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PCNA, Ki-67, p53, bcl-2 and prognosis in intraoral squamous cell carcinoma of the head and neck

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Abstract. Eighty patients with primary intraoral squamous cell carcinomas of the head and neck, with a follow-up of 4–14 years were analysed for clinical outcome in relation to immunohistochemical expression of PCNA, Ki-67, p53, bcl-2 and presence of mutations in the p53 gene. The tumour site was not associated with the different parameters calculated. PCNA and Ki-67 labelling showed median values of 56% and 32%, respectively, and neither antigen was of predictive value. Fiftyfive percent of the tumours expressed p53, and 38 (48%) had mutations in the p53 gene. No association between the presence of p53 protein or mutations in the p53 gene and clinical outcome was found. Bcl-2 positivity was detected in a minor fraction (10%) of the tumours.

Keywords: Immunohistochemistry, mutation analysis, prognosis, head and neck, squamous cell carcinoma

1. Introduction

In the diagnostic characterisation of tumours a major problem is to define parameters of clinical significance regarding therapy response and prognosis, which would provide the means for more individually based treatment. For squamous cell carcinoma of the head and neck (SCCHN) the prognostic significance of various parameters has been thoroughly studied, indicating the presence of regional metastasis as a main prognostic factor [10,23,43]. Tumour cell kinetic properties have raised much interest, and for some tumours the cell proliferation rate has been shown to be of predictive value [31]. The prognostic impact of cell proliferation can, however, vary within a specific group of tumours due, for example, to differences in tumour grade [31].

A frequently used method to study cell proliferation is immunohistochemical detection of cell cycle related antigens such as Ki-67 and PCNA (proliferating cell nuclear antigen). The Ki-67 antigen, which is present in the G1, S, G2 and M-phases of the cell cycle but absent in G0 cells [12,13], can provide information on the fraction of actively cycling cells [4]. In certain malignant tumours the number of tumour cells positive for Ki-67 coincided with the estimated tumour proliferation rates [11].

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In a recent study Ki-67 expression was of no predictive value in a group of SCCHN, consisting of a mixture of oral, laryngeal and pharyngeal tumours, as well as neck dissection specimens [33].

In normal tissues and lymphoid neoplasms expression of PCNA can be used as a cell kinetic marker, whereas the relation between PCNA expression and cell proliferation in some neoplasias can be lost [18]. The use of PCNA as a proliferation marker can thus be questioned, since growth factors have been shown to cause overexpression of PCNA in some tumours without a concomitant cell proliferation [24]. Several tumour types have been studied concerning PCNA expression and prognosis, giving different results [1,9,17,38,41]. In mucoepidermoid carcinoma and breast carcinoma expression of PCNA was indicated to be an important parameter in predicting survival [1,9,17,38], whereas no correlation to prognosis could be seen in malignant melanoma and a group of mainly laryngeal and pharyngeal SCCHN [20,41].

Mutations in the p53 tumour suppressor gene are the most common genetic alteration found in SCCHN [37], and overexpression of the p53 protein has been demonstrated in 34–80% of the tumours studied [5,8,15,22,29,40]. The normal p53 product functions as "guardian of the genome" with the ability to stop cells with DNA-damage in late G1 phase [21]. Significantly higher cell proliferation, as judged by Ki-67 expression, has been seen in well differentiated p53 immunopositive oral carcinoma [40], but no prognostic information was given by p53 overexpression [19]. In our previous studies of SCCHN no relationship between either p53 expression or p53 mutation and cell proliferation judged by *in vivo* incorporation of IdUrd was found [28,29], and no correlation between immunohistochemically detectable p53 protein and mutation in the p53 gene was present [29]. A mutation in the p53 gene leads to an increase in protein half life facilitating immunohistochemical detection. But an increase in half life can also be achieved by binding of wild type p53 protein to other proteins or by disturbance in the degradation pathway [42].

