ANTICANCER RESEARCH *21:* 2889-2894 (2001)

P53 Protein Expression in Oral Squamous Cell Carcinoma. Survival Analysis

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Abstract. *Background: The present study aimed to analyze the pattern of p53 expression and its influence on survival in patients with oral squamous cell carcinoma (OSCC). Materials and Methods: An immunohistochemical technique with BP53-12 antibody was performed on stored tissue from 78 patients with OSCC (intraoral cancer n=40; lip cancer n=38). The nuclear and cytoplasmic extension of p53 staining was assessed. Clinical and histopathological data were gathered and the patient survival was analyzed. Results: 57.7% (n=45) of the OSCCs expressed p53, with nuclear expression in 52.6% (n=41) of cases and cytoplasmic expression in 24.4% (n=19). The OSCCs with extensive nuclear expression of p53 showed dissociated patterns of invasion of adjacent tissues (p<0.05). A greater extension of cytoplasmic expression of p53 most commonly appeared in tumors that were better-differentiated (p<0.005), more keratinized (p<0.01) and with less nuclear atypia (p<0.05). The parameters that significantly influenced survival of patients were tumor localization (p<0.01), size (p<0.0001), lymph node invasion (p<0.0001), clinical stage (p<0.0001), differentiation degree (p<0.01) and nuclear grade (p<0.01). Conclusion: The expression of p53 protein did not behave as a marker of prognostic value in patients with OSCC.*

The most frequently documented genetic change that appears in human cancer is that occurring on the short arm of chromosome 17 (17p) in the region that contains the TP53 gene (1,2). The TP53 gene is a tumor suppressor gene that codifies a protein of 53 kilodaltons molecular weight, which arrests the cell cycle at the late G1-phase in cells that carry sublethal damage in their genome until their complete repair or induces apoptosis in cells with irreparable damage, thus avoiding the development of cell clones with severe DNA damage (3).

Alterations of the TP53 gene prevent its function as a genome guardian (4) and allow the survival of cells with damaged DNA. The p53 protein with normal function, also called wild-type p53, has a short half-life and is therefore theoretically not detectable by immunohistochemistry. After the point-mutation of TP53 gene at a concrete point the mutated protein stabilizes, accumulates in the nucleus and becomes immunohistochemically detectable (5). While p53 has classically been considered to be localized in the nucleus (6), its cytoplasmic expression has been documented in some tumors (7-9). Furthermore, in tumors at certain sites, p53 status has demonstrated predictive value as a prognostic factor for the progression of the disease and the survival of cancer patients (2). However, findings in OSCC are sparse and contradictory on these issues. The present study aimed to analyze the pattern of p53 staining in a series of OSCCs and to determine the influence of p53 expression on the survival of patients with oral cancer.

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Key Words: Oral squamous cell carcinoma, p53, survival analysis.

Materials and Methods

The present study used stored formalin-fixed paraffin-embedded tissue from patients treated for oral and lip cancer before 1992 at our hospital. To avoid sample selection bias, fifty patients with oral cancer and 50 patients with lip cancer were randomly selected for the study from a list of medical histories. Criteria for inclusion in the study were: 1) diagnosis of squamous cell carcinoma of the oral cavity or lip; 2) finalization of initial treatment before 1992; and 3) availability of medical history and operative specimen of the primary tumor. Criteria for exclusion were: 1) loss of patient follow-up in the three months before the data collection; 2) Missing TNM, tumor stage or localization data; or 3) non-availability of paraffin-embedded tumor specimen.

Application of the above criteria led to the exclusion of 22 out of the initially selected group of 100 patients, so that the final study sample comprised 78 patients with squamous cell carcinoma of the oral cavity (n=40) or lip (n=38). The age was 62.7±12.2 years (mean ± standard deviation). The hospital medical record of each patient in the study yielded data on the tumor localization, gross appearance of the tumor, size of primary tumor, invasion of lymph nodes, distant metastasis, tumoral stage, and months of survival since the initial treatment.

