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Prognostic Value of PCNA and Cytokeratins for Radiation Therapy of Oral Squamous Cell Carcinoma

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In a retrospective pilot study we compared morphologically the first biopsies of 20 patients suffering from oral squamous cell carcinoma (OSCC) where preoperatively performed radiation therapy [gamma rays (cobalt-60)] was successful with 20 patients who underwent the same therapy without the expected success. All specimens were formalin fixed and paraffin embedded. We performed haematoxylin and eosin staining and immunohistochemistry (ABC-method) applying broad spectrum cytokeratin, cytokeratin (CK) 1 + 2, 3 + 6, 13 and anti-proliferating cell nuclear antigen (PCNA, PC10). The specimens of the patients with success of the radiotherapy showed statistically higher levels of PCNA positive tumour cells, measured by a computerised morphometric analysis system (VIDAS). These specimens showed significantly less CK 3–6 positive tumour cells than the specimens of the patients with failure of this therapy. The difference in the content of proliferating cell nuclear antigen might explain the different results of the performed radiation therapy. The difference in cytokeratin 3 + 6 expression might be linked with the different amount of benign hyperproliferation. Prospective studies are planned to prove the results.
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INTRODUCTION

ORAL SQUAMOUS cell carcinoma (OSCC) still has a bad prognosis [1, 2]. Studies, however, have shown the benefit of a combined surgical and radiation therapy [3, 4]. Preoperatively performed, radiation therapy might have the additional effect to devitalise tumour masses in the tumour periphery and to avoid the distribution of vital tumour cells intraoperatively [5]. The use of gamma rays (cobalt-60) is an established radiation therapy for oral squamous cell carcinoma. We tried to find out whether the pathologist can—by the means of immunohistochemistry—give more information about the tumour to help the radiologist to plan an optimal therapy so that "non-responders" could be avoided [6]. We used immunohistochemistry with some cytokeratins of the squamous cell type [7]. Furthermore the proliferation marker anti-PCNA, which can be applied to formalin fixed, paraffin embedded specimens was used.

MATERIALS AND METHODS

Specimens

All patients suffering from OSCC of 1988–1991 of our department were re-evaluated. Most patients had received preoperatively, radiation therapy (gamma rays [cobalt-60]) in a hyperfractionated course. This was performed in the department of radiation therapy, Institute of Radiology, Hamburg University. From this group, 20 patients who showed tumour rest in the operative specimens were selected (group A). Furthermore 20 patients, whose operative specimens did not show any tumour rest were selected as a control group (group B, Table 1). Additionally, the tumour specimens of the patients with insufficient radiation therapy result (group A) were compared before (A_0) and after (A_1) radiation therapy.

Technique

All tumour specimens were fixed in neutral buffered formalin, embedded in paraffin and dried at 56°C for 20 min. Sections were dewaxed in xylene and hydrated through graded ethanols to water. Haematoxylin and eosin staining was performed. Immunohistochemistry was performed using monoclonal anti-cytokeratin 1 + 2 (Amersham, Buckinghamshire, U.K.; 1:5), polyclonal anti-keratin 3 + 6 (DAKO, Carpinteria, California, U.S.A.; 1:400), monoclonal anti-cytokeratin 13 (Progen, Heidelberg, Germany; 1:10), monoclonal mouse anti-human cytokeratin MNF 116 (broad spectrum cytokeratin) (DAKO, Glostrup, Denmark; 1:100) and the monoclonal mouse anti-prolifetating cell nuclear antigen (PCNA) (PC10) (Dako, Glostrup, Denmark; 1:15).

For immunohistochemistry conventional 4 μm histological sections from the paraffin blocks were dried overnight at room temperature. Endogenous peroxidase activity was blocked with methanolic peroxidase for 30 min. After washing for 5 min in running tap water, the slides were digested for 30 min at 37°C in a prewarmed solution of pronase (Sigma, St Louis, Missouri, U.S.A.). This was terminated by washing the slides in phosphate buffered saline (PBS) buffer for 5 mins. Sections were then treated with 10% normal horse serum/PBS buffer for 20 minutes at room temperature and incubated for 30 min at 4°C with the primary antibody.

