p16 Expression in Squamous Carcinomas of the Tongue

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Summary

Background: 81 patients with tongue carcinomas were studied to determine: 1) the proportion of carcinomas with altered p16 expression; 2) whether loss of p16 is an early carcinogenic event; 3) whether p16 expression alterations influence the prognosis. **Methods**: 50/81 cases could be analysed by immunohistochemistry. **Results**: Tumours were p16– in 32% (16/50) and p16+ in 68% (34/50) of patients; 32.3% (11/34) of p16+ tumours presented 1–10% of tumour cells as positive, 14.7% (5/34) 11–40% as positive, and 59.2% (18/34) presented 41–100% of tumour cells as positive. Adjacent non-tumoural epithelium (ANTE) was available in 33 of the 50 immunohistochemically analysed specimens. ANTE was normal in 25 cases and dysplastic in 8 cases. In normal ANTE, p16 expression was positive in 16% (4/25) and negative in 84% (21/25) of cases. p16 expression was negative in all dysplastic ANTE samples (8/8). **Conclusions**: Changes in p16 expression are frequent in tongue cancer and can be detected at very early stages of carcinogenesis. Nevertheless, in our study neither the absence nor the degree of p16 expression influenced the survival of patients.

Zusammenfassung

Hintergrund: 81 Patienten mit Zungenkarzinom wurden untersucht, um zu bestimmen, 1. wie hoch der Anteil der Karzinome mit verändertem p16-Vorkommen ist; 2. ob der Verlust von p16 im Krebsfrühstadium geschieht; 3. ob Veränderungen des p16-Vorkommens die Prognose beeinflussen. **Methoden:** 50 von 81 Fällen konnten durch Immunhistochemie analysiert werden. **Ergebnisse:** Bei 32% der Patienten (16 von 50) waren die Tumoren p16– und bei 68% (34 von 50) waren sie p16+; 32,3% (11 von 34) der p16+-Tumoren wiesen 1–10% der Tumorzellen als positiv auf, 14,7% (5 von 34) wiesen 11–40% als positiv auf, und 59,2% (18 von 34) wiesen 41–100% der Tumorzellen als positiv auf. Anhängendes nicht tumoröses Epithel (ANTE) war bei 33 von 50 immunhistochemisch analysierten Proben vorhanden. ANTE war in 25 Fällen normal und in 8 Fällen dysplastisch. Im normalen ANTE war das p16-Vorkommen in 16% der Fälle (4 von 25) positiv und in 84% (21 von 25) negativ. Das p16-Vorkommen war in allen dysplastischen ANTE-Proben (8 von 8) negativ. **Schlussfolgerungen:** Veränderungen des p16-Vorkommens sind bei Zungenkrebs häufig und können in sehr frühem Stadium der Karzinogenese erkannt werden. Dennoch beeinflusste in unserer Studie weder das Fehlen noch der Grad des p16-Vorkommens das Überleben der Patienten.

Introduction

Oral squamous cell carcinomas (OSCCs) account for approximately 4% of neoplasms in men and 2% in women [1]. Although the oral cavity is readily accessible and frequently examined, late diagnosis of OSCC is frequent [2] and is associated with a low 5-year survival rate. Poor outcomes in these patients have prompted researchers to investigate the prognostic value of various clinical and pathologic parameters [3]. Several studies have pointed out the inadequacy of the TNM system for prognosis in OSCCs [4], because some large tumours progress well whereas some smaller tumours cause the patient's death. With this background, many research groups have been involved in the search for clinical, histopathologic, and molecular factors related to the prognosis of OSCCs [5].