A 3-5 μm tissue section of each paraffin block was stained with hematoxilin-eosin. The histopathological analysis was always performed by the same specialist pathologist and included the following parameters, evaluated as noted in parentheses below: degree of tumor differentiation (well- differentiated, moderately-differentiated, poorly- differentiated); keratin production (absent: 0 keratin pearls/40X field; minimum: 1 keratin pearl/40X field; moderate: 2 keratin pearls/40X field; intense ≥ 3 keratin pearls/40X field); nuclear grade (Grade I: ≥ 75% mature nuclei;

0250-7005/2001 $2.00+.40

Grade II: 25-75 % mature nuclei and Grade III: < 25% mature nuclei). Number of mitosis/8 fields at 40X (0-1, 2-5, > 5); intensity of peritumoral inflammatory infiltrate (mild, moderate or severe); and pattern of tumoral infiltration (solid tumoral masses with blunt edges, cords of neoplastic cells, small groups of neoplastic cells, dissociated neoplastic cells) (10).

Immunohistochemical study. A 3-5 μm tissue section of each paraffin block was used for the immunohistochemical analysis. After de-paraffinization and dehydration, the endogenous peroxidase was blocked by incubation for 30 minutes in 0.3% H_2O_2 in methanol. Non-specific binding was inhibited by incubation with 1:20 dilution of normal horse serum for 60 minutes. Sections were incubated in 1:50 and 1:100 dilution of monoclonal anti-p53 protein antibody Bp53-12 overnight at 4°C in a humidified chamber. Biotinylated anti-mouse IgG (Vector Lab., USA) was applied at a dilution of 1 :200 for 30 minutes, followed by streptavidin peroxidase (Vector Lab., USA) at 1:100 for an additional 30 minutes. Finally, the peroxidase activity was developed by the 3,3'-diaminobenzidine hydrogen peroxidase reaction. Primary antibody was replaced by normal goat serum and a known p53-positive squamous cell carcinoma was used for negative and positive controls, respectively. Antigen retrieval methods were used in this study. The results of the immunoreactivity were evaluated and classified according to the cytoplasmic and nuclear reactions observed. The nuclear and cytoplasmic p53 staining was recorded as negative (0% of cells stained), mild (<25%), moderate (25-50%) or extensive (>50%).

Statistical analysis. The mean, standard deviation and percentages were calculated. The Chi-square test was applied for the comparison of qualitative independent distributions. The Spearman non-parametric correlation coefficient was used for the comparison between quantitative, ordinal or dichotomous variables. Survival was analyzed by the actuarial method (11). All data were processed with the SPSS programme for Windows, version 6.01 (SPSS Inc, Chicago, Illinois, USA) (12).

Results

The clinical outcome of the 78 patients in the present study are shown in Table I. The results of the histopathological study are listed in Table II.

In the immunohistochemical analysis, 57.7% of the OSCCs expressed p53; in 10.2% of these (8 cases) the p53 expression was diffuse throughout the tumor, while in 47.5% (37 cases) the expression was localized in some areas of the tumor (Figure 1). The remaining cases were negative for p53. Separate results for nuclear and cytoplasmic expression are shown in Table III. There was no statistically significant association between p53 expression and tumor size, invasion of lymph nodes, or clinical stage. There was a significant positive correlation ($p<0.05$) between the extent of p53 expression and the pattern of tumoral infiltration, so that tumors with more intense nuclear expression more frequently infiltrated adjacent structures in the form of dissociated neoplastic cells. The extent of cytoplasmic expression of p53 significantly correlated with the degree of tumoral differentiation ($p<0.05$) (Figure 2), the keratin production ($p<0.01$) and the cytological grade ($p<0.01$). Thus, tumors that expressed p53 in the cytoplasm in wide areas of the neoplasia were more frequently well-differentiated and highly keratinized and showed less nuclear atypia compared with tumors not expressing p53 in the cytoplasm. There was no correlation between p53 expression and the other parameters analyzed.

Table I. *Clinical manifestations of the 78 tumors studied.*

Variable	Distribution	n (%)
Tumor location[a]	Base of tongue	8 (10.4%)
	Lateral tongue	11 (14.3%)
	Base of tongue + pharynx	1 (1.3%)
	Floor of mouth	6 (7.8%)
	Gingiva	6 (7.8%)
	Retromolar Trigonum	4 (5.2%)
	Retrocomissural space	2 (2.6%)
	Lower lip	35 (45.5%)
	Upper lip	3 (3.9%)
	Palate	1 (1.3%)
Gross appearance	Exophytic	24 (30.8%)
	Endophytic	3 (3.8%)
	Ulcerated	48 (61.5%)
	Verrucous	3 (3.8%)
Size of primary tumor (S)	T1	30 (38.5%)
	T2	18 (23.1%)
	T3	9 (11.5%)
	T4	21 (26.9%)
Involvement of lymph nodes (N)	N0	63 (80.8%)
	N1	10 (12.8%)
	N2A	1 (1.3%)
	N2B	3 (3.8%)
	N2C	1 (1.3%)
Distant metastasis (M)	M0	78 (100%)
	M1	0 (0%)
Clinical stage	I	30 (38.5%)
	II	15 (19.2%)
	III	10 (12.8%)
	IV	23 (29.5%)

[a]: unknown in 1

The survival of the patients is shown in Table IV. Overall, the patients had a mean survival of over 120 months. The parameters that significantly influenced survival are shown in Table V. The expression of p53 did not significantly influence the survival of the patients studied.