Expression of PCNA and p53 has shown in esophageal carcinoma and astrocytic neoplasms a strong correlation [16,35], whereas no such correlation was found in SCCHN [28]. By inducing the Cip1 protein, p21, wild type p53 protein can indirectly and selectively inhibit PCNA activity in DNA replication, whereas the PCNA function in nucleotide excision-repair is unaffected [36]. Wild type p53 protein can also induce apoptosis, a process that can be blocked by the bcl-2 protein by diversion of p53 activity from induction of apoptosis to induction of growth arrest [6]. In an immunohistochemical study of so-called stem cell SCCHN bcl-2 was found in 40% of the tumours accompanied by strong proliferation [3]. No data concerning bcl-2 expression and prognosis in SCCHN are so far available.

The aim of the present work was to evaluate the possible prognostic significance of a number of cell proliferation and apoptosis associated parameters in a large number of retrospective material consisting only of primary intraoral SCCHN with long clinical follow-up. None of the parameters studied, i.e., Ki-67, PCNA, p53 and bcl-2 was found to have any obvious impact on prognosis for this patient group. Nor did the values calculated for the parameters differ with respect to tumour site.

2. Material and methods

2.1. Material

Formalin fixed and paraffin embedded samples from 80 tumours with a follow-up between 4–14 years were included in the study. Thirtysix tumours were located in gingival/buccal mucosa, 18 in tongue, 16 in the floor of the mouth, six in tonsil/mesopharynx and the remaining four in epipharynx. Forty-three (54%) of the patients were women and 37 men, with an overall mean age of 66 years (range 32–91).

2.2. Antibodies and staining

Five-μm sections were cut from each block, and left at room temperature overnight. For detection of PCNA, Ki-67, p53 and bcl-2, respectively, the following antibodies were used: PC10, a mouse monoclonal antibody (Novocastra Laboratories Ltd., Newcastle, UK), recognising an epitope within aa 111–125 of the PCNA protein [34]; MIB-1, a monoclonal antibody against Ki-67 (BioGenex, San Ramon, CA, USA) [25]; DO7 a monoclonal antibody (Novocastra) recognising a denaturation resistant epitope between amino acids 1 and 45 in wild type as well as mutant p53 protein [39]; and M0887, a monoclonal antibody (Dakopatts A/S, Denmark) against bcl-2. The PCNA and p53 antibodies were diluted 1 : 50, the Ki-67 antibody 1 : 25 and the bcl-2 antibody 1 : 70. As secondary antibody for PCNA, p53 and bcl-2 an alkaline phosphatase conjugated rabbit anti-mouse antibody (D 314; Dakopatts) was used, and for detection of the Ki-67 antibody a super sensitive Multi Link Kit (BioGenex) was applied. For visualisation of the staining reactions fast red (Sigma Chemical Co, St Louis, MO, USA) was used.

Preincubation of slides in methanol was performed before staining with the PC10 antibody, and when staining with the Ki-67 and bcl-2 antibodies slides were pretreated in 10 mM citrate-buffer using a microwave oven at full effect (900 W) for 2.5 min and at 350 W for 9 min. Slides were left to cool for 20–30 min and then rinsed in distilled water. Incubation with the primary antibodies was carried out at 4 °C overnight. After rinsing in TRIS buffer, slides were incubated with the secondary antibody at room temperature for 45 min, and with the Multi Link Kit according to the supplier's recommendation.

2.3. Immunohistochemical evaluation

For PCNA and Ki-67 stained slides a labelling index (LI) was calculated as earlier described in detail [27]. In brief, a 10×10 square grid comprising 121 cross points was fitted into the eyepiece of the microscope using an objective lens of $\times 40$, and cells showing a distinct nuclear staining were counted in 5–10 randomly chosen fields, in most cases covering the whole tumour. The average LI was calculated as the percentage of positively stained nuclei falling in the crossing between two lines of the grid. The immunohistochemical evaluation and calculation of LI was performed by one of the authors (KN). Control calculations of LI showed an intra-observer variation that was not statistically significant.

For p53 and bcl-2, tumours were graded positive or negative, where tumours with only occasional p53 positive cells were considered negative. For bcl-2 the group of positive tumours was so small, that a proper evaluation of different LIs was not considered relevant.

