively. MIB1 labeling indices of the tumors ranged between 18% – 64% in p53 positive cases, and 10% – 53% in p53 negative ones. The difference was not statistically significant. Close spatial coexpression of p53, mdm2 and MIB1 immunoreactivity was observed at the invasive front of the carcinomas and in the basal and suprabasal layers of the non-tumorous epithelium in all p53 positive cases. However, the MIB1 expression was similarly increased at the invasive margins in carcinomas lacking immunohistochemically detectable p53 alterations. Our results strongly suggest that p53 overexpression does not necessarily correspond to increased rate of proliferation, but rather to mdm2 overexpression and is largely dependent on the anatomical site in case of small and localized squamous cell carcinomas of the head and neck region. (Pathology Oncology Research Vol 2, No1–2, 37–42, 1996)

Key words: p53; mdm2; MIB1; immunohistochemistry; wet autoclave pretreatment; head and neck cancer

Introduction

Wild-type p53 has been recognized as an important supervisor of cell proliferation in order to maintain genomic stability. Upon recognizing DNA damage it may arrest the cells in cycle or initiate apoptosis, depending on the extent of DNA injury or the type of cell.[12,16] Mutated or functionally inactivated p53 protein fails to exert these regulatory functions and may actively contribute to abnormal cell growth.[11,15] P53 dysfunction has also recently been attributed to the formation of stable complexes with other proteins, such as viral proteins,[13,25,26] members of the heat shock proteins,[14] or mdm2 oncoprotein.[20] Most of these inactivated p53 complexes, due to their increased stability, can be detected by anti-p53 antibodies.

P53 has been detected immunohistochemically in 32-54% of oral cancers and 44-84% of laryngeal cancers. Controversial results were published on the association of p53 anomalies with Ki-67 (a proliferation marker) in squamous cell cancers of the head and neck area.[28,21] As mentioned, the activity of wild type p53 can be inhibited by mdm2,[20,31] however, in head and neck cancers no amplification of the mdm2 gene has been detected, yet.[2]

In this study, 36 squamous cell carcinomas of the larynx and the oral cavity were immunohistochemically stained with a panel of anti-p53 antibodies, anti-mdm2 and MIB1 antibody (which detects Ki-67 antigen in cycling cells), in order to topographically locate these proteins. Such an approach can provide indirect evidence of potential functional links between the involved molecules.

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Materials and Methods

Formalin fixed and paraffin embedded tumor tissues from 36 squamous cell carcinomas of the larynx and the oral cavity with a tumor stage of $T_2N_0M_0$, R_0 (UICC) were retrieved from the files of the Department of Pathology, University of Innsbruck and the Department of Pathology, University of Münster. 2 µm thick serial sections were cut from the paraffin blocks, mounted on poly-L-lysine coated glass slides, and dried overnight at 37°C. Afterwards, sections were dewaxed in xylene, rehydrated in a series of alcohols, and finally rinsed in distilled water.

Autoclave pretreatment

Sections were immersed in plastic Coplin jars containing citrate-buffer (0.01M Na-citrate, pH 6.0) and incubated in a Gössner Laborautoklav (GLA-40-2) at 120°C for 5 minutes. Afterwards, sections were cooled to room temperature (30 minutes) and briefly rinsed with 0.05 M Tris-HCl buffer, pH 7.4. (For details see Bánkfalvi et al.[5])

Primary antibodies

CM-1: a rabbit polyclonal antiserum raised against full-length human p53 expressed in *E. Coli;*[19] *PAb1801:* a murine monoclonal antibody that recognizes a denaturation resistant epitope at the N terminus of both wild-type and mutant p53 proteins between amino acids 32-79;[4] *DO7:* a murine monoclonal antibody that recognizes an epitope between amino acids 1-45 both in wild-type and mutant forms of p53 protein;[27] *PAb240:* a murine monoclonal antibody that reacts with a common conformation dependent, denaturation resistant epitope in mutant p53 variants (amino acids 213-217)[10], originally designed for frozen sections; *anti-mdm2:* a murine monoclonal antibody IF2, recognizing an epitope in the amino terminal portion of the human mdm2 protein; *MIB1:* an anti-Ki-67 equivalent murine monoclonal antibody.[7]

The four anti-p53 antibodies were purchased from Medac (Hamburg), the MIB1 antibody from Dianova (Hamburg) and the anti-mdm2 antibody from Oncogene Science (Hamburg).

