Prognostic significance of $p21^{WAF1/CIP1}$, $p16^{INK4a}$ and CD44s in tongue cancer

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Abstract. The role of lost or reduced expression of p21, p16 and CD44s in the survival of tongue cancer patients was investigated. Tumours and adjacent non-tumour epithelia (ANTE) from 36 patients with tongue cancer were retrospectively studied by immunohistochemistry using monoclonal antibodies against p21, p16 and CD44s proteins. Expression of p21, p16 and CD44s and their relationship with clinical and pathological parameters were analyzed. Of 36 patients, 12 (33.33%) developed recurrence and 12 died of the disease (mean survival, 25.5 months). In four cases (11.1%), concomitant low expression (<50% of tumour cells) of p21, p16 and CD44s was detected but had no effect on survival or recurrence in the univariate analysis. In the multivariate analysis, low expression of CD44s was the sole prognostic factor related to survival (p=0.01, hazards ratio: 0.749). There was no expression of p21, p16 or CD44s in ANTE from 3 out of 24 cases studied, and this finding was related to recurrence in the univariate analysis. In the multivariate analysis, low expression of CD44s in ANTE was again the sole factor related to recurrence (p=0.002, hazards ratio: 0.028). In conclusion, low expression of CD44s is related to tumour cell invasiveness and may be of clinical relevance as a prognostic factor.

Introduction

Cellular programs of proliferation, differentiation, senescence and apoptosis are closely linked to cell cycle regulation, and alterations of the cell cycle machinery have been described as a hallmark of cancer development (1). Molecular mechanisms controlling progression through the restriction point (R), such as the retinoblastoma protein (pRB), are of paramount importance in this context (2). Hypophosphorylated pRB inhibits S phase entry (cell arrest near R point) by forming complexes with E2F transcription factors that actively repress transcription of DNA synthesis-regulating genes (3). Upon pRB phosphorylation, pRB/E2F complexes are disrupted and E2F can activate these promoters, allowing the advance of cells to late G1 and DNA synthesis. Therefore, pRB or members of this regulatory pathway are critical molecular targets for malignant transformation, and there is growing evidence that the pRB pathway is rendered dysfunctional in oral cancer (2). Down-regulation of $p16^{INK4a}$, a member of the INK family of CDK inhibitors (CDKis), has been described in oral cancer, and $p16^{INK4a}$ inactivation is believed to be an early event in oral carcinogenesis (4-8). Some authors reported that loss of $p16^{INK4a}$ is correlated with a poor prognosis in oral cancer patients (9-11), whereas others found no relationship with survival (12). In addition, some studies found loss of $p21^{WAF1/CIP1}$, a member of CIP/KIP family of CDKis, to be a very early event in oral carcinogenesis (13-17). However, the prognostic significance of $p21^{WAF1/CIP1}$ expression is controversial, with several studies identifying it as a prognostic factor (18-20) and others finding no association (17,21-24).

Besides alterations in their cell-cycle control mechanisms, tumour cells must have the ability to invade adjacent and distant tissues. Although the phenotypic changes that increase the capacity of tumour cells for invasion are not well known (25), alterations in the expression of intercellular adhesion molecules on the tumour cell surface have been implicated (26-28). The CD44 molecule has been shown to be a major factor in cell-cell interactions and adhesion, and our group found that lost or reduced expression of CD44s was an early event in oral carcinogenesis (29) and a marker of a poor prognosis with an independent influence on survival (30).

The development of many solid tumours, including oral cancer, is the result of a multi-step process of accumulated genetic alterations that render tumour cells capable of proliferating and invading adjacent tissues; therefore, the associated phenotypic changes in these cells may have prognostic value. The objective of the present study was to investigate the prognostic role in tongue cancer of the

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Table I. Clinical and pathological data from patients with tongue cancer.