2.4. PCR and SSCP (*single strand conformation polymorphism*) analysis

DNA was extracted from the paraffin blocks, and PCR/SSCP analysis of exons 5–9 of the p53 gene was performed the same way as earlier described in detail [29]. All mutations found in exon 8 were further sequenced. This was due to the unexpected dominance of mutations in exon 8 as well as the concordance in aberrant gel pattern in many of these tumours.

2.5. Statistical analysis

For analysis of crude survival, curves were constructed using the method of Kaplan and Meier, with statistical significance determined by the Chi square test. For PCNA and Ki-67 expression tumours

were divided into three almost equal groups referring to LI, and for p53 expression two groups were analysed; p53 positive and p53 negative tumours, respectively. The bcl-2 positive group of tumours was too small (only 8 cases) to allow any meaningful survival analysis. For comparison of calculated values between different tumour sites the Kruskal Wallis and log-rank test were used.

3. Results

3.1. Immunohistochemical evaluation

Seventy-six of the 80 tumours showed a positive staining for PCNA. In the remaining four tumours the staining reaction did not work. PCNA positive cells were found in the basal and parabasal layers of the tumours, no staining was seen in keratinised areas. PCNA expressing cells could also be found in the basal and parabasal layers of normal adjacent epithelium. PCNA staining was clearly nuclear and, although a slight difference in staining intensity was seen in most tumours, cells showing nuclear staining were recorded positive irrespective of intensity. In some areas of the epithelium a weak to distinct reactivity in the cytoplasm could also be seen. This cytoplasmic staining was most probably unspecific, and did not affect calculation of LI since only cells with nuclear staining were included in the calculation. LI for PCNA showed a median value of 56% (range 14–93%, Table 1).

Material from 77 tumours could successfully be analysed for expression of the Ki-67 antigen, and positive cells were located in the same areas as PCNA positive cells, but the Ki-67 expressing cells were mainly restricted to the most basal layers. The number of Ki-67 positive cells was therefore in most tumours lower than the amount of cells expressing PCNA, with a median value for LI of 32% (range 11–60%, Table 1). No significant correlation was found between Ki-67 and PCNA labelling indices.

Forty-four of the 80 tumours (55%) expressed the p53 protein. Only cells with a clear nuclear staining were considered positive. Staining intensity did not markedly differ between tumours. Cells positive for p53 were located in the same areas as PCNA and Ki-67 expressing cells, but were only occasionally detected in normal adjacent epithelium. Forty-five mutations in the p53 gene were found distributed in 38 of the tumours since seven tumours had two mutations. At sequencing, 17 of the mutations were represented by a novel non random deletion in exon 8 [30]. SSCP detected mutations

Table 1
Summary of immunohistochemical data regarding Ki-67, PCNA, p53 and bcl-2 and SSCP data on p53 gene alterations

	Median LI/Ki-67*	Median LI/PCNA*	p53* IHC+	p53* SSCP+	bcl-2 pos.*	No
All	32	56	55	48	10	80
LI/Ki-67 $>$ median	–	60	50	45	3	38
LI/Ki-67 $\leqslant$ median	–	52	59	46	18	39
LI/PCNA $>$ median	36	–	55	55	8	38
LI/PCNA $\leqslant$ median	30	–	58	42	11	38
p53, SSCP+	32	59	50	–	8	38
p53, SSCP−	32	54	60	–	12	42
p53, IHC+	30	56	–	43	9	44
p53, IHC−	33	57	–	53	11	36

*Given as %.

were found in a similar frequency in p53 positive and negative tumours, in agreement with our previous study [29] (Table 1). Thus, no association between p53 overexpression and p53 gene mutations could to demonstrated.

Sections from 78 tumours were stained for bcl-2 protein, and eight of these tumours showed a distinct cytoplasmic staining (Table 1). The staining pattern was heterogeneous within the tumours, i.e., in some areas only basal cells were stained, whereas in other areas staining in all cell layers was seen.

3.2. Subgrouping of tumours with respect to localisation

The complete group of 80 tumours was subdivided into five groups depending on localisation, and a comparison of calculated values was made. No statistically significant difference was seen for PCNA, Ki-67, p53 protein or p53 mutations between the different locations. Concerning bcl-2 expression, a difference between various sites could be seen. The group of bcl-2 positive tumours, however, consisted only of eight tumours, and the data must therefore be interpreted with care (Table 2).