Immunohistochemical staining procedure

Optimal dilutions of the primary antibodies after autoclave pretreatment have been previously tested: CM-1 1:20.000, DO7 1:500, PAb1801 1:500, PAb240 1:50, anti-mdm2 1:10, MIB1 1:500 in 9% RPMI containing 9% heat inactivated human serum. Incubations with the primary antibodies were carried out overnight (14-16 hours) at 4°C. Secondary immunostaining reactions were performed according to standard alkaline phosphatase- anti-alkaline phosphatase (APAAP) protocol using rabbit-anti-mouse Ig 1:30 in TBS for the monoclonal antibodies, and a mouse anti-rabbit Ig 1:125 in TBS for the polyclonal CM1 antibody, both for 30 minutes, at room temperature. Afterwards, incubation with a mouse-APAAP complex was carried out in 1:100 diluted TBS for 60 minutes at room temperature. The secondary reagents were purchased from Dako (Copenhagen). For the PAb240, DO7 and mdm2 antibodies, a "double APAAP" reaction was performed (with one repeat for 10 minutes for both the secondary antibody and the APAAP-complex). Colour development was carried out using a freshly prepared Fast Red solution containing naphthol-As-biphosphate (Sigma) for 30 min at room temperature. For the mdm2 reactions, an NBT/BCIP (nitro blue tetrazolium, 5-bromo-4-chloro-3-indolyl phosphate) chromogen solution was applied for 30 minutes in the dark in a humidified chamber. Finally, slides were rinsed in tap water, counterstained with Mayer's haematoxilin and mounted in Kaiser's glycerine-gelatine.

Control: In negative controls, the primary antibodies were omitted. One gastric and one breast cancer sample with known p53 mutations and overexpression were used as positive controls for p53. Normal tonsil sections were unsed for MIB1 controls.

Immunohistochemical assessment

All immunohistochemical reactions were assessed at the invasive edge of the respective tumors. Estimation of the proliferative tumor cell fraction was performed at a 400-fold magnification using a 10x10 eyepiece grid in a Zeiss microscope. The number of positively stained tumor cell nuclei was determined in at least 5 areas per tumor, each containing at least 100 tumor cells. The labeling indices (LIs) were expressed as percentages of all cells counted. P53 scoring was performed as recommended by Fischer *et al.*[9]: "strong, moderate, weak, scattered, cytoplasmic and negative." Mdm2 immunoreactivity was estimated as follows: +++ more than 50% positive cells, ++ 10-50% positive cells, + less than 10% positive cells, "neg" no immunoreactivity detectable. Additionally, both p53 and mdm2 were assessed in the dysplastic and normal-looking mucosa adjacent to the respective tumors.

Results

Immunostaining with anti-p53 antibodies

Immunohistochemically detectable p53 was almost exclusively confined to cell nuclei. Additional intracytoplasmatic reactions could be observed in 2 cases using PAb240 and in one case using PAb1801 antibodies. Immunoreactivity with all four anti-p53 antibodies could be detected in 8/22 oral- and in 8/14 laryngeal carcinomas. We considered a tumor as "p53 positive" if it reacted with at least one anti-p53 antibody even if the reaction was designated as