Discussion

In the present study, 57.7% of the OSCCs were positive for p53. These results, obtained by the immunohistochemical

Table II. *Histological parameters of the 78 tumors studied.*

Variable	Distribution	n (%)
Degree of differentiation[a]	WDSCC	30 (40.5%)
	MDSCC	35 (47.3%)
	PDSCC	8 (10.8%)
	Fusocellular	1 (1.4%)
Keratin production[a]	Absent	1 (1.4%)
	Minimum	7 (9.5%)
	Moderate	26 (35.1%)
	Maximum	40 (54.1%)
Cell pleomorphism[b]	Grade I (low)	28 (38.4%)
	Grade II (moderate)	31 (42.5%)
	Grade III (marked)	14 (19.2%)
Number of mitoses[b]	0-1/8 fields/40X	36 (49.3%)
	1-2/8 fields/40X	6 (8.2%)
	2-5/8 fields/40X	12 (16.4%)
	> 5/8 fields/40X	19 (26.0%)
Inflammatory infiltrate[b]	Mild	13 (17.8%)
	Moderate	30 (41.1%)
	Intense	30 (41.1%)
Tumor infiltration pattern[b]	Solid	30 (41.1%)
	Cord	23 (31.5%)
	Small groups of tumor cells	19 (26.0%)
	Dissociated cells	1 (1.4%)

WDSCC: well-differentiated squamous cell carcinoma; MDSCC: moderately-differentiated squamous cell carcinoma; PDSCC: poorly-differentiated squamous cell carcinoma.
[a]: unknown in 4; [b]: unknown in 5.

Table III. *Expression of p53 in the 78 tumors.*

Variable	*Distribution*	*n (%)*
Nuclear expression	None	37 (44.6%)
	Mild	15 (20.3%)
	Moderate	12 (16.2%)
	Extensive	14 (18.9%)
Cytoplasmic expression	None	59 (74.3%)
	Mild	1 (1.4%)
	Moderate	6 (8.1%)
	Extensive	12 (16.2%)

Table IV. *Survival analysis of patients included in the study.*

Survival time (months)	No. of patients Starting/Excluded/Dead	Acumulated survival proportion (se)[a]
0-12	76/0/5	0.9342 0.028)
12-24	71/1/13	0.7619 (0.049)
24-36	57/0/4	0.7085 (0.052)
36-48	53/0/2	0.6817 (0.054)
48-60	51/0/1	0.6684 (0.054)
60-72	50/11/0	0.6684 (0.054)
72-84	39/3/0	0.6684 (0.054)
84-96	36/2/0	0.6684 (0.054)
96-108	34/7/0	0.6684 (0.054)
102-120	27/25/0	0.6654 (0.054)
> 120	2/2/0	0.6654 (0.054)

[a]: Standard error. Mean survival over 120 months.

detection of the p53 protein, are within the range of recently published findings (13-20). These studies and our own results reveal a marked variability in the percentage of OSCCs that express p53, from 33% to 100% (15-17). This variability may be accounted for by various factors. Not all p53 gene alterations imply an accumulation of the protein and, unlike missense mutations, nonsense mutations do not cause an accumulation (21,22). Thus, immunohistochemistry does not appear to be a sensitive method of detecting p53 gene alterations that do not imply protein accumulation (23). Furthermore, different monoclonal antibodies have been employed with variable sensitivities for the recognition of the p53 protein. False positive results can be obtained as a result of the possible detection of physiological amounts of p53 derived from an up-regulation caused by the influence of a noxa on the cell genome (23,24). The stabilization and accumulation of p53 can result from its binding to certain viral proteins such as the large T-antigen from SV40 virus (25), the E1B antigen from adenovirus type 5 (26), or the oncoprotein E7 of human papillomavirus type 16 (27,28). However, in this last case the loss of p53 function is associated with a positive immunohistochemical result equivalent to a missense mutation (20). It must also be borne in mind that the detection of p53 immunoreactivity in tumoral tissue is not devoid of subjectivity, since the pathologist must determine whether a case of borderline expression should be considered positive or negative (29). Nevertheless, despite the variability of the results cited above, we believe that p53 protein expression in an OSCC implies the participation of this protein in the tumorigenesis of the OSCC through an alteration of the codifying gene.