Clinical data		Pathological data	
Variables	N (%)	Variables	N (%)
Clinical T		Pathological T	
T_1	12 (33.3)	T_1	11 (30.5)
T_2	12 (33.3)	T_2	14 (38.8)
T_3	6 (16.6)	T_3	5 (13.8)
T_4	6 (16.6)	T_4	6 (16.6)
Clinical N		Pathological N	
N_0	18 (50)	N_0	21 (58.3)
N_1	12 (33.3)	N_1	10 (27.7)
N_{2a}	2 (5.5)	N_{2b}	5 (13.8)
N_{2b}	4 (11.1)		
M		Pathological stage	
M_0	36 (100)	I	9 (25.0)
M_1	0	II	5 (13.8)
		III	10 (27.7)
		IV	12 (33.3)
Local recurrence		Extracapsular spread	
No	24 (66.6)	No	33 (91.6)
Yes	12 (33.3)	Yes	3 (8.3)
Death		Degree of differentiation	
No	24 (66.6)	Well-differentiated	22 (62.8)
Yes	12 (33.3)	Moderately-differentiated	12 (34.2)
		Poorly-differentiated	1 (2.8)
		Surgical margin involvement	
		Free	33 (91.6)
		Positive	3 (8.3)

concurrent loss of three proteins, two related to cell cycle regulation ($p21^{WAF1/CIP1}$ and $P16^{INK4a}$) and one to cell adhesion (CD44s).

Patients and methods

The study, approved by the institutional human ethics committee of our university, included 36 patients with squamous cell carcinoma of the tongue treated at our University Hospital from 1990 to 1996 and followed up until 1998. Immunohistochemistry was used to assess expression of CD44s (standard form of CD44), $p21^{WAF1/CIP1}$ and $p16^{INK4a}$. The mean age of patients was 58.3 years (range 34-88 years) and 29 were males.

Hospital medical records of the patients were searched to gather data on clinical T and N values (cervical lymph node involvement determined by clinical methods), presence of distant metastasis according to IUAC and AJCC criteria (31), type of treatment, local tumour recurrence after primary therapy and survival.

Pathologic data and tumour thickness measurements were obtained by hematoxylin-eosin staining of formalin-fixed paraffin-embedded operative tissue sections. Pathologic T value and involvement of cervical lymph nodes (pathologic N) were assessed according to IUAC and AJCC criteria (31). Extranodal spread of the tumour, degree of differentiation and the status of the surgical margin were also evaluated. Tumour thickness was measured using a method reported elsewhere (32). Histopathological studies were all carried out by a single pathologist (IRA), who also evaluated adjacent non-tumour epithelium (ANTE).

For the immunohistochemical staining, 4-μm sections were cut from the paraffin blocks. After blocking endogenous peroxidase with H_2O_2 in methanol for 30 min, sections were immersed in citrate buffer (pH 6.0) in a microwave-resistant container. Monoclonal antibodies (MoAbs) against CD44s (Clone DF1485; Dako Corporation, Carpinteria, CA), $p21^{WAF1/CIP1}$ and $p16^{INK4a}$ (Santa Cruz Biotechnology Inc., Santa Cruz, CA) were used. Sections were incubated overnight. Immunoperoxidase detection was employed using the ABC method (Dako Corporation) and diaminobenzidine substrate. Counter-staining was performed with haematoxylin. Antigen retrieval methods were used. Staining of infiltrating lymphocytes was considered as positive internal control for CD44s