***Table 1.* p53, mdm2 and MIB1 immunoreactions at the invasive front of laryngeal carcinomas**

Case	CM1	PAb1801	DO7	PAb240	mdm2	MIB1 (LI%)
1.	moderate	strong	strong	weak	++	50
2.	weak	weak	neg	neg	+	57
3.	weak	neg.	weak	neg	neg	41
4.	weak	weak	weak	neg	+	42
5.	moderate	moderate	moderate	moderate	+	23
6.	moderate	moderate	moderate	moderate	++	60
7.	moderate	moderate	moderate	moderate	+	18
8.	weak	strong	strong	strong	++	58
9.	moderate	moderate	moderate	moderate	++	27
10.	moderate	moderate	moderate	weak	++	64
11.	moderate	moderate	moderate	weak	++	35
12.	neg	neg	neg	neg	neg	27
13.	neg	neg	neg	neg	+	34
14.	neg	neg	neg	neg	neg	47

"weak". Thus, the total rate of p53 positive oral and laryngeal carcinomas was 9/22 and 11/14, respectively. The immunohistochemical results are summarized in *Table 1 and 2*.

The four anti-p53 antibodies revealed in most cases similar reaction patterns. CM1, PAb1801 and DO7 antibodies were found to be negative in only 1 tumor, whereas PAb 240 was negative in 3 tumors. If stained, the quantity of p53 positive cells was within the same range using the different antibodies. Different scores were mainly due to the variability in staining intensity. The remaining p53 negative cases (3/11 laryngeal and 13/22 oral carcinomas) completely lacked p53 positive tumor cell nuclei.

P53 positive tumor cells accumulated predominantly at the periphery of the invading edge of the carcinomas whereas the more differentiated and keratinizing central areas were consistently negative (*Fig.1*). The only grade 3 tumor showed a diffuse staining pattern (*Fig.2*). In the adjacent non-tumorous mucosa, consisting of dysplastic and normal-looking epithelial components, p53 positivity was detected either focally, in cell clusters, or diffusely distributed in the basal and parabasal layers of the epidermis (*Fig.3*). Positive p53 immunoreactions could be observed in the adjacent normal-looking epithelium in 13 oral and in 7 laryngeal cases, irrespective of the p53 immunophenotype of the tumor. The same reactivity pattern was observed in dysplasias (positivity: 15 oral and 8 laryngeal cases).

***Table 2.* p53, mdm2 and MIB1 immunoreactions at the invasive front of oral squamous cell carcinomas**

Case	CM1	PAb1801	DO7	PAb240	mdm2	MIB1 (LI%)
1.	neg	moderate	moderate	weak	++	42
2.	moderate	strong	moderate	weak	++	53
3.	moderate	strong	moderate	weak	++	37
4.	strong	strong	strong	strong	++	35
5.	moderate	moderate	weak	moderate	++	44
6.	strong	strong	moderate	moderate	+	40
7.	strong	strong	moderate	strong	++	40
8.	moderate	moderate	weak	weak	++	35
9.	strong	strong	moderate	moderate	+	53
10.	neg	neg	neg	neg	neg	52
11.	neg	neg	neg	neg	+	45
12.	neg	neg	neg	neg	neg	33
13.	neg	neg	neg	neg	+	10
14.	neg	neg	neg	neg	neg	46
15.	neg	neg	neg	neg	+	10
16.	neg	neg	neg	neg	neg	25
17.	neg	neg	neg	neg	neg	10
18.	neg	neg	neg	neg	neg	42
19.	neg	neg	neg	neg	neg	35
20.	neg	neg	neg	neg	neg	44
21.	neg	neg	neg	neg	++	48
22.	neg	neg	neg	neg	neg	53

Figure 1. *p53 positive tumor cells accumulate at the invasive zone of well differentiated squamous cell carcinomas (PAb 1801 antibody, x65)*

MIB1 immunostaining

The distribution of MIB1 positive tumor cells was similar to p53 positivity. The MIB1 labeling indices (LI), calculated at the invasive edge of the tumors, are shown in *Tables 1 and 2*. In the adjacent normal mucosa, MIB1 reaction was confined to the basal or occasionally to the