We found that 24.4% of the tumors in our study expressed p53 in the cytoplasm *versus* 52.6% that expressed it in the nucleus. This concurs with previous studies on OSCCs that used the monoclonal antibodies PAb 1801, PAb 421 and PAb

Table V. *Influence of variables on survival of 78 patients with oral cancer.*

Variable/categories	Survival at 36 months	Accumulated survival ± se at 60 months	Comparison z,p[1]
Primary localization			
Other	0.5183±0.080	0.4405±0.079	z=20.376,
Lower lip	0.9429±0.039	0.9429±0.039	p<0.001
Clinical stage			
I/II	0.9308±0.039	0.9075±0.044	z=27.265,
III/IV	0.4063±0.087	0.3438±0.084	p<0.0001
Size of tumor			
T1	0.8922±0.046	0.8487±0.053	z=19.952,
T2/T3/T4	0.4138±0.091	0.3793±0.090	p<0.0001
Involvement of lymph nodes (N)			
No	0.8048±0.047	0.7719±0.054	z=15.434,
Yes	0.2857±0.121	0.2143±0.110	p<0.0001
Degree of differentiation			
WDSCC	0.8621±0.064	0.8276±0.070	z=5.648,
MDSCC/PDSCC	0.5619±0.075	0.5385±0.075	p<0.01
Nuclear grade			
Grade I (low)	0.8519±0.068	0.8519±0.068	z=6.481,
Grade II (moderate)/ Grade III (marked)	0.5948±0.074	0.5262±0.075	p<0.01

[1]Lee-Desu test, with 1 degree of freedom; z: contrast statistic, p: statistical significance. The survival curves of the two categories considered (showing survival at 36 and 60 months) are compared for each variable.

420 (30,31). A study using the antibody BP53-12, employed in the present work, documented the cytoplasmic expression of the p53 protein in salivary gland lesions (32). However, the significance of the cytoplasmic localization of p53 is debatable. Some studies that used the PAb 421 antibody on breast cancer and oral cancer samples interpreted the cytoplasmic localization of p53 as cross-reactivity with keratin polypeptides (33-35). For some authors, cytoplasmic reactivity with PAb 1801 antibody reflects an escape of p53 protein from the nucleus during the fixation or mutation process in the p53 gene (34). Zerrahn *et al.* (36) pointed out that wild-type p53 protein is fundamentally localized in the nucleus, whereas around 60% of the mutated protein is localized in the cytoplasm. The cytoplasmic expression of p53 has been documented in rat cells transformed by mutant p53 genes (37). The binding of a mutant form of p53 protein to heat shock cytoplasmic proteins has also been reported to be responsible for the cytoplasmic localization of p53 (38). The possibility of artifact cytoplasmic staining as a result of inappropriate fixation has been reported by Gusterson *et al.* (39). However, other authors have focused on the cytoplasmic expression of p53 as a prognostic factor, and its association with a poor prognosis in colorectal adenocarcinoma has been documented (7,9). To the best of our knowledge, no studies have been published on the prognostic value of the cytoplasmic localization of p53 in oral cancer. On the other hand, there are contradictory reports on the prognostic value of the nuclear expression of p53 in patients with OSCC, which was associated with a negative prognosis by some authors (20,40-48) but was found to have no prognostic value by others (49-55), although only two studies (45,51) actually included a survival analysis. Neither the nuclear nor cytoplasmic expression influenced the survival of the patients in our series. However, we have shown that the meaning of these expressions is different, because the cytoplasmic expression of p53 occurred with significantly greater frequency in better differentiated ($p<0.05$) and more keratinized neoplasias with less nuclear atypia ($p<0.05$), whereas nuclear expression was more frequent in tumors that invaded with dissociated patterns ($p<0.05$).

Further large studies on oral cancer are required to elucidate the different implications of the cytoplasmic and nuclear immunostaining of p53 and to determine the prognostic value of the expression of this protein in OSCC.

