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Identification of site-specific prognostic biomarkers in patients with oral squamous cell carcinoma

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The purpose of the present study was to identify site-specific prognostic biomarkers in patients with oral squamous cell carcinoma (OSCC). For this purpose, Epidermal growth factor receptor (EGFR), Stat3, H-ras, c-myc, p53, cyclin D1, p16, Rb and Bcl-2 were localized immunohistochemically in buccal mucosa carcinoma (n=74) and tongue carcinoma (n=61) patients. Expression of markers was compared between buccal mucosa and tongue carcinoma and assessed for their prognostic value in site-specific manner. On comparison, only cyclin D1 showed significant difference in expression with higher accumulation in tongue tumors (r=+0.177, p=0.039). Moreover, univariate survival analysis showed that in buccal mucosa patients, loss of p16 and overexpression of H-ras were significant prognosticators for relapse-free survival (RFS) and overall survival (OS), respectively. However, in Cox multivariate analysis, they lost their significance after adjusting for significant clinicopathological parameters. On the other hand, in tongue cancer patients, Cox multivariate analysis showed that for RFS, Stat3 and c-myc, and for OS, Stat3, Bcl-2 and p53 were significant prognosticators after adjusting for significant confounding factors. Our findings indicated that buccal mucosa and tongue carcinoma exhibit different biological behavior which is reflected in prognosis. Therefore, this approach might be helpful to precisely identify patients for more effectively tailored treatment strategy.

Key words: oral squamous cell carcinoma; site-specific biomarkers; immunohistochemistry; biological behavior; prognosis.

Oral squamous cell carcinoma (OSCC), a frequent neoplasm, is displaying the most dynamic growth among the top ten malignancies [1]. In recent decades, an escalation in the incidence of this malignancy has been observed in certain areas of the world [2]. In India too, it represents 35-40% of all malignant tumors and is the leading cause of death. This tumor entity is characterized by an aggressive growth pattern, and it has unpredictable biological behavior and unfavorable prognosis. It constitutes heterogeneous group of tumors, originating from different anatomic sites of the oral cavity. Furthermore, tumors from these different sites, often invading more than one, each one with their own particular problems regarding management [3]. Also, it could be shown that various anatomic sites of oral cavity exhibit different risk factors [4] and oncogenic alterations [5] which are reflected in differences in growth pattern, clinical behavior of the tumors and prognosis. This indicates that for OSCC patients, anatomic site of the tumor is an important factor for disease outcome and treatment. However, till to date, disease stage and grade of tumor have profound influence on disease outcome for these patients and the influence of anatomic site is less clear. Hence, for an accurate measurement of prognosis, it is clinically important to identify site-specific prognostic biomarkers that can accurately reflect the biological aggressiveness of disease and provide more precise prognostic and therapeutic characteristic of individual tumor.

In most studies, squamous cell carcinomas of the oral cavity are considered as one tumor entity, although they behave differently depending on anatomic site of the tumor [6, 7]. Furthermore, the site of attack within oral cavity can be varying between geographical areas. In most South and South-East Asian countries including India, buccal mucosa represents the most common anatomic site for cancer development followed by carcinoma of the tongue as compared to Western Countries where cancer of the tongue is more common [8, 9]. Amongst all intra-oral sites, carcinoma of the tongue behaves entirely different. It seems to grow faster than other sites of the oral cavity and, more frequently metastasize to cervical lymph nodes with a poorer prognosis [10]. Moreover, an analysis of the Surveillance, Epidemiology, and End-Results (SEER)

Abbreviations: ABComplex – Avidin-biotin-complex, APES – 3-amino-propyltriethoxy silane, DAB – 3,3´diaminobenzidine tetrahydrochloride, DPX – Dibutyle phthalate xylene, HR – Hazard Ratio, TBS – Tris buffer saline

registry data for head and neck squamous cell carcinomas (HNSCC) demonstrated that site-specific differences in this entity may also be seen in the varied treatment approaches [11]. However, the causes for such differences in tumor behavior remain enigmatic.