The p16 gene is localised in chromosome 9p21 and codifies the synthesis of protein p16. In normal cells, hypophosphorylate pRb protein activity prevents cell transition through the G1 checkpoint by sequestering transcription factor E2F, which is required to activate S phase genes. An opposite functional effect on pRb is exerted by the formation of cyclin complexes D/CDK4 and/or D/CDK6, which give rise to the phosphorylation of pRB half-way through G1 phase, thus cancelling its cell-cycle suppressor function. The p16 protein acts as inhibitor of CDK4 and CDK6, maintaining pRb hypophosphorylated and active in its cell-cycle repressor function [6]. Some studies demonstrated that the growth of neoplastic squamous cell lines with homozygous deletions of the p16 gene is inhibited by exogenous p16, supporting the biological activity of p16 as tumour suppressor [7]. Alterations of the p16 gene may give rise to a lack of protein p16, so that the cell loses this important mechanism of cell cycle control [8, 9].

p16 gene alterations have been reported in a large proportion of carcinomas of the upper aerodigestive ways [10] and are regarded as an early event of carcinogenesis in the head and neck [11]. The present work aimed to establish the proportion of carcinomas of the tongue with altered expression of protein p16, to determine whether this alteration is an early event in the carcinogenesis, and to investigate the prognostic utility of alterations in protein p16 expression.

Patients and Methods

Eighty-one consecutive patients with OSCC of the tongue, diagnosed and treated at our referral centre before 1996, were included in the present study. The patients' clinical data were collected from the hospital medical records, including: clinical T; pathologic T (from pathology report); clinical N; presence of distant metastasis (M) according to IUAC and AJCC criteria [12]; presence of locoregional tumour recurrence; and time (in months) between treatment and recurrence. The status of the patient at the time of the study was assessed as 'alive and disease-free', 'alive with disease', or 'death from cancer of the tongue'. Patients who died from other causes were classified according to their status at time of death. The 5-year survival time was calculated in months. 8 patients were lost to the follow-up.

Histopathologic (haematoxylin-eosin) study of tissue sections of the operative specimen was undertaken to determine the pathologic N according to IUAC and AJCC criteria [12] and to assess the extracapsular spread of the tumour.

For the immunohistochemical analysis, tissue sections of the operative specimen were fixed in formol and embedded in paraffin, endogenous peroxide activity was blocked by immersing sections in methanol containing 0.3% (v/v) hydrogen peroxide for 20 min. Sections were then washed in phosphate-buffered saline (PBS). Non-specific binding was blocked by incubating sections with 1% (w/v) bovine serum albumin (BSA) for 1 h. Sections were incubated with primary antibody at 1:100 dilution (0.1 µg/ml) overnight at 4 ºC. C-20 antibody (Santa Cruz Biotechnology, Santa Cruz, CA) was used for detecting p16 protein. The primary antibody was detected using avidin-biotin complex and 3,3´-diaminobenzine tetrahydrochloride as the chromogen. In the negative controls, primary antibody was replaced by PBS. HeLa cell line was used as positive control for p16. The p16 staining patterns were scored using previously published criteria [13]. Cytoplasmic activity was disregarded and only nuclear staining above any cytoplasmic background was considered to be evidence of the protein. Tumours were regarded as p16+ if any tumour cells showed nuclear immunoreactivity. The immunoreactivity was classified into four categories according to the percentage of positive cells: 0 = negative, 1 = 1–10%, 2 = 11–40%, and 3 = 41–100%.

When detected in the sample, non-tumoural epithelium adjacent to the tongue cancer (adjacent non-tumoural epithelium – ANTE) was assessed as normal, hyperplastic, or dysplastic. The immunohistochemical expression of p16 protein in ANTE was categorised as negative, exclusively localised in basal cell layer, or localised in both basal and suprabasal layers.

Statistical Analysis

The disease-specific survival rate was determined by the Kaplan-Meier product-limit actuarial method. Comparison of two or more survival curves was performed with the log-rank test. Prognostic factors were evaluated using univariate analysis and multivariate analysis with the Cox proportional hazards regression model. Correlations were considered significant when their overall p values were < 0.05.

Results

81 patients with cancer of the tongue were studied: 64 males and 17 females, aged 37–87 (mean 58) years. Table 1 displays major patients' characteristics.