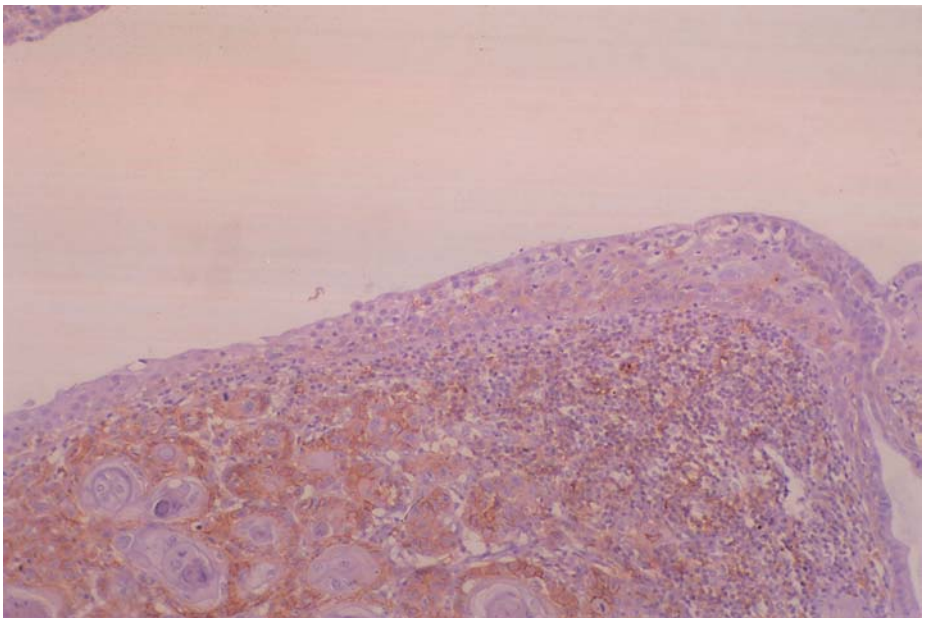

Figure 1. Intense CD44 expression in cohesive tumoral cell groups.

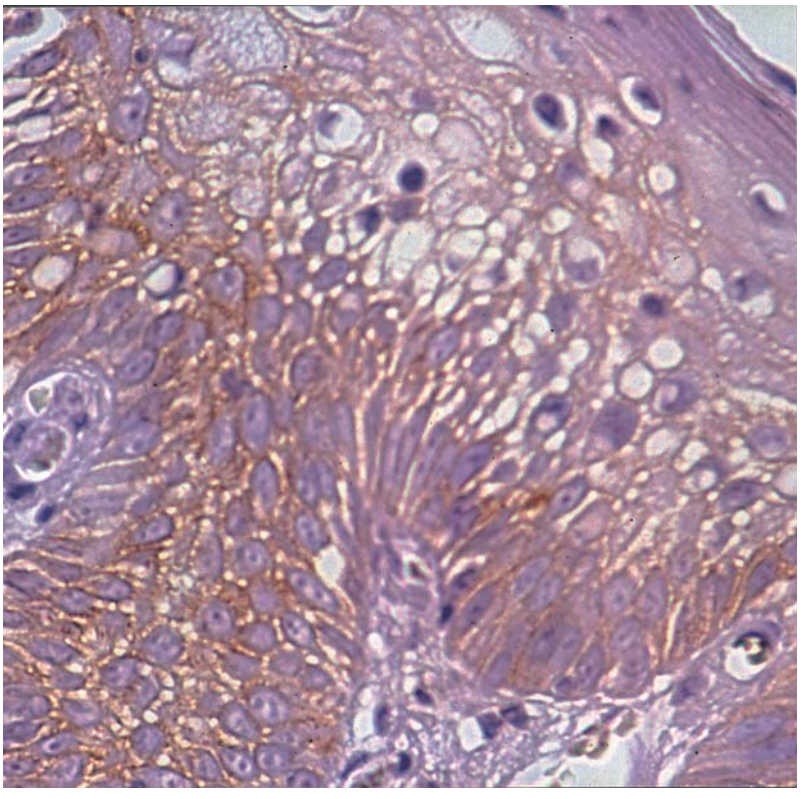

Figure 2. Basal and suprabasal CD44 expression in an adjacent non-tumoral epithelium.

expression. A section of a squamous cell carcinoma from a previous study with intense positivity for all three MoAbs was used as an additional positive control, and a carcinoma section that did not receive the primary antibodies was used as negative internal control. Membrane expression was considered for immuno-histochemical evaluation of CD44s, whereas only nuclear staining above any cytoplasmic background was considered for evaluation of $p21^{WAF1/CIP1}$ and $p16^{INK4a}$ proteins. Tumour expression was determined by calculating the percentage of positive malignant cells with respect to the total number of cells encountered in 20 representative high-power fields. The intensity of the staining was disregarded. Groups were formed according to the percentage of positive cells (0-24, 25-49, 50-74 and ≥75%) (Fig. 1). Protein expression in ANTE was also evaluated. Following criteria used in previous studies (34-38), CD44s expression in basal and parabasal layers with absence of expression in superficial layers of ANTE was considered

Table II. Immunohistochemical results in the tongue cancers under study.