Recently, our group has demonstrated the prognostic significance of molecular markers in OSCC patients with early and advanced stage of disease [12]. Most of published studies have shown prognostic significance of biological markers in patients with OSCC, however, there have been relatively few published series that specifically addressed site-wise analysis for disease prognosis [13, 14, 15, 16]. Therefore, the present study sought to identify significant prognostic biomarkers in a site-wise manner in patients with OSCC. We examined protein expression of epidermal growth factor receptor (EGFR), an intracellular signal transducer and activator of transcription 3 (Stat3), transcription factors H-ras and c-myc, cell cycle regulators p53, cyclin D1, p16, Rb and anti-apoptotic marker, Bcl-2 in buccal mucosa and tongue carcinoma patients and assessed their prognostic value in a site-wise manner. Also, we compared expression of biomarkers between these two sites of the oral cavity. Moreover, we evaluated the correlation between clinicopathological parameters and buccal mucosa and tongue carcinoma tumors. To our best knowledge, this is the first report that has analyzed the mentioned biomarkers in a multiparametric approach for detecting prognostic biomarkers in a site-specific manner.

Patients and methods

Patients. A total of 135 untreated histologically confirmed OSCC patients evaluated at The Gujarat Cancer and Research Institute, Ahmedabad, India, between 2000 and 2003, were enrolled in this study. Amongst, 74 patients had carcinoma of the buccal mucosa and 61 patients had oral tongue cancer. Written consent of the patients who underwent surgery at the Department of Surgical Oncology was obtained, prior to primary tumor tissue collection. Tumor portion selected by the pathologist was used for block preparation which was then subsequently used for routine H&E examination and immunohistochemical localization. Post-operative treatment included radiotherapy and chemotherapy, instituted by the Radiotherapy and Medical Oncology units, respectively. The detailed clinical history (age, gender, anatomic site, family history of cancer, habit, pTNM stage, histopathological findings, treatment given, appearance of recurrence/metastases, survival time, both, relapse-free and overall, and disease outcome) was noted from the case files maintained at the Medical Record Department of the institute. The disease was staged according to the criteria of the International Union Against Cancer pTNM classification [17]. The tumors were histologically graded based on the criteria of Broders [18].

Table 1. Antibodies used in the study and the staining pattern

Antigen	Primary antibody	Dilution	Staining pattern
EGFR	Monoclonal, clone 111.6 (Neomarkers, CA, USA)	1:50	Membranous
Stat3	Monoclonal, clone SC-8019 (Santa Cruz Biotechnology, USA)	1:100	Nuclear
H-ras	Monoclonal, clone SC-29 (Santa Cruz Biotechnology, USA)	1:50	Cytoplasmic
c-myc	Monoclonal, clone9E11 (Novocastra Laboratories, UK)	1:50	Cytoplasmic
p53	Monoclonal, clone DO-7 (Dako, Glostrup, Denmark)	1:50	Nuclear
cyclin D1	Monoclonal, clone p2D11F11 (Novocastra Laboratories, UK)	1:50	Nuclear
p16	Monoclonal , clone SC-166 (Santa Cruz Biotechnology, USA)	1:150	Nuclear
Rb	Monoclonal, clone Rb1 (Dako, Glostrup, Denmark)	1:40	Nuclear
Bcl-2	Monoclonal, clone 124 (Dako, Glostrup, Denmark)	1:80	Cytoplasmic

For survival analysis, 89 patients (buccal mucosa; n=46 tongue; n=43) who could be followed for a minimum period of 2 years or until death within that period were included. Both, relapse-free survival (RFS) and overall survival (OS) were evaluated in patients with buccal mucosa and tongue carcinoma.

Immunohistochemistry. Immunohistochemistry of biomarkers (EGFR, Stat3, H-ras, c-myc, p53, cyclin D1, p16, Rb, and Bcl-2) was localised in primary tumors as described previously [12]. Briefly, 4μm thick paraffin embedded tissue sections were deparaffinised and rehydrated in graded ethanol. Endogenous peroxidase activity was quenched with methanol containing by 3% hydrogen peroxide for 20 min. To retrieve antigenicity, the sections were heated in 10 mM citrate buffer (pH 6.0) for 20 min. For EGFR, 1% trypsin treatment was given. Non-specific conjugation was blocked with normal rabbit serum (DAKO, Glostrup, Denmark). For immunostaining, the primary antibody at a given dilution in TBS was applied to the sections which were then incubated overnight, at 4°C. Primary antibodies and the dilutions used in the study are shown in Table 1.The specific immune reaction was detected using the avidin-biotin peroxidase complex technique. As positive controls, formalin-fixed paraffin-embedded tissue sections with intense staining for the given marker were included with each staining procedure. For negative control, the primary antibody was replaced with normal serum. All the sections were scored independently, by two individual observers, in a blinded fashion. The sections were scored by assessing the site of staining (nuclear, Stat3, p53, cyclin D1, p16, Rb; membranous, EGFR and cytoplasmic, H-ras, c-myc, Bcl-2) and using a semiquantitative score ranging from negative to 3+ (negative staining: 0-10% of cells positive, 1+: 11-30% of cells positive, 2+: 31-50% of cells positive , 3+: >50% of cells positive) was used. For statistical evaluations the scores 1+, 2+ and 3+ were incorporated in the positive group. For Rb

and p16 loss of expression was observed and the threshold considered for Rb and p16 negativity was absence of staining in ≤10% of cells.