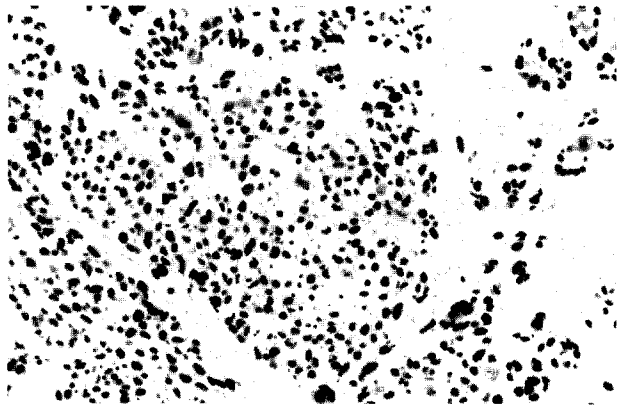

Figure 2. *Diffuse distribution of p53 positive tumor cells in a Grade 3 carcinoma (CM1 antibody, x100)*

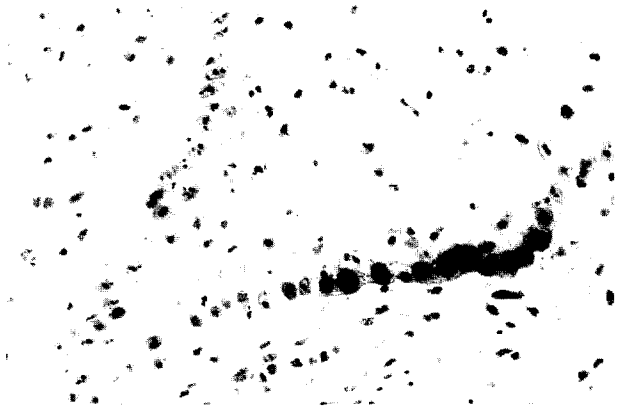

Figure 3. Focal accumulation of p53 positive cells along the basal layer of normal looking epithelium adjacent to a squamous cell carcinoma (PAb 1801 antibody, x250)

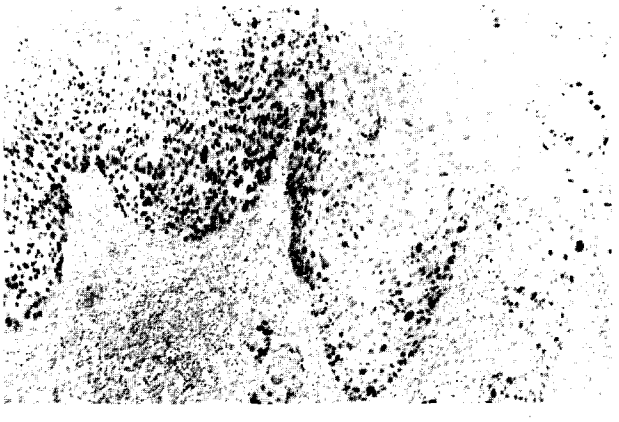

Figure 4. MIB 1 positive cells in normal- (right side) and in dysplastic epithelium (left side) (x65)

parabasal layers. In areas of dysplasia or in situ carcinomas, a suprabasal or panepithelial MIB1 reactivity was observed. (*Fig.4*).

Comparison of the anti-p53 and MIB1 immunostainings

Both p53- and MIB1 positive tumor cells were predominantly accumulated at the invasive front of the carcinomas. In all p53 positive tumors examined, p53 immunoreactivity was associated with MIB1 expression in a close spatial localisation (*Fig.5 a,b*), but MIB1 positivity could also be observed without immunohistochemically detectable p53. If p53 immunoreactivity was present, it was localised similarly to MIB1 positive cells in the normal and dysplastic mucosa compared.