Because of the poor state of preservation or inadequacy of the paraffin-embedded tissue samples for 31 of these patients, only 50 of the operative specimens underwent immunohistochemical analysis. Of these, 32% (16/50) showed no p16 expression and 68% (34/50) showed variable degrees of p16 expression (table 2).

33 of the immunohistochemically analysed specimens contained ANTE. Table 3 shows the results of the immunohistochemical determination of p16 expression in the ANTE. Of the 25 samples with normal ANTE, 84% (21/25) expressed no epithelial p16 and always accompanied tumours that also showed total or partial loss of p16 expression. The 8 cases with dysplastic ANTE also expressed no p16 and always accompanied tumours with partial or total loss of p16 expression.

There was no locoregional recurrence in 63% (46/73) of patients who had been followed up, recurrence in tongue in 23.3% (17/73), and recurrence in neck lymph nodes in 13.7%

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Table 1. Major patients' characteristics (gender, T, N, pathologic N, M variables, tumor stage and extracapsular spread)

Variable		Frequency	
		n	%
Gender	male	64	79.0
	female	17	21.0
T[a]	T1	22	28.2
	T2	33	42.3
	T3	10	12.8
	T4	13	16.6
N[a]	N0	46	59.0
	N1	18	23.1
	N2[a]	3	3.8
	N3	11	14.1
Pathologic N[a]	N0	50	64.1
	N1	17	21.8
	N2A	2	2.6
	N2B	7	9.0
	N2C	1	1.3
	N3	1	1.3
M[a]	M0	78	100
	M1	0	0
Tumour stage[a]	I	18	22.2
	II	22	27.2
	III	15	18.5
	IV	23	28.4
Extracapsular spread	yes	16	57.1
	no	12	42.9

[a]Missing: 3.

Table 2. Degree of p16 expression in p16-positive carcinomas of the tongue

Degree of expression[a]	n	%
1	11	32.3
2	5	14.7
3	18	52.9

[a] Percentage of p16+ cancer cells: 1 = 1–10%; 2 = 11–40%; 3 = 41–100%.

Table 3. Immunohistochemical expression of p16 in non-tumoural epithelium adjacent to the tongue cancer (ANTE)

Variable	n	%
Absence of ANTE	17	34.0
NB+	1	3[a]
NBS+	3	9[a]
D+	0	0[a]
N–	21	63.6[a]
D–	8	24.2[a]
Total	50[b]	

NB+ = p16+ expression in basal layer of normal ANTE;
NBS+ = p16+ expression in basal and suprabasal layers of normal ANTE; D+ = p16+ dysplastic ANTE; N– = p16– normal ANTE; D– = p16– dysplastic ANTE.
[a] Percent of analysed ANTE samples.
[b] Total of immunohistochemically analysed tumours.

(10/73). The mean time between treatment and recurrence in patients who had been followed up was 15.7 (range 1–84) months. 8 patients were lost to the follow-up.

The mean survival time to death was 57.1 months (95% confidence interval: 48.2–66.0).

According to the univariate analysis, the survival of the patients was influenced by the following variables: T ($p < 0.01$), N ($p < 0.05$), pathologic N ($p < 0.05$), extracapsular spread ($p < 0.05$), and locoregional recurrence ($p < 0.01$). Neither the absence nor the degree of p16 expression influenced the survival of patients in the present series (fig. 1).