Variables	N (%)
p21	
+	20 (55.5)
++	5 (13.8)
+++	9 (25.0)
++++	2 (5.5)
p16	
+	26 (72.2)
++	7 (19.4)
+++	3 (8.3)
CD44	
+	4 (11.1)
++	7 (19.4)
+++	12 (33.3)
++++	13 (36.1)

+, <25% positive tumour cells; ++, 25-49% positive tumour cells; +++, 50-74% positive tumour cells; ++++, ≥75% positive tumour cells.

normal (positive) (Fig. 2). The immunohisto-chemical staining of $p16^{INK4a}$ and $p21^{WAF1/CIP1}$ proteins in ANTE was categorised as positive or negative (13,17).

The association of the different prognostic factors with recurrence and death was assessed by means of hazards ratio (HR) and its 95% confidence interval. Cox regression analysis was used to adjust for potential confounders and the assumption of proportional hazards was tested. To assess the statistical significance of a trend in multivariable analyses, the indices were introduced into the Cox model as a continuous variable (38). To determine the variables to be included in the Cox multivariate regression analysis, standard procedures were followed to control for confounding factors (39).

Table III. Clinical and pathological variables with influence on survival in the statistical analysis.

Variable	N	Death (%)	Crude HR (95%CI)
Clinical T			
I and II	24	3 (12.50)	1 (reference)
III and IV	12	9 (75.0)	9.820 (2.570-37.513)
Pathological T			
I and II	25	4 (16.0)	1 (reference)
III and IV	11	8 (72.7)	20.96 (4.14-105.97)
Extracapsular spread			
No	33	9 (27.2)	1 (reference)
Yes	3	3 (100)	3.994 (1.06-15.0)

Results

The distribution of the clinical, pathological and immunohistochemical variables is shown in Tables I and II. One-third of the patients (12 cases) presented tumour recurrence in oral cavity and 12 died of the cancer. Mean time between primary surgery and local recurrence was 10.9 months (median 7.5, interquartile range 5.5-9). Mean survival of patients who died of tongue cancer was 25.5 months after treatment (median 19, interquartile range 12-37). $p21^{WAF1/CIP1}$, $p16^{INK4a}$ and CD44s were expressed in <25% of tumour cells in 20

Table IV. Statistical analysis of the influence on survival of the immunohistochemical variables under study.

Variable	N	Death (%)	Crude HR (95% CI)	Adjusted[a] HR (95% CI)
p16				
≤50% cells +	33	9 (27.3)	1 (reference)	1 (reference)
>50% cells +	3	3 (100)	5.03 (1.32-19.20)	2.17 (0.39-12.16)
p21				
≤50% cells +	25	7 (28.0)	1 (reference)	1 (reference)
>50% cells +	11	5 (45.5)	1.54 (0.49-4.92)	1.00 (0.23-4.40)
CD44				
≤50% cells +	11	9 (81.8)	1 (reference)	1 (reference)
>50% cells +	25	3 (12.0)	0.10 (0.02-0.33)	0.54 (0.25-36.66)
p16+p21+CD44				
≤50% cells +	4	3 (75.0)	1 (reference)	1 (reference)
>50% cells +	32	9 (28.1)	0.33 (0.08-1.27)	3.05 (0.25-36.66)

[a]Adjusted for CD44, tumour thickness, pathological T and extranodal spread.

Table V. Immunohistochemical expression of p21, p16 and CD44s in the non-tumour epithelium adjacent to tumour.

Variables	N (%)[a]
p21	
-	21 (87.5)
+	3 (12.5)
p16	
-	21 (87.5)
+	3 (12.5)
CD44s	
-	5 (20.8)
+	19 (79.1)

[a]Only 24 of the 36 studied tumours presented non-tumour epithelium adjacent to tumour.

Table VI. Clinical and pathological variables with influence on recurrence in the statistical analysis.