Correlation with clinicopathological parameters

No statistically significant correlations[30] were observed between both p53 overexpression and MIB1 labeling indices with histopathological and clinical parameters (age of the patients, localisation, clinical and pathological staging and histological differentiation grade of the tumors). (Data not shown).

Immunostaining with the anti-mdm2 antibody

Mdm2 reactions were confined to the nuclei of cells both in tumorous and in non-tumorous epithelium and resembled the spatial distribution of the p53 and MIB1 reactions (*Fig.6*). Mdm2 positivity could be detected in all p53 positive oral- and in all, except two, p53 positive laryngeal carcinomas. Among the p53 negative tumors 4/12 oral- and 1/3 laryngeal cancers expressed mdm2 positivity. All but one revealed scattered distribution (+). In non-tumorous mucosa adjacent to carcinomas, mdm2 immunoreactivity could be found in 13 laryngeal and in 18 oral samples (*Fig.7*).

Correlation between p53 and mdm2 expression

Statistically significant co-expression of p53 and mdm2 proteins was found both in the carcinomas and in the adjacent non-tumorous mucosa ($chi^2 = 31.5$, $p = 0.0001$).

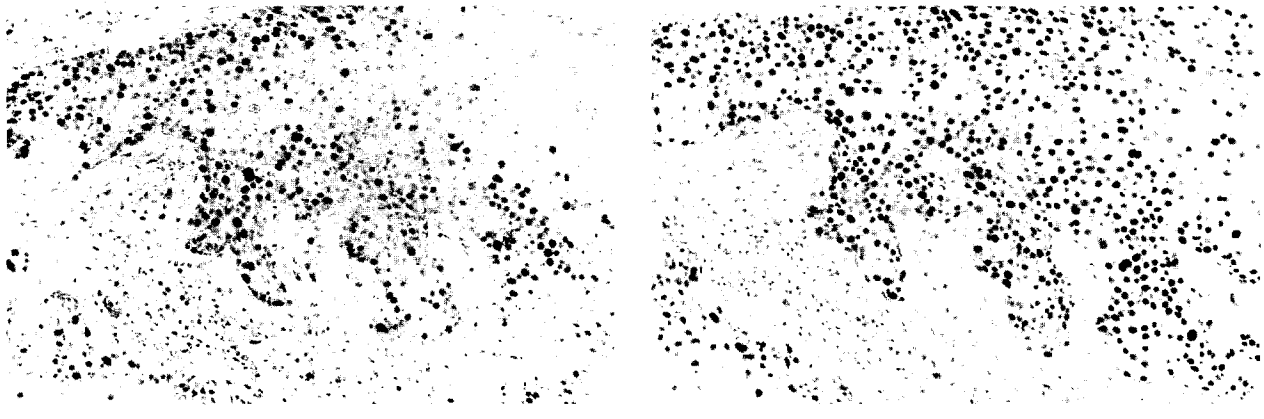

Figure 5. a, b Colocalisation of p53 positive (5a) and MIB1 positive tumor cells (5b) at the invasive tumorfront. The amount of p53 positivity exceeds that of the MIB1 (DO7 and MIB1 antibodies, x100)

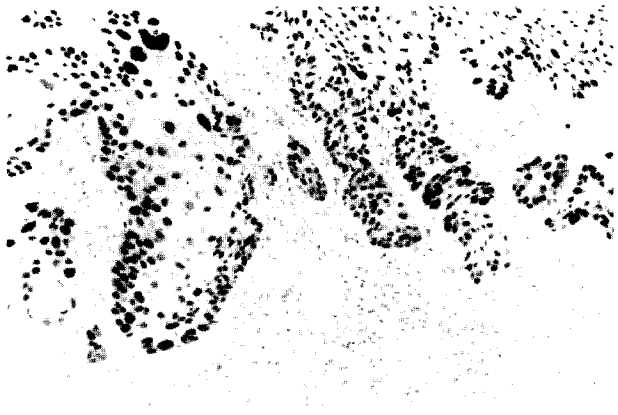

Figure 6. The distribution of mdm2 immunoreactivity resembles to that of p53 and MIB1 in squamous cell carcinomas (x100)