Statistical analysis. The data were analyzed statistically using SPSS software (release 10; Chicago, IL, USA, 1999). Two-tailed Chi-square test was used to assess the associations between two parameters. Correlation between two parameters was calculated using Spearman's correlation coefficient (r) method. For relapse-free survival and overall survival, univariate and multivariate analysis was performed by Cox regression using 'enter' method. All clinicopathologic variables significant in univariate analysis to RFS and OS were adjusted for in multivariate analysis. Hazard Ratio (HR) with 95% confidence interval (CI) was used to assess the prognostic value of biomarkers for relapse-free and overall survival. Survival rates (RFS and OS) were estimated according to the Kaplan-Meier survival estimates. p value ≤0.05 was considered significant.

Results

Details of clinicopathological parameters and their association with buccal mucosa and tongue carcinoma. The details of clinicopathological parameters and their association with buccal mucosa and tongue carcinoma patients are depicted in Table 2A and 2B. The age range observed for buccal mucosa and tongue carcinoma patients was 28 to 75 years and 27 to 78 years, respectively, with a median of 45 years. The male: female ratio for both anatomic sites was similar and it was 3:1. Eighty-five percent and 77% of buccal mucosa and tongue carcinoma patients had habit of tobacco (chewing, smoking or both), respectively. Regarding treatment modalities and disease outcome, no significant difference was observed between buccal mucosa and tongue carcinoma tumors (Table 2A). Further, amongst pathological variables, except for histological grade of the tumors, none of the parameter such as tumor size, nodal status, tumor stage, keratin formation, lymphatic and vascular permeation showed significant difference between buccal mucosa and tongue carcinoma tumors. Seventy-five percent of tongue carcinoma patients had higher histological grade compared to 43% (32/74) of buccal mucosa patients (χ^2=14.18, df=1, r=+0.324, p=0.0001; Table 2B).

Incidence of biomarkers and their correlation with buccal mucosa and tongue carcinoma. Protein expression of markers and their association with buccal mucosa and tongue carcinoma tumors is summarized in Table 3. In buccal mucosa patients, the percentage positivity of EGFR, Stat3, H-ras, c-myc, p53, cyclin D1 and Bcl-2 proteins was 55%, 43%, 45%, 61%, 46%, 16% and 44%, respectively. The loss of expression of p16 and Rb was noted in 64% and 65%, respectively. Similarly, in tongue carcinoma patients, the percent positivity noted was 49%, 44%, 39%, 67%, 49%, 31% and 43% for EGFR, Stat3, H-ras, c-myc, p53, cyclin D1 and Bcl-2, respectively. For p16 and Rb, loss of expression observed was 54% and 75%, respectively (Table 3).

On comparison of biomarker expression, cyclin D1 was the only marker which showed significant difference in the expression between buccal mucosa and tongue carcinoma. Accordingly, 41% (25/61) of tongue cancer patients showed higher accumulation of cyclin D1 compared to 24% (18/74) of buccal cancer patients (χ^2=4.27, df=1, r=+0.178, p=0.039, Table 3).

Survival analysis. A total of 89 patients (buccal mucosa; n=46 and tongue carcinoma; n=43) were included in survival analysis. Using Cox proportional hazard regression model, univariate and multivariate survival analysis was performed to identify site-specific significant prognostic biomarkers with an independent effect on risk of recurrence or death from cancer. For RFS, 12 months median was observed with a range of 1.16 to 39.14 months and 4.15 to 27.11 months in buccal mucosa and tongue carcinoma patients, respectively. For OS, in buccal mucosa patients the median observed was 15 months with a ranged of 2.19 to 39.14 months and for tongue cancer patients, 16 months median was noted with ranged of 5.0 to 27.11 months.