Discussion

We found a total absence of p16 expression in 32% of the tongue tumours analysed in our series (16/50) and a loss of p16 expression in a variable proportion of tumour cells in the remaining 68% of cases (34/50). Reports of p16 status in squamous carcinoma of head and neck vary widely, although p16 disorders were very frequent in some series [14, 15]. Total loss of the immunohistochemical expression of p16 was reported in 83% of a series of squamous cell carcinomas of head and neck [7] and in 63% of a series of oral tumours [11]. The discrepancies in the findings of these studies may result from differences in the patient populations or in the detection techniques. Determination of the status of the p16 gene is difficult, and the multiple mechanisms to inactivate p16 require extensive genetic studies, including investigation to detect homozygous deletion and point methylations and mutations [16, 17]. Furthermore, the standard methods to determine homozygous deletions and methylation status need large amounts of tumour DNA of excellent quality [18, 19]. Even when a large amount of tumour DNA is available, homozygous deletions can be easily missed because of the masking effect of non-cancerous cells. Reed et al. [7] showed a good correlation between immunohistochemical and genetic analyses and confirmed immunohistochemical analysis to be a simple method to detect p16 inactivation regardless of the genetic mechanism involved. Their report inspired our adoption of immunohistochemical analysis for the present study. These findings and the present results confirm the importance of p16 gene alterations in carcinogenesis in the head, neck and oral cavity. However, unlike some other authors [11], we found no prognostic role for the tumour expression of p16. We recorded no significant differences in mean survival time between patients with posi-
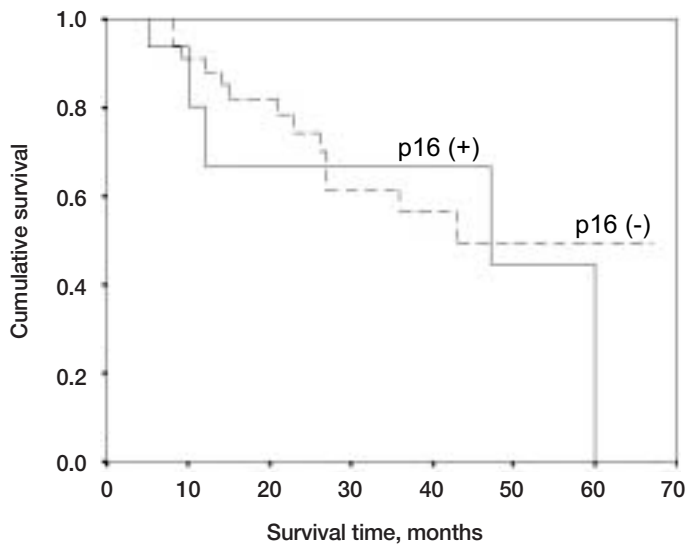
Fig. 1. Kaplan-Meier curve of disease-free survival corresponding to p16–and p16+ patients.

tive and those with negative p16 expression (45.5 vs. 40.5 months). In the present series, survival was only influenced by the clinical T, clinical N, and pathologic N, parameters, by extracapsular spread, and by locoregional recurrence, all long known to be prognostic factors [3].

We highlight the frequency of loss of p16 expression in non-

tumoural epithelium adjacent to the tongue cancers in the present series. Similar results have been reported in premalignant lesions of the tongue [11] and lung, and in dysplastic nevus lesions [20].

All of these findings support the inclusion of p16 gene alterations among early carcinogenic events in the multi-step genetic progression model for head and neck cancer proposed by Califano et al. [13]. These authors reported that 30% of a series of histopathologically benign hyperplastic lesions were composed of cell clones with genetic alterations that characterise head and neck squamous cell cancer (SCC), suggesting that these histopathologically benign lesions are on the genetic path to malignant transformation. The present finding of loss of p16 expression in 84% (21/25) of normal ANTE samples and partial or total loss of expression in all the accompanying tumours is consistent with the above observation and indicates that p16 gene alterations appear early in carcinogenesis of the tongue. The fact that all of the ANTE samples with epithelial dysplasia (8 cases) showed loss of p16 expression may indicate the development of a cell clone population that acquired a p16 genetic alteration during earlier phases of the carcinogenesis, giving them a growth advantage over other epithelial cells that preserve a normally functioning p16 control system.

In conclusion, protein p16 alterations are common in cancers of the tongue and can be detected in very early phases of the carcinogenesis.

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