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Prognostic value of p53 expression and histopathological parameters in squamous cell carcinoma of oral tongue

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Abstract

The TNM staging system is helpful but not enough to determine prognosis of the patients with squamous cell carcinoma of the oral tongue. T-stage alone is not suggestive for prediction of occult nodal metastases. For this reason, histopathological examination of 70 patients with squamous cell carcinoma of the oral tongue was done retrospectively. The histological differentiation, tumour thickness, perineural and lymphovascular space invasions, the amount of lymphocyte infiltration and pattern of tumour invasion were examined. Immunohistochemical examination was used to determine p53 immunoreactivity as well. The effect of these histopathological parameters and p53 immunoreactivity on nodal metastases and locoregional recurrence were analyzed using the chi-squared test. In terms of nodal metastases the only statistically significant difference between the two groups was tumour thickness, either <9 mm or >9 mm ($p<0.05$, $\chi^2 = 17.182$). Tumour thickness, perineural invasion, lymphovascular space invasion, the amount of lymphocyte infiltration all correlated statistically with locoregional recurrence ($p<0.05$, $\chi^2 = 6.293$ for tumour thickness; $p<0.06$, $p = 0.054$ for perineural invasion; $p<0.05$, $\chi^2 = 8.689$ for lymphovascular space invasion; $p<0.05$, $\chi^2 = 5.320$ for lymphocyte infiltration). The immunoreactivity of p53 correlated significantly with larger primary tumour size ($p<0.05$, $\chi^2 = 5.440$, lymph node metastases ($p<0.05$, $\chi^2 = 4.093$) and with pathological tumour stage ($p<0.05$, $\chi^2 = 5.713$).

These results reveal that the above-mentioned histological parameters and p53 determination could be used for handling a specimen from an anterior tongue squamous cell carcinoma.

Key words: Mouth neoplasms; Squamous cell carcinoma; Genes, p53; Prognosis

Introduction

Selecting the proper treatment for squamous cell carcinoma (SCC) of the tongue is quite difficult since there have been several treatment modalities proposed by several authors (Spiro and Strong, 1971; Callery *et al.*, 1984; Cunningham *et al.*, 1986; Francheschi *et al.*, 1993). This is particularly true for small tumours because there is more controversy concerning the treatment type of T1 and T2 tumours, since the occult metastasis rate is less than T3 and T4 tumours. The decision to make an elective neck dissection is subject of debate (Jones *et al.*, 1992).

Although wide local excision of tongue tumours is usually possible, the recurrence rate is higher than those of pharyngeal and laryngeal tumours. Mostly, the recurrences occur in the neck due to the high rate of occult metastases (Jones *et al.*, 1992).

The high rate of recurrence is probably multifactorial. It would be easier to predict the presence of occult metastases and probability of recurrence if these factors were better known (Maddox, 1984).

The first histological classification of SCC by Broders in 1926 put SCC's into four categories according to the histological parameters of tumour population including the degree of keratinization, the presence of intercellular bridges, nuclear pleomorphism, and the number of mitoses. Recently, Jacobsson proposed a semi-quantitative grading scheme for SCC that incorporates multiple observations describing histological parameters of both the tumour cell population and the host tumour interface; such as mode of invasion, vascular invasion and lymphocytic response. In addition to these histological parameters, perineural invasion, lymphatic space invasion, tumour thickness are all well known prognostic histological parameters (Crissman *et al.*, 1984).

The TNM staging system is intended to provide a uniform terminology and to improve therapeutic decision making. But TNM stage alone is not predictive for recurrence and survival; and should be evaluated together with histhopathological parameters (Bryne *et al.*, 1992).