3.3. Correlation to prognosis

PCNA or Ki-67 expression was not associated with crude survival (Figs 1 and 2). Also, no significant difference was found in crude survival for patients with p53 positive or negative tumours, although a tendency towards a worse prognosis could be seen for patients with 53 positive tumours ($p = 0.143$, Fig. 3).

LIs for PCNA or Ki-67 were similar for p53 positive and negative tumours, and gave no predictive information within the p53 subgroups.

3.4. Mutation analysis

Regarding p53 gene mutations, no association was seen between the presence of mutations and clinical outcome (Fig. 4). As mentioned above, a significant fraction of the mutated tumours showed the same deletion in exon 8, but even if these cases were excluded no prognostic information was given by the p53 mutational status. Furthermore, the presence of a p53 mutation seemed to be of no relevance for tumour cell proliferation as judged by Ki-67 or PCNA expression.

Although the group of bcl-2 positive tumours was very small, it can be noted that all except one of the bcl-2 positive cases had a Ki-67 LI below median.

Table 2
Median values for the different parameters with respect to localisation

Localisation	p53/IHC+ %	p53 mut+ %	PCNA %	Ki-67 %	bcl-2+ %	No
All	55	48	56	32	10	80
Gingiva/bucca	53	47	55	30	6	36
Tongue	56	56	63	36	6	18
Floor of mouth	44	62	58	36	6	16
Tonsil/mesopharynx	83	17	50	28	40	6
Epipharynx	75	100	52	28	50	4

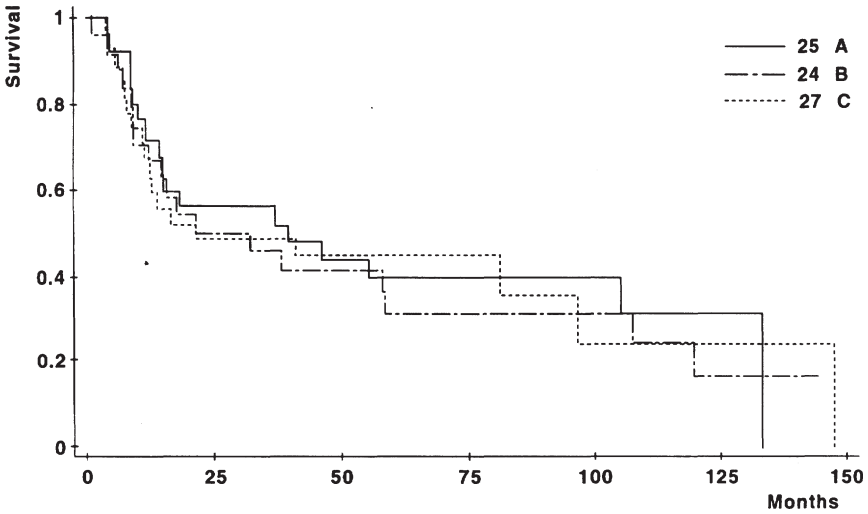

Fig. 1. Crude survival for PCNA expression. Patients were divided into three groups according to LI; group A: LI 14–47%, group B: LI 48–65% and group C: LI 66–93%. No statistically significant difference in crude survival was found between the groups.