Variable	N	Recurrence (%)	Crude HR (95%CI)
Clinical T			
I and II	24	4 (16.6)	1 (reference)
III and IV	12	8 (66.6)	5.82 (1.17-19.53)
Pathological T			
I and II	25	5 (20.0)	1 (reference)
III and IV	11	7 (63.6)	6.50 (1.84-22.95)
Extracapsular spread			
No	33	9 (27.2)	1 (reference)
Yes	3	3 (100)	3.84 (1.02-14.39)
Tumour thickness			
<7 mm	15	2 (13.3)	1 (reference)
≥7 mm	21	10 (47.6)	5.156 (1.11-23.85)

(55.56% of series), 26 (72.22%) and 4 (11.11%) cases, respectively. The relationship between survival and clinical, pathological and immunohistochemical variables is shown in Tables III and IV. Four patients (11.11%) displayed staining in <50% of the tumour cells for the three proteins studied ($p21^{WAF1/CIP1}$, $p16^{INK4a}$ and CD44s). No statistically significant association was found between low expression of all three proteins and survival (Table IV). Low tumoral expression of CD44s showed a significant relationship with survival that persisted after adjusting for other predictors of mortality (HR=0.04, 95% CI=0.01-0.30, p=0.01).

Fifteen patients (41.67%) showed low expression of both $p21^{WAF1/CIP1}$ and $p16^{INK4a}$, but this was not significantly associated with the clinical and pathological parameters studied, including local recurrence (HR=0.841, 95% CI=0.30-2.33, p=0.74) and survival (HR=0.839, 95% CI=0.28-2.50, p=0.75).

ANTE was studied in 24 out of the 36 patients (66.6%). Immunohistochemistry results are described in Table V. Three patients showed negative expression of all three studied proteins in ANTE and all presented with local recurrence; this association was significant (HR=0.03, 95% CI=0.00-0.29, p=0.002). Local recurrence was observed in only 5 out of the remaining 21 patients. Furthermore, local recurrence occurred earlier (mean of 7 months versus 28.8 months; p=0.03) in patients with loss of expression of $p21^{WAF1/CIP1}$, $p16^{INK4a}$ and CD44s in ANTE. In the multivariate analysis, only loss of CD44s in ANTE was independently related to local recurrence (HR=0.1, 95% CI=0.02-0.45, p=0.005) (Tables VI and VII).

Table VII. Statistical analysis of the influence on recurrence of the immunohistochemical variables under study.

Variable	N	Recurrence (%)	Crude HR (95% CI)	Adjusted[a] HR (95% CI)
p16				
≤50% cells +	33	9 (27.3)	1 (reference)	1 (reference)
>50% cells +	3	3 (100)	5.12 (1.34-19.52)	1.98 (0.39-10.0)
p21				
≤50% cells +	25	7 (28.0)	1 (reference)	1 (reference)
>50% cells +	11	5 (45.5)	1.66 (0.52-5.30)	2.11 (0.50-8.78)
CD44				
≤50% cells +	11	8 (72.7)	1 (reference)	1 (reference)
>50% cells +	25	4 (16.0)	0.15 (0.04-0.50)	0.10 (0.02-0.45)
P16+p21+CD44				
≤50% cells +	4	3 (75.0)	1 (reference)	1 (reference)
>50% cells +	32	9 (28.1)	0.31 (0.83-1.18)	2.97 (0.40-21.80)

[a]Adjusted for CD44, tumour thickness, pathological T and extranodal spread.

Discussion

Acquisition by neoplastic cells of an invasive phenotype is a critical event during the multi-step process of head and neck carcinogenesis (40). Alterations in some cell-adhesion molecules have been associated with basal membrane rupture and in-depth invasiveness of submucosal tissues (28,41-44). It appears that cancer with alterations in cell-cycle control mechanisms and adhesion molecules may carry a worse prognosis. The present study investigated whether alterations of proteins $p21^{WAF1/CIP1}$ and $p16^{INK4a}$ with concomitant alteration of adhesion molecule CD44s are of prognostic relevance in patients with tongue cancer.