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p53 mutations are the most common genetic abnormalities known for human cancers (Hollstein *et al.*, 1991; Kelly and Johnson, 1994). Mutation of the p53 gene can inactivate its tumour suppressor activity. In normal cells the p53 protein has a very short half-life and cannot be detected immunohistochemically. In contrast, the mutant forms are more stable and thus have an extended half-life and can be detected using immunohistochemical methods (Chen *et al.*, 1990; Ayhan *et al*, 1992). There are several studies concerning the relation of p53 mutations with tumorigenesis and tumour progression in several human cancers such as lung, head and neck, breast, colorectal, thyroid etc. (Kelly and Johnson, 1994; Hosal *et al.*, 1997).

This study was conducted to determine the prognostic value of immunohistochemically determined abnormal p53 expression and some histopathological parameters in SCC of oral tongue.

Materials and methods

The original histological slides of 70 patients with SCC of the oral tongue treated at Hacettepe University Medical Faculty were evaluated blindly without knowing the prognosis of the patients. Patients with positive surgical margins and patients with no follow-up were excluded from the study. Nine patients with follow-up periods less than three years were not included in survival analysis. Files of the patients were then examined and data for follow-up periods, recurrences and survival was collected. There were 38 male, 32 female patients; ages ranging between 21–78 (mean 52.13 ± 12.38) for males and 23–76 (mean 51.59 ± 12.37) for females. Patients were staged according to the American Joint Committee On Cancer 1992 criteria; 20 patients had stage I, 18 Stage 2, 19 stage 3, and 13 stage 4 tumours.

Histological examination

All original histological slides were re-evaluated for presence of perineural invasion, lympho-vascular space invasion, degree of differentiation which was graded as 'poor', 'moderate' or 'well-differentiated' based on the degree of keratinization, nuclear pleomorphism, and presence or absence of intracellular bridges. Tumour thickness was measured microscopically from the mucosal surface of the tumour to the point of furthest penetration into the tissue and were grouped as those with the depth <2 mm, those with depth between 2–9 mm, and those with the depth >9 mm. The invasion pattern of tumour is determined and typed as 'A' if there is pushing borders; as 'B' if infiltration is as large tumour cords, bands or strands, and as 'C' if infiltrating as widespread small groups and small cells. Lymphocytic infiltration of advancing tumour border is evaluated and graded as poor (+), moderate (++) and marked (+++), using arbitrary criteria.

Immunohistochemical examination

From formalin-fixed paraffin embedded blocks, 6 μm sections were made on silicone-coated slides. All tumour sections were deparaffinized and dehydrated in xylol and different degrees of alcohol. They were then rinsed in phosphate buffered saline (PBS) and then antigenic retrieval was accomplished using citrate buffer solution of pH 6. Peroxidase activity was blocked using absolute methanol containing 0.3% H_2O_2. After non-specific protein blockage, sections were incubated with primary monoclonal anti-p53 antibody (1/40 dilution, Novocastra, Clone 1801).

Labelling and visualization were performed as described previously (Ayhan *et al.*, 1992) using biotin-conjugated anti-mouse serum (Vectastain, Vector, Burlingaine CA) and 0.1 per cent diaminobenzidine and counterstaining was done. As a positive control each staining procedure contained a section of ovarian carcinoma known to be p53(+). Those sections having nuclear brown staining were considered to be p53 positive. p53 immunoreactivity was graded as (+) if less than 50 per cent of the tumour cells are stained positively, and (++) if more than 50 per cent of the tumour cells stained positively.

Statistical analyses

The effects of all the histological parameters on recurrence is analysed by both univariate and multivariate statistical analysis methods. Chi square test and Fisher's exact test was used for univariate analyses; Cox regression model was used for multivariate analyses.