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Table 1. List of patients

Sex	Age	Tumour localisation	Case no.
Patient group A			
m	53	Tongue	90/6798
m	43	Floor of the mouth	89/4295
m	46	Floor of the mouth	89/5227
m	41	Floor of the mouth	90/4562
m	43	Mandibular angle	89/1264
m	45	Floor of the mouth	89/5676
f	83	Palate	90/1450
m	64	Floor of the mouth	89/5674
f	64	Mandible	90/2675
m	51	Mandible	89/2162
f	65	Tongue	89/4535
m	48	Tongue	89/1114
m	49	Mandible	88/2107
f	55	Tonsil	89/5180
f	74	Floor of the mouth	89/4562
f	70	Tongue	89/2937
f	61	Mandible	89/5528
m	52	Floor of the mouth	89/1323
m	48	Tongue	90/47
f	53	Mandible	88/4624
Patient group B			
f	80	Palate	88/1163
m	52	Floor of the mouth	89/4477
m	51	Floor of the mouth	89/3440
f	55	Floor of the mouth	88/4712
m	40	Mandible	88/1469
m	71	Floor of the mouth	88/3653
m	63	Mandible	89/254
m	70	Floor of the mouth	88J6195
m	48	Tongue	89/3380
m	62	Floor of the mouth	89/2684
m	66	Floor of the mouth	88J1508
m	56	Floor of the mouth	88/4239
m	53	Mandible	89/1022
m	51	Floor of the mouth	89/5068
m	55	Tongue	88/636
f	60	Mandible	88/4322
m	58	Maxilla	88J23364
m	86	Tongue	88J17587
m	49	Maxilla	88J14667
m	56	Floor of the mouth	88J15351

Slides were washed with PBS buffer and overlaid with biotinylated horse antimouse/anti-rabbit immunoglobulin antibody (1:200) (Vector) for 30 min followed by a second PBS buffer wash. Sections were reacted with streptavidin–biotin complex for 30 min.

Colour was developed with 3′–5′ diaminobenzidine and H_2O_2 for 30 min. Slides were washed in tap water, counterstained for 30 s in Harris haematoxylin, developed in diluted ammonia water, dehydrated through graded alcohols, cleared in xylene and mounted with permount.

Morphological evaluation

All tumours were reclassified according to Broders's classification [8] and to the UICC [9]. The tumour invasion was measured microscopally on the slide. With the immunohistochemistry we performed a semiquantitative analysis estimating the percentage of positive tumour cells in five degrees (0%, 1–25%, 26–50%, 51–75%, 76–100%).

Computerised morphometry

With the anti-PCNA antibody we performed a computerised morphometric analysis, using the microscope Axioskop (Zeiss, Germany), B/W camera BC-2 with camera control Unit AVT-Horn (Germany), the image analysis computer VIDAS (Kontron, Germany) with software Vidas X2.0 (Kontron) and application program Prolifex 1.0 (Zeiss). We used two colour Monitors EUM 1481A (Mitsubishi/Japan). With this setting at least 10 areas of each tumour slide were measured. The positively stained areas were automatically analysed and interactively controlled so that only positively stained tumour cell nuclei were counted. By this means an index of proliferation was gained:

Index of proliferation: (stained tumour cell nuclei/total tumour area) × 100.

Statistical evaluation

Statistical evaluation was performed with help of the Institute of Statistical Analyses, University Hospital, Hamburg University (The authors thank PD Dr. Rehpenning for his help). The discontinuous data of both groups (A and B) were checked by the "Freeman-Halton-Test", the continuous data (index of proliferation) were checked by the "Duncan multiple range test" for statistical significance. P was always set up as 0.05. Finally a stepwise logistic regression was performed. The results of the tumour specimens of the patients with insufficient result of radiation therapy pre- and postradiation therapy were checked up with the Wilcoxon test [10, 11].

RESULTS

Comparison of the two patient groups before radiation therapy (A_0 and B)

There were no differences between both groups of patients concerning age of the patients, sex, or localisation of the tumour. In most cases a G2 tumour was diagnosed (see Table 2). The mean of the tumour thickness was 4.6 mm with standard deviation of 2.8. The immunohistochemistry showed no significant different results concerning antibodies cytokeratin (CK) 1+2, CK 13, or broad-spectrum cytokeratin (Tables

Table 2. Grading of the tumours

	G1	G2	G3	G4
Group A_0	6	14	0	0
Group B	2	18	0	0

Table 3. Results of staining with antibody anti-cytokeratin 1+2

	0%	1–25%	26–50%	51–75%	75–100%
A_0	2	8	7	2	1
B	7	5	3	3	2

Table 4. Results of staining with antibody anti-cytokeratin 13

	0%	1–25%	26–50%	51–75%	75–100%
A_0	0	10	5	3	2
B	2	13	3	2	0

Table 5. Results of staining with antibody anti-cytokeratin (broad spectrum)

	0%	1–25%	26–50%	51–75%	75–100%
A_0	0	0	1	2	16
B	0	0	0	3	15

Table 6. Results of staining with antibody anti cytokeratin 3+6

	0%	1–25%	26–50%	51–75%	75–100%
Group A_0	0	0	2	2	16
Group B	0	1	6	6	7

$P=0.0174$ (Freeman–Halton-Test).