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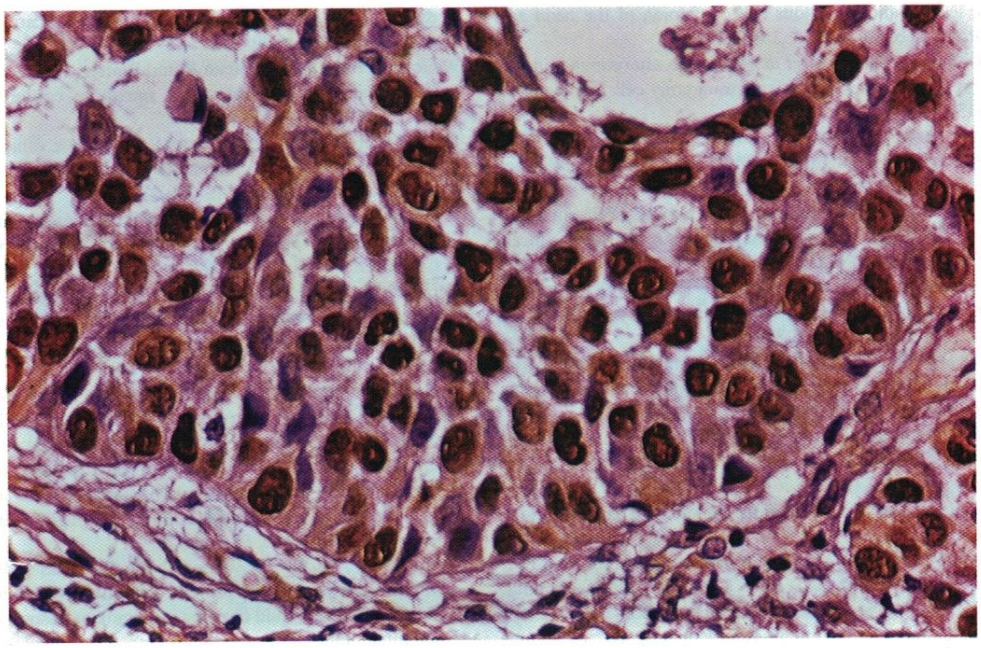

Figure 1. *p53 expression in the nucleus of tumor cells. (IMH staining, 40×).*

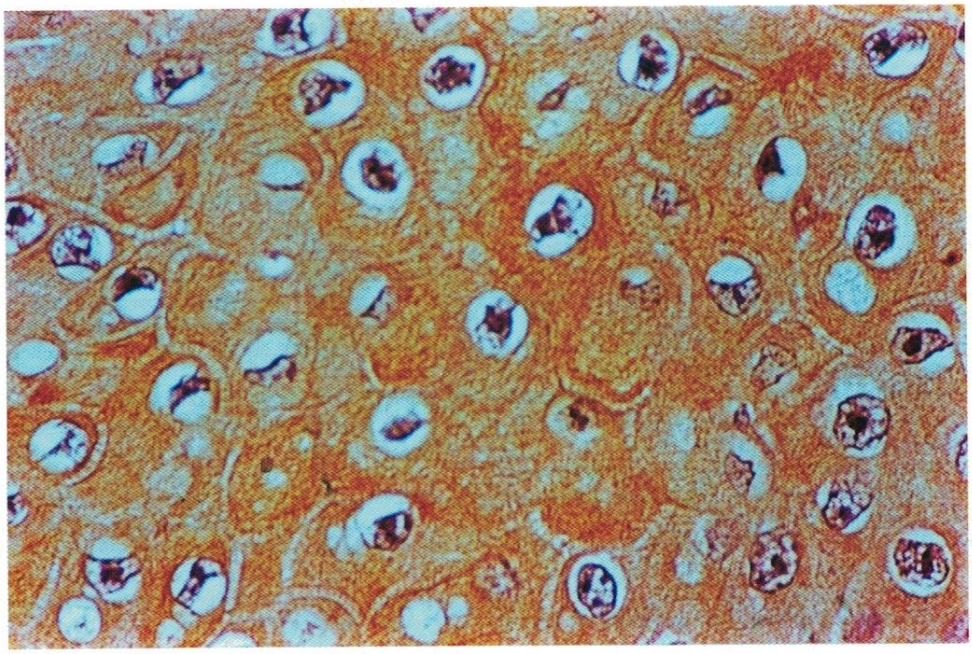

Figure 2. *p53 expression in the cytoplasm of tumor cells. (IMH staining, 40×).*

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Received February 12, 2001
Accepted May 7, 2001