Results

Twenty (28.5 per cent) of the 70 patients had locoregional recurrence. Nine patients with follow-up periods less than three years were not included in statistical analyses for recurrence. When the statistical analyses using univariate analyses of all the parameters mentioned are performed, it was seen that tumour thickness, perineural invasion, lympho-vascular space invasion, the amount of lymphocyte infiltration are all correlated statistically with loco-

TABLE I

EFFECT OF TUMOUR THICKNESS, PERINEURAL INVASION, LYMPHO-VASCULAR SPACE INVASION AND LYMPHOCYTE INFILTRATION ON LOCOREGIONAL RECURRENCE

	Recurrence + (n = 20)	Recurrence − (n = 41)
<9 mm	8	30
>9 mm	12	11
Perineural inv. +	6	4
Perineural inv. −	14	37
Lymphovasc. inv. +	10	6
Lymphovasc. inv. −	10	35
(+) lymphocyte. inf	12	12
(++) and (+++) lymphocyte inf.	8	29

$p = 0.054$ for perineural invasion.
$p<0.05$ for other parameters.

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TABLE II
EFFECT OF PATTERN OF INVASION, CLINICAL TUMOUR STAGE AND NODAL METASTASES ON RECURRENCE

	Recurrence +	Recurrence −	Total
Type A invasion	4	21	25
Type B invasion	10	17	27
Type C invasion	6	3	9
T1–2	12	35	47
T3–4	8	6	14
N0	3	25	28
N+	17	15	32

$p<0.005$ for all parameters.

regional recurrence (Table I). There were eight patients with a tumour thickness less than 9 mm and 12 patients more than 9 mm in the group with recurrences. The numbers were 30 and 11 consequently in the group with no recurrence ($\chi^2 = 6.293$, $p<0.05$). Six of the patients with recurrence had perineural invasion, while 14 of them did not. These numbers were four and 37 in group without recurrence ($p = 0.054$). Ten patients with lymphovascular space invasion had recurrence, six did not. The numbers were 10 and 35 consequently in patients with no lymphovascular invasion ($\chi^2 = 8.689$, $p<0.05$). Twelve patients with poor lymphocytic infiltration had locoregional recurrence while only eight with moderate and marked lymphocyte infiltration recurred. These numbers were 12 and 29 consequently in patients with no recurrence ($\chi^2 = 5.320$, $p<0.05$).

When the effect of pattern of invasion, clinical tumour stage and nodal metastases on recurrence was analysed (Table II), six of the nine patients with type C invasion pattern had recurrence, the numbers were 10 and 27 for type B; and four and 25 for type A invasion pattern. Type 'C' invasion pattern was statistically significantly different from types A and B invasion patterns in patients with the recurrence ($p<0.05$). There was no statistical difference between patients with and without recurrence in terms of tumour differentiation. (Data not shown) $p<0.05$, $\chi^2 = 0.206$). Advanced T stage and nodal metastases correlated statistically with locoregional recurrence as well. ($p = 0.316$ for T stage, $p<0.05$, $\chi^2 = 12.087$ for nodal metastases.)

Nineteen (27.14 per cent) of the 70 patients were p53 (+). Eleven of the p53 positive tumours contained more than 50 per cent of positive cells (++) while eight contained less than 50 per cent of positive cells (+). There was no statistically significant difference between groups, in terms of sex and age of patients and p53 positivity for both (+) and (++) groups. There was no correlation with p53 immunoreactivity for both (+) and (++) groups and histological differentiation either ($\chi^2 = 1.647$, $p<0.05$). Sixty-one patients had regular follow-up, 20 of which later developed locoregional recurrence. Four of the patients with recurrence and 10 of those without recurrence were immunoreactive with p53. There was no statistical difference between these groups ($\chi^2 = 0.147$, $p>0.05$). On the other hand both (+) and (++) p53 immunoreactivity correlated significantly with larger primary tumour size ($\chi^2 = 5.440$, $p<0.05$), existence of lymph node metastases ($\chi^2 = 4.093$, $p<0.05$) and advanced pathological tumour stage ($\chi^2 = 5.713$, $p<0.05$) (Table III).

TABLE III
CORRELATION OF P53 IMMUNOREACTIVITY WITH TUMOUR STAGE, METASTASIS, AND PATHOLOGICAL STAGE*

	Total	p53(−)	p53 immunoreactive (+)	(++)
T1–2	48	39	3	6
T3–4	22	12	5	5
N0	31	27	2	2
N+	35	23	5	7
Stage 1–2	27	24	2	1
Stage 3–4	43	27	6	10

$p<0.05$ for all parameters.
*+patients did not have neck dissections, so total number of patients in case of lymph node analysis is 66.