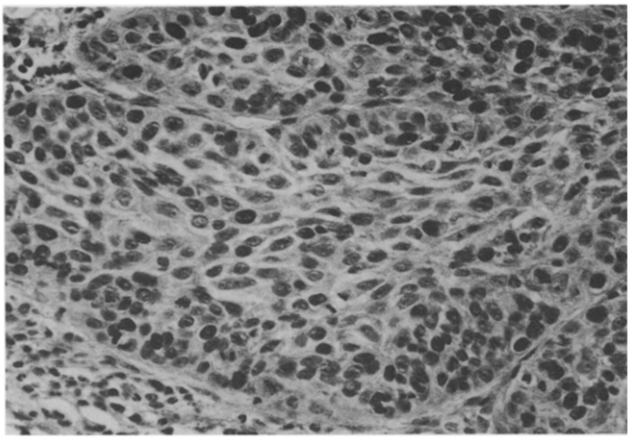

Fig. 1. Oral squamous cell carcinoma (G2) of the maxilla, 49 year old male patient, before radiation, radiation with success (group B), (ABC): PCNA, 400 ×.

3–5). The comparison of the results with cytokeratin 3+6 showed significant different results with more positive tumour cells in group A (Table 6) ($P=0,0174$).

The index of proliferation measured by PCNA and by VIDAS showed significantly higher values in group B (success of radiation therapy) (Fig. 1) than in group A (Fig. 2) (Table 7). The result of proliferation Index (Comparison between group A_0 and group B) was:

	A_0	B
mean value	3.95	7.56
S.D.	3.28	4.54

Table 7. Index of proliferation (stained areas of PCNA positive tumour cell nuclei, measured by VIDAS)

Group A_0	Group B
0.0	0.0
0.4	0.6
0.95	1.99
0.99	2.19
1.1	2.76
1.2	3. 51
1.48	5.99
1.52	6.14
2.14	7.16
2.24	8.58
2.48	8.72
3.04	9.24
4.06	9.35
4.26	9.55
6.26	10.38
6.53	10.53
7.23	11.73
8.11	13.6
10.49	13.87
10.6	15.25

$P=0.0075$.

The mean value of the proliferation index for group A was half as high as for group B. This difference was highly statistical significant ($P=0.0075$, Duncan Test).

In the stepwise logistic regression the statistical significances of the difference in CK 3+6 expression as well as the PCNA value, measured by VIDAS were reassured.

Comparison of the first biopsy (A_0) and the operation specimen (A_1) (both with tumour) of the patient group A with insufficient radiation therapy success.

Six of the 20 tumours were less differentiated after radiation therapy. The tumour stroma was more fibrous after radiation therapy. The amount of cytokeratin 13 positive tumour cells was smaller after radiation therapy. In many cases the PCNA-level was higher after radiation therapy (Figs 3 and 4). After radiation the tumours seemed to be less differentiated than before radiation (Table 8). The comparison of the index of proliferation using anti-PCNA did not significantly differ.

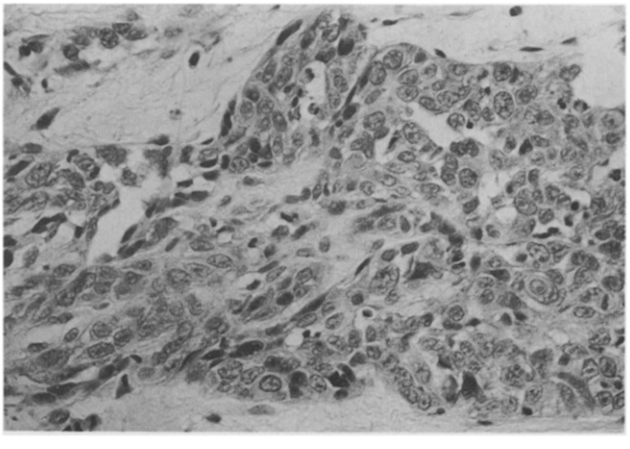

Fig. 2. Oral squamous cell carcinoma (G2) of the mandible, 53 year old female patient, before radiation, radiation without success (group A_0), (ABC): PCNA, 400 ×.