Low expression (<50% of tumour cells) of all three proteins ($p21^{WAF1/CIP1}$, $p16^{KIP4a}$ and CD44s) was observed in four of the present series of patients with tongue cancer (11.1% of the tumours) but no relationship with survival was found. It cannot be concluded, therefore, that alterations in the expression of all of these proteins is related to a worse prognosis. In fact, only low CD44s expression emerged as a factor independently related to survival in the multivariate analysis. Most authors also found no relationship between survival and lower expression of $p16^{INK4a}$ (45-52) or $p21^{WAF1/CIP1}$ (17,21-24,51). Nevertheless, this is a controversial issue, with other researchers reporting a relationship between these proteins and survival (9,18-20,53). In contrast, there is a wide consensus that loss of CD44 expression is an adverse prognostic factor in head and neck cancer (30,33,37,54,55). We believe that absent or low expression of the proteins $p21^{WAF1/CIP1}$ and $p16^{INK4a}$ may contribute to carcinogenesis by preventing progression from G1 to S phase of the cell cycle. However, the overall prognosis may be related to more aggressive phenotypes determined by losses or alterations in cell adhesion molecules. Cell adhesion molecule CD44 has been reported to modulate cell migration, invasiveness and metastatic ability. Several experimental studies support this view. Thus, Sato *et al* (42) studied the invasive gain of several oral cancer cell lines after treatment with antibodies against CD44v9. Cell lines with higher expression of CD44v9 (HSC 2 and 3) increased their invasive potential after the treatment, whereas cell lines with weak expression did not (HSC 4 and KB). Moreover, Sato *et al* (41) demonstrated a significant reduction in the invasive ability of cell line HSC 4 after transfection of the CD44v9 gene. Ogawa *et al* (44) found no differences in proliferation capacity between verrucous carcinoma and well-differentiated OSSC of the tongue but recorded a difference in CD44v9 expression, which was more frequently found in verrucous carcinoma. They concluded that this difference may be responsible for the variation in invasive and metastatic capacity between these tumour types. Verrucous carcinoma probably proliferates in large masses of cohesioned cells that are hampered, as a result of CD44-mediated adhesion, from passing through the basal membrane and invading surrounding tissues. Although our group previously demonstrated that depth of invasion was an important factor related to the survival of patients with tongue cancer (32), Cox multivariate analysis identified CD44s as the only independent factor related to survival (29). We believe that the reduced expression of CD44s increases tumour cell invasiveness and may explain the worse survival detected in patients with tumours of greater thickness.

The appearance of tumour tissue after primary treatment is a dismal prognostic factor that can correspond to local recurrence, cervical node metastasis, second primary tumours (SPT) or second field tumours (SFT) (56). In our series, tumour tissue reappeared in the oral cavity in 12 patients (33.3%). Studies of clinicopathological data and molecular profiles (loss of heterozygosity and TP53 mutations) are required to differentiate among local recurrence of the primary tumour, regional metastasis, SPT and SFT (40,56). Thus, a common genetic pattern may explain a distant (metastasis) or local recurrence (secondary to involvement of surgical margins after treatment of primary tumour). Only 3 of our 36 cases presented tumour at the surgical margin, and this finding was not related to overall survival or recurrence rate. Hence, although no molecular study of these tumours (LOH and TP53 mutations) was performed, the recurrences observed in this study might have been largely due to SFT (partially shared genetic profile) or SPT (different genetic profiles) (56-58). In this context, we detected a significant association between concomitant losses of expression of $p21^{WAF1/CIP1}$, $p16^{KIP4a}$ and CD44s in the ANTE and the post-treatment reappearance of tumour tissue in oral cavity after treatment. Thus, tumour tissue reappeared in all 3 cases (100%) with no expression of any of these proteins but in only 5 of the remaining 21 cases (23.8%), and the reappearance was earlier in the former than in the latter (7 months vs. 28.8 months). However, only loss of CD44s expression in the ANTE was independently related to tumour tissue reappearance in the multivariate analysis.

Alterations in cell cycle regulation proteins appear to be a precondition for the progression of premalignant clones, increasing cell proliferation and replacing normal cells, thereby expanding the premalignant field. However, loss of cell adhesion, i.e., loss of CD44 and other adhesion molecules (43), may be necessary to develop a definitively malignant phenotype with the ability to invade adjacent tissues. The present results suggest a critical role for these invasion-related molecular processes in the prognosis of tongue cancer patients.

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