TABLE IV
RELATIVE RISKS AND 95% CI FOR NODAL METASTASES, TUMOUR STAGE AND PERINEURAL INVASION

Variable	Relative risk	95% CI lower	upper
N1	1.8233	.3440	9.6647
N2	4.3320	1.0555	17.7791
N3	14.6599	3.3387	64.3700
Perineural invasion	3.1245	1.0837	9.0083
T2	1.9765	.5577	7.0047
T3	2.6338	.6832	10.1539
T4	28.7364	2.9090	283.8743

When multivariate analyses using the Cox Regression Model was performed, and age, sex, T and N stage, p53 status, perineural invasion, lymphovascular space invasion, lymphocyte infiltration, mode of invasion, histological differentiation, tumour thickness were taken as independent variables; nodal metastases ($p = 0.022$), perineural invasion ($p = 0.0464$) and T stage ($p = 0.0530$) were found to be the most important parameters effecting recurrence. N0 for nodal metastases, T1 for tumour stage and absence of perineural invasion for perineural invasion was taken as baseline level, and the relative risks of other levels and 95 per cent CI are given in Table IV. When only histological parameters are included and the effects of T and N stage eliminated; lymphovascular space invasion ($p = 0.0048$) and tumour thickness was found to be the most important factors effecting recurrence. Also absence of lymphovascular space invasion and tumour thickness less than 9 mm were taken as baseline levels and the risk ratios of lymphovascular space invasion and tumour thickness >9 mm found (for lymphovascular space invasion rr = 3.9002, 95 per cent CI = 1.5517 to 9.8033, and for tumour thickness >9 mm, rr = 2.5643 and 95 per cent CI = 1.0270 to 6.4028).

Discussion

Cure rates for SCC of oral tongue with available treatment modalities have not improved significantly over the last few years (Shah *et al.*, 1976). There may be some increase of survival due to better diagnostic

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tools resulting in the early detection of tumour, but it is still not at an acceptable level. The recurrence rate even for T1–T2 tumours is very high, varying between 16–42 per cent (Mohit-Tabatabai *et al.*, 1986; Spiro *et al.*, 1986). The local and regional recurrence rate in our series when those patients with positive excision margins are excluded is 28.5 per cent. Although this rate is somewhat similar with other series, it is still dissatisfying.

There are several therapeutic measures reported to decrease the recurrence in SCC of the oral tongue, and they mostly depend upon determination of high risk tumours for recurrence. The additional therapeutic measures such as chemotherapy and or radiotherapy either pre- or post-operatively given to high risk patients are known to affect prognosis in a positive manner. These additional therapeutic measures are not suitable for every patient with SCC of the oral tongue because of their hazardous side-effects. So the main point is to determine patients with high risk of locoregional recurrence, and use the adjunctive treatment tools for those patients (Shah *et al.*, 1976).

Kirita *et al* (1994), reported that the most important risk factors for local recurrence of SCC of the oral tongue were endophytic tumour growth, invasion pattern of tumour, and tumour within 5 mm of excision border, also mentioning that risk of recurrence is not higher for T3–T4 tumours than T1–T2 tumours if excision margin is clear. Bryne *et al.* (1992), found that the effect of tumour differentiation of deep invasive margins is very important.

Brown *et al.* (1989), in multivariate analysis of prognostic factors found that tumour thickness, tumour stage and perineural invasion were the most important parameters affecting the survival. Spiro *et al.* (1986), reported this cut-off value for tumour thickness as 2 mm, which also predicts lymph node metastases. This value varied from 1.5 mm to 6 mm in different studies (Mohit-Tabatabai *et al.*, 1986; Urist *et al.*, 1987). According to Close *et al.* (1987), intravascular invasion was more predictive and well correlated with locoregional metastases than tumour thickness or perineural invasion. Other researchers also stressed the importance of the existence of perineural invasion and suggested that elective neck dissection and/or radiotherapy should be considered in addition to primary tumour resection if there was perineural invasion (Goepfert *et al.*, 1984; Brown *et al.*, 1989).