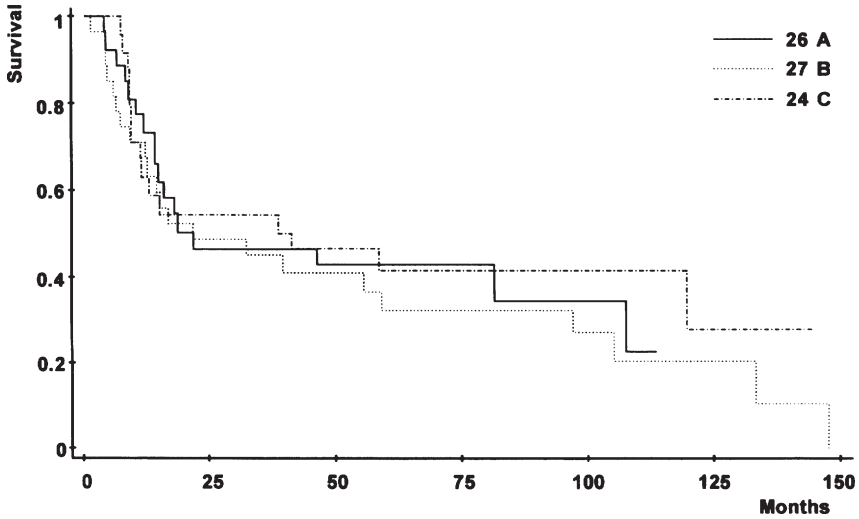

Fig. 2. Crude survival for Ki-67 expression. Patients were divided into three groups; group A: LI 11–24%, group B: LI 25–38% and group C: LI 39–60%. No significant difference in crude survival was found between the three groups.

4. Discussion

In the present study the prognostic significance of a number of proliferation and apoptosis associated proteins were studied in a group consisting only of intraoral SCCHN. Neither PCNA nor Ki-67 expression seemed to have any impact on the prognosis which is in agreement with previous reports using immunohistochemistry on the whole group of SCCHN [20,33]. No relationship between these two cell cycle related proteins was observed in contrast to an earlier study by Jones et al. [20] who did find a strong correlation. In Jones' study, however, only four of 37 SCCHN were oral carcinomas, and accordingly their results were achieved mainly from laryngeal and pharyngeal tumours and are

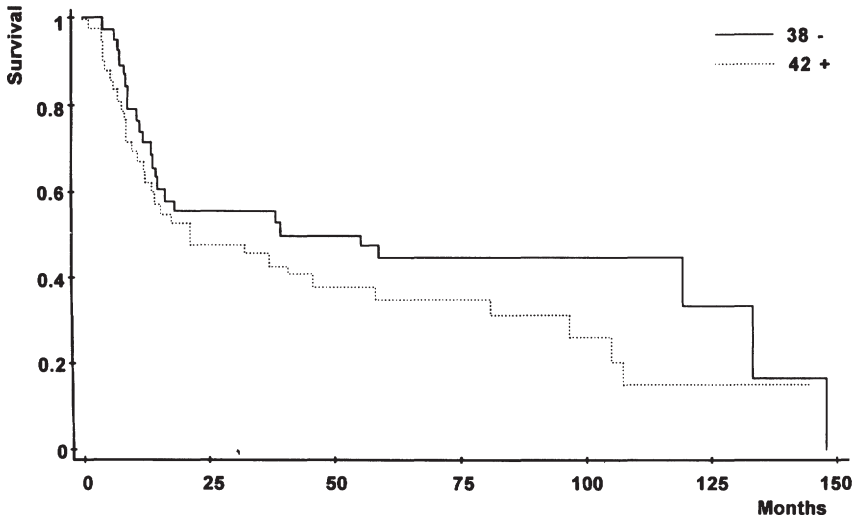

Fig. 3. Crude survival for p53 expression. Patients were divided into two groups: one with p53 positive and the other with p53 negative tumours. No significant difference was found between the groups, though a tendency for worse survival could be seen for patients with p53 positive tumours.

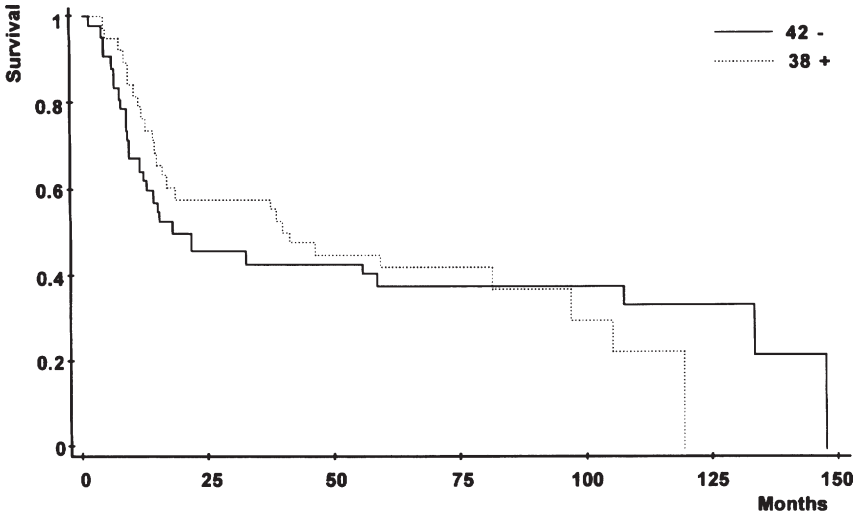

Fig. 4. Crude survival for p53 mutations, including the 17 tumours with the new non random deletion. No difference in crude survival was seen between tumours with and without mutation.