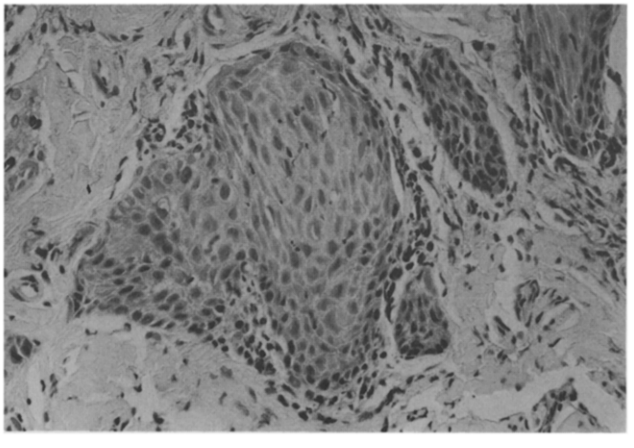

Fig. 3. Oral squamous cell carcinoma (G2) of the palate, 83 year old female patient, before radiation, radiation without success (group A_0), (ABC): PCNA, 400 ×.

Table 8. Group A (failure of radiation therapy). Change of differentiation and cytokeratin-expression before/after radiation therapy

	Amount of degrees	Changes to lower degree			No change	Changes to higher degree		
		+3	+2	+1	0	−1	−2	−3
Differentiation (GI–GIV)	4	0	1	6	13	0	0	0
Anti-cytokeratin 1+2	5	0	2	6	7	3	2	0
Anti-cytokeratin 3+6	5	0	2	1	15	2	0	0
Anti-cytokeratin 13	5	1	2	7	8	1	1	0
Anti-broad spectrum cytokeratin	5	0	1	2	15	1	1	0

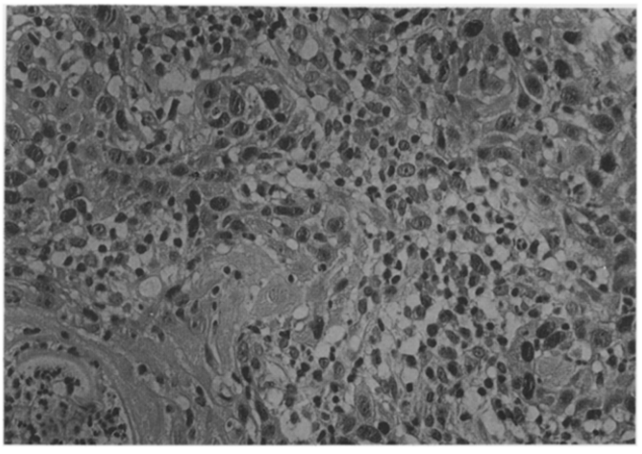

Fig. 4. Oral squamous cell carcinoma (G3) of the palate, 84 year old female patient (the same patient as fig. 3.): after radiation, radiation without success (group A_1), (ABC): PCNA, 400 ×.

DISCUSSION

The present study was designed as a pilot study to find out whether a prospective study including a larger group of patients is likely to be useful. This pilot study has shown some hints for prognostic factors:

(1) The tumour specimens of the patients with success of radiation therapy showed significantly less cytokeratin 3+6 than the group with tumour rest. Whether this might be linked with findings that cytokeratin 6 is associated with (benign) hyperproliferation [12] can only be presumed.

(2) The tumour specimens of the patients with success of radiation therapy showed a significantly higher proliferation index, employing the anti-PCNA antibody and the VIDAS system than the group without success of radiation therapy. This difference was highly statistically significant with the mean value of proliferation index twice as high as the control group. We think that this is the most important finding of the present pilot study.

The anti-PCNA antibody has become a powerful tool in measuring cell proliferation [13]. As shown in the literature the anti-PCNA antibody is linked with S, G2, and M-phase fraction of tumour cells of gastrointestinal lymphomas [14] and in malignant lesions of epithelial origin in the oral cavity and the skin [15]. It might be suggested that even in the present case the more proliferative tumour cells responded better to radiation therapy with gamma rays. In this study however, the amount of PCNA was not strictly correlated to the histological grading as it was shown in other studies on other tumours [16], so that a new information was gained by the anti-PCNA antibody for oral squamous cell carcinoma. From other studies we have learned that anti-PCNA has the same value as Ki-67 [17]. However with anti-PCNA retrospective studies on routinely paraffin embedded materials are possible. Therefore this method might be of high practical value. There is some hope that for planning a radiation therapy of oral squamous cell carcinoma the growth fraction, measured by PCNA and gaining the proliferation index can be taken into account. It can only be presumed that for less proliferative tumours perhaps neutron radiation therapy would be more effective as performed for salivary gland tumours [18].

Further prospective studies with more cases should follow to prove the results of this pilot study.

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