Host response to a malignancy is considered to be a possible mechanism to predict prognosis in some studies as well. One of the signs of host response to a tumour is histological finding of lymphocyte infiltration around the tumour cells. Hiratsuka *et al* (1984) reported a significant reverse correlation with lymphocyte infiltration and lymph node metastases. Totterman *et al.* (1978), indicated that there is a wide spread, although not complete, absence of paralysis of cytotoxic cells inside the inflammatory infiltrates of advanced cancers.

In our study, tumour thickness >9 mm is found to be a statistically significant factor affecting recurrence. Although our patients could be grouped using different cut-off values, the statistical analyses were appropriate in two groups as those <9 mm and those >9 mm. In multivariate analysis, tumour thickness itself was not among the most important parameters determining risk of recurrence, but it was the only parameter to predict occult metastases significantly ($p<0.05$, $\chi^2 = 17.182$). Univariate statistical analysis revealed that, presence of perineural invasion and lymphovascular space invasion correlated significantly with recurrence. The type C invasion pattern was also significantly different than type A and B invasion patterns for the prediction of recurrence. The amount of lymphocyte infiltration correlated inversely with recurrence. T stage of the tumour and nodal metastases again significantly affected the recurrence. Age and sex of the patients, and Broders histological differentiation grading did not have significant impact on recurrences.

Ogden *et al.* (1992) and Leedy *et al.* (1994) were the pioneers who found mutant p53 in tongue SCC. In several studies in the medical literature correlation of p53 positivity with tumour progression and metastasis was shown and p53 was considered as a prognostic factor. Ayhan *et al.* (1992) in colorectal tumours, Tahara *et al.* (1993) in gastro-intestinal tract malignancies, revealed the correlation of p53 and advanced tumour stage. The same correlation was found in thyroid malignancies (Hoşal *et al.*, 1997) and several others as well. But Leedy *et al.* (1994), found no difference between mutant p53 expression in metastatic and non-metastatic tongue tumours. Field *et al.* (1991) confirmed the same result in their study on head and neck tumours. One thing in common to all those studies is that p53 mutation is an important event in tumorigenesis. Whether mutation of p53 occurs early in tumorigenesis or not is another point of discussion. In several studies concerning head and neck pre-malignancies, lesions were shown to have mutant p53 protein (Warnakulasuriya and Johnson, 1992). Thus they claimed that p53 mutation is an early event in tumorigenesis and could be used as a tumour marker.

In this study p53 positivity was found to be statistically correlated with nodal metastases and advanced tumour stage. Finding of p53 positivity in both primary and metastatic lymph nodes in metastatic cases also suggest that p53 mutations occur prior to metastases and promote metastatic potential of that clone of cells. In one of our patients an area of in situ carcinoma revealed p53 positivity proving that p53 immunoreactivity could be used to detect early tumours as well.

When multivariate analyses for effects of age, sex, T and N stage, differentiation, perineural and lymphovascular invasion, pattern of invasion, amount of lymphocyte infiltration, tumour thickness, pattern of invasion, p53 status on recurrence were examined, perineural invasion, T stage and nodal metastases were found to be the most important parameters for predicting recurrence. When T stage

and nodal metastases were eliminated from the analyses and only histological findings were taken into account, lymphovascular invasion was the most important prognostic parameter.

The results of this study prove that histopathological parameters are important for the prediction of recurrence and p53 status gives important clues to determine the aggressive behaviour of the tumour and its metastatic potential. With the prediction of recurrence and occult metastases and possibility of aggressive behaviour early in the treatment planning, adjuvant treatment modalities could be considered, thus a better survival with lower rate of recurrence could be achieved.

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