Discussion

Commonly, squamous cell carcinomas with the same histological pattern often vary in their behavior according to the anatomical localisation within the head and neck region. Different prevalence of p53 positive tumors at different sites within this region has been already described.[22] The significance of the invasive edge of the tumors in determining the malignant potential of squamous cell carcinomas of the head and neck has been also established.[5,6] Immunohistochemically detectable p53 positivity of laryngeal tumors has been reported in 44% - 84% of invasive carcinomas[22,8], in 50% of in situ carcinomas and in 28% of the non-progressive lesions.[21] Additionally, p53 protein overexpression has been frequently detected both in dysplastic and normal-looking mucosa in the vicinity of carcinomas.[1,8,22] In oral squamous cell carcinomas, p53 positivity has been reported in a prevalence range between 30-54%[18,23] with frequent occurrence in the adjacent non-tumorous mucosa.[2] Our result with 79% p53 immunopositivity in laryngeal and 40% in oral squamous cell carcinomas is in accordance with previous observations indicating an anatomical site preference of p53 positive tumors within the head and neck region.

The evaluation and results of such studies could be hampered by the availability of antigens. We have recently shown that immunohistochemical demonstrations of p53 protein in routinely formalin-fixed and paraffin-embedded tumor tissues depends largely on antigen retrieval.[24]

The four different anti-p53 antibodies used in this study were directed against different epitopes or conformational variants of the p53 protein. The expression intensity of these p53 variants within a given tumor reflects more inter- than intratumoral heterogeneity. Our finding that even in p53 negative carcinomas a p53 immunoreactivity can exist in neighbouring mild to severe dysplasias or in normal-looking basal or parabasal epithelial cells is in accordance with the results of Nees *et al.*[22]

Immunohistochemically detectable p53 protein accumulation within the cells marks a dysfunction of the p53 system. The coexpression of p53 and mdm2 in the carcinomas suggests, that beside mutations of the p53 gene, which occur in about 70% of head and neck cancers,[2,17] complex formation between the two proteins may contribute to the inactivation and accumulation of the p53. P53 overexpression in non-neoplastic cells could represent, in theory, a controlled inhibition of cell proliferation due to DNA damage, or in dysplasias the accumulation of functionally inactivated p53 protein as an early sign of uncontrolled proliferation.

It is intriguing that an increased cell proliferation rate can be observed at the invasive front of squamous cell carcinomas of the larynx and oral cavity and in basal and/or suprabasal layers of the non-tumorous mucosa regardless of whether immunohistochemically demonstrable p53 protein accumulation is present or not. However, this result might simply indicate that the regulation of cell proliferation is not entirely p53 dependent.

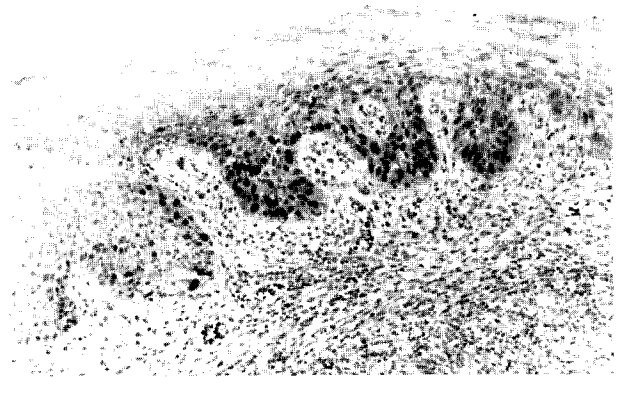

Figure 7. Focal mdm2 immunoreactivity in mild dysplasia (the same area revealed p53 positivity as well) (x100)