not comparable to our data from oral carcinomas only. In our hands the expression of at least one of the two parameters, Ki-67 and PCNA, was unrelated to tumour cell growth. In many tumours PCNA positive cells were found in parabasal layers with more differentiated cells, which can be explained by the long half life of PCNA making it detectable also in cells that had left the cell cycle. In contrast, Ki-67 expression was restricted to the most basal and proliferative compartment of the epithelium similar to findings in benign tissues. Regarding PCNA, the possibility of a growth factor induced and cell cycle unrelated expression must also be taken into consideration [18,24]. In summary, these data indicate that PCNA can be questioned as a reliable marker of cell proliferation in intraoral SCCHN. It can not be stated from the present study that Ki-67 is a useful cell growth marker either.

By using PCNA/Ki-67 immunohistochemistry it is not possible to make a definite distinction between actively proliferating cells and cells arrested in the cell cycle. Therefore, if cell cycle arrest or transit out of active cell cycle are common events in SCCHN usual PCNA and Ki-67 stainings might give a false representation of the growth fraction. Consequently, in studies of cell cycle related antigens arrested cells must be considered as a confounding factor. A double labelling approach using, e.g., Ki-67 and a marker for resting cells such as statin [2] could perhaps solve this problem by distinguishing arrested cells from proliferating cells. Dynamic cell kinetic measurements can be made after *in vivo* labelling with thymidine analogues, like iodo- and bromodeoxyuridine, and estimation of the potential tumour doubling time (T_{pot}) can be made. T_{pot} estimations can give useful clinical information and in a previous study using iododeoxyuridine labelling we found that prognostic information might be achieved by analysis of dynamic cell kinetic parameters [27].

The p53 protein exerts a check point control in late G1 phase, preventing replication of cells with DNA damage [21], and a mutation in the gene will likely result in a defective G1 control. In tumours with p53 gene mutations a higher proliferation rate could be expected in comparison with wild type p53 tumours. In the present study no association between the presence of a p53 mutation and the expression of proliferation associated antigens was demonstrated. One explanation for this could be that the mutation was present in only a minor fraction of the tumour cells giving no measurable effect on cell kinetic parameters. It must also be remembered that there are other regulators of cell cycle progression effective in the G1 phase, like the Rb-protein and cyclins D and E [14,32]. The p53 induced cyclin dependent kinase inhibitor p21 interacts with a number of proteins including cdks, cyclin D1 and PCNA leading to cell cycle arrest [7,32,36]. However, p21 can also be induced in a p53 independent pathway [26]. No relationship between p53 protein expression and presence of mutations was found, which can be explained by a large fraction of nonsense mutations and the fact that the antibody used detected wild type as well as mutated p53 protein [27,29]. Our data indicated that regarding intraoral SCCHN the p53 status is of limited importance for regulation of cell kinetic events as well as for prognosis.

The data from the present study of intraoral SCCHN seemed to rule out a prognostic significance of some frequently used cell proliferation and apoptosis associated parameters. Expression of the bcl-2 protein in SCCHN has previously only been reported once, showing a much higher percentage of bcl-2 positive tumours in a subgroup of SCCHN than in our study [3]. The bcl-2 positive tumours studied by Bosch et al. were judged as stem cell tumours with a high proliferation rate, whereas all our bcl-2 positive tumours were regular squamous cell carcinomas.

The group of intraoral SCCHN comprises tumours from different locations, such as tongue, floor of mouth, gingiva/buccal mucosa and tonsil. We could, however, show that the expression of PCNA, Ki-67, p53 protein and presence of p53 mutations were independent of tumour site.

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