References

1. *Anwar K, Nakakuki K, Imai H, Naiki H and Inuzuka M:* Overexpression of p53 protein in human laryngeal carcinoma. Int J Cancer 53:952-956, 1993.
2. *Ahomadegbe JC, Barrois M, Fogel S, Le Bihan ML, Douc-Rasy S, Duvillard P, Armand JP and Riou G:* High incidence of p53 alterations (mutation, deletion, overexpression) in head and neck primary tumors and metastases: absence of correlation with clinical outcome. Frequent protein overexpression in normal epithelium and in early non-invasive lesions. Oncogene 10:1217-1227, 1995.
3. *Bánkfalvi A, Navabi H, Bier B, Böcker W, Jasani B and Schmid KW:* Wet autoclave pretreatment for antigen retrieval in diagnostic immunohistochemistry. J Pathol. 174:223-228, 1994.
4. *Banks L, Mathlashewski G, Crawford LV:* Isolation of human p53 specific monoclonal antibodies and their use in the study of human p53 expression. Eur J Biochem. 159:529-534, 1986.

5. *Bryne M, Koppang HS, Lilleng R and Kjarheim A:* Malignancy grading of the deep invasive margins of oral squamous cell carcinomas has high prognostic value. J Pathol 166:357-361, 1992.
6. *BryneM, Jenssen N and Boysen M:* Histological grading in the deep invasive front of T1 and T2 glottic squamous cell carcinomas has high prognostic value. Virchows Arch 427:277-281, 1995.
7. *Cattoretti G, Becker MHG, Key G, Duchrow M, Schlüter C, Galle J and Gerdes J:* Monoclonal antibodies against recombinant parts of the Ki-67 antigen (MIB1 and MIB3) detect proliferating cells in microwave-processed formalin-fixed paraffin sections. J Pathol 168:357-363, 1992.
8. *Dolcetti R, Doglioni C, Maestro R, Gasparotto D, Barzan L, Pastore A, Romanelli M and Boiocchi M:* p53 overexpression is an early event in the development of human squamous cell carcinoma of the larynx: genetic and prognostic implications. Int J Cancer 52:178-182, 1992.
9. *Fischer CJ, Gillett CE, Vojtesek B, Barnes DM and Millis RR:* Problems with p53 immunohistochemical staining: the effect of fixation and variation in the methods of evaluation. Br J Cancer 69:26-31, 1994.
10. *Gannon JV, Greaves R, Iggo R and Lane DP:* Activating mutations in p53 produce a common conformational effect. A monoclonal antibody specific for the mutant form. EMBO J 9:1595-1602, 1990.
11. *Hollstein M, Sidransky D, Vogelstein B and Harris CC:* p53 mutations in human cancers. Science: 253:49-53, 1991.
12. *Kastan MB, Onyekwere O, Sidransky D and Vogelstein B, Craig RW:* Participation of p53 protein in the cellular response to DNA damage. Cancer Res 51:6304-6311, 1991.
13. *Lane DP and Crawford LV:* T antigen is bound to a host protein in SV40 transformed cells. Nature 278:261-263, 1979.
14. *Lehman TA, Bennett WP, Metcalf RA, Welsh JA, Ecker J and Modali RV:* p53 mutations, ras mutations and p53-heat shock protein complexes. Cancer Res 51:4090-4096, 1991.
15. *Levine AJ, Perry A, Chang A, Silver A, Dittmer D, Wu M and Welsh D:* The 1993 Walter Hubert lecture: The role of the p53 tumour-suppressor gene in tumorigenesis. Br J Cancer 69:409-416, 1994.
16. *Lowe SW, Ruley HE, Jacks T and Houseman DE:* p53-dependent apoptosis modulates the cytotoxicity of anticancer agents. Cell 74:957-968, 1993.
17. *Maestro R, Dolcetti R, Gasparotto D,Doglioni C, Pelucchi S, Barzan L, Grandi E and Boiocchi M:* High frequency of p53 gene alterations associated with protein overexpression in human squamous cell carcinoma of the larynx. Oncogene 7:1159-1166, 1992.
18. *Matthews JB, Scully C, Jovanovic A, Van der Waal I, Yeudall WA and Prime SS:* Relationship of tobacco/alcohol use to p53 expression in patients with lingual squamous cell carcinomas. Oral Oncol Eur J Cancer 29B: 285-289, 1993.
19. *Midgley CA, Fisher CJ, Bartek J, Vojtesek B, Lane DP and Barnes DM:* Analysis of p53 expression in human tumours: an antibody raised against human p53 expressed in *E. coli*. J Cell Sci 101:183-189, 1992.
20. *Mohmand J, Zambetti GP, Olson DC, George D and Levine AJ:* The mdm2 oncogene product forms complex with the p53 protein and inhibits p53 mediated transactivation. Cell 69:1237-1245, 1992.
21. *Munck-Wikland E, Edström S, Jungmark E, Kuylenstierna R, Lindholm J and Auer G:* Nuclear DNA content, proliferating cell nuclear antigen (PCNA) and p53 immunostaining in predicting progression of laryngeal cancer *in situ* lesions. Int. J. Cancer 56:95-99, 1994.
22. *Nees M, Homann N, Discher H, Andl T, Enders C, Herold.Mende C, Schumann A and Bosch FX:* Expression of mutated p53 occurs in tumor-distant epithelia of head and neck cancer patients: a possible molecular basis for the development of multiple tumors. Cancer Res 53:4189-4196, 1993.
23. *Ogden GR, Kiddie RA, Lunny DP and Lane DP:* Assessment of p53 protein expression in normal, benign and malignant oral mucosa. J Pathol. 166:389-395, 1992.
24. *Piffkó J, Bánkfalvi A, Öfner D, Joos U, Böcker W and Schmid KW:* Immunohistochemical detection of p53 protein in archival tissues from squamous cell carcinomas of the oral cavity using wet autoclave antigen retrieval. J Pathol 176:69-75, 1995.
25. *Piffkó J, Bánkfalvi A, Öfner D, Berens A, Tkotz T, Joos U, Böcker W and Schmid KW:* Expression of p53 protein in oral squamous cell carcinomas and adjacent non-tumorous mucosa of the floor of the mouth: an archival immunohistochenical study using wet autoclave pretreatment for antigen retrieval. J Oral Path Med 24:337-342, 1995.
26. *Scheffner M, Werness BA, Huibregste JM, Levine AJ and Howley PM:* The E6 oncoprotein encoded by human papilloma virus type 16 and 18 promotes degradation of p53. Cell 63:1129-1136, 1990.
27. *Slootweg PJ, Koole R and Hordijk GJ:* The presence of p53 protein in relation to Ki-67 as cellular proliferation marker in head and neck squamous cell carcinoma and adjacent dysplastic mucosa. Oral Oncol, Eur J Cancer 30B: 138-141, 1994.
28. *Vojtesek B, Bartek J, Midgley CA and Lane DP:* An immunochemical analysis of the human nuclear phosphoprotein p53: New monoclonal antibodies and epitope mapping using recombinant p53. J Immunol Methods 151:237-244, 1992.
29. *Warnakulasuriya KAAS and Johnson NW:* Association of overexpression of p53 oncopreotein with the state of cell proliferation in oral carcinoma. J Oral Pathol Med 23:246-250, 1994.
30. *Wilkinson L:* SYSTAT: The system of statistics. Evanston, IL, Systat Inc., 1989.
31. *Wu X, Bayle JH, Olson D and Levine AJ:* The p53-mdmd2 autoregulatory feedback loop. Genes Dev 7:1126-1132, 1993.