Relapse-free survival. In buccal mucosa patients, univariate analysis for RFS demonstrated that besides age (HR=3.51, 95% CI=1.18-10.48, p=0.024), nodal status (HR=3.01, 95% CI=1.26-7.21, p=0.013), tumor stage (HR=3.83, 95% CI=1.53-9.57, p=0.004) and histological grade of the tumor (HR=2.38, 95% CI=1.00-5.66, p =0.050), the significant marker was p16 (HR=3.06, 95% CI=1.02-9.14, p=0.045; Table 4). However, in Cox multivariate analysis, p16 lost its significance after adjusting for age, nodal status, tumor stage and histological grade of the tumor.

Similarly, in tongue carcinoma patients, univariate analysis demonstrated that besides tumor size (HR=3.39, 95% CI=1.46-7.85, p=0.004), tumor stage (HR=3.73, 95% CI=1.62-8.58, p=0.002), lymphatic permeation (HR=2.42, 95% CI=1.05-5.55, p=0.036) and vascular permeation (HR=3.21, 95% CI=1.18-8.76, p=0.022), the significant biomarkers were Stat3 (HR=4.22, 95% CI=1.64-10.86, p=0.003), c-myc (HR=3.88, 95% CI=1.31-11.48, p=0.014) and p53 (HR=3.66, 95% CI=1.42-9.39, p=0.007). In these patients, Cox multivariate analysis showed that after adjusting for tumor size, stage, lymphatic and vascular permeation, Stat3 (HR=2.98, 95% CI=1.05-8.41, p=0.039) and c-myc (HR=3.71, 95% CI=1.12-12.27, p=0.031) remained significant risk predictors for RFS (Table 4).

Overall survival. Univariate survival analysis for OS in buccal mucosa patients indicated that besides tumor size (HR=3.51, 95% CI=1.35-9.13, p=0.010), nodal status (HR=4.16, 95% CI=1.58-10.90, p=0.004), tumor stage (HR=8.63, 95% CI=2.46-30.19, p=0.001) and lymphatic permeation (HR=3.06, 95% CI=1.18-7.97, p=0.021), only H-ras (HR=3.06, 95% CI=1.17-7.96, p=0.022) was the significant marker to predict poor overall survival (Table 5). However, when Cox multivariate analysis was performed, H-ras lost its significance after adjusting for tumor size, tumor stage, lymph node status and lymphatic permeation.

Table 2A. Correlation between clinicopathologic parameters and patients with buccal mucosa and tongue carcinoma

	Total patients	Buccal mucosa	Tongue	Correlation r	p value
Characteristics	No (%)	No (%)	No (%)		
Total	135	74	61		
Age				-0.006	0.943
Range (Median 45 years)		28-75 years	27-78 years		
≤45	77 (57)	42 (57)	35 (57)		
>45	58 (43)	32 (43)	26 (43)		
Gender				-0.022	0.801
Female	34(25)	18 (24)	16 (26)		
Male	101(75)	56 (76)	45 (74)		
Tobacco habit				-0.104	0.232
Absent	25 (18)	11 (15)	14 (23)		
Present	110 (82)	63 (85)	47 (77)		
Family history				+0.056	0.521
Absent	126 (93)	70 (95)	56 (92)		
Present	09 (07)	04 (05)	05 (08)		
Treatment				-0.044	0.613
Surgery (S) followed by	62 (46)	33 (44)	29 (47)		
Radiotherapy (RT)	57 (42)	31 (42)	26 (43)		
Chemotherapy (CT)	03 (02)	02 (03)	01 (02)		
RT + CT	13 (10)	08 (11)	05 (08)		
Disease status (N=89)				+0.078	0.466
No recurrence	45 (51)	25 (54)	20 (47)		
Recurrence	44 (49)	21 (46)	23 (53)		
Recurrence pattern				+0.213	0.164
Local recurrence	23 (52)	13 (62)	10 (43)		
Regional recurrence	19 (43)	08 (38)	11 (48)		
Distant recurrence	02 (05)	00 (00)	02 (09)		
Disease outcome				+0.050	0.641
Alive	54 (61)	29 (63)	25 (58)		
Died	35 (39)	17 (37)	18 (42)		

Table 2B. Correlation between clinicopathologic parameters and patients with buccal mucosa and tongue carcinoma

	Total patients	Buccal mucosa	Tongue	Correlation r	p value
Characteristics	No (%)	No (%)	No (%)		
Total	135	74	61		
Tumor size				-0.049	0.572
T1/T2	85 (63)	45 (61)	40 (66)		
T3/T4	50 (37)	29 (39)	21 (34)		
Nodal status				+0.036	0.683
Absent	91 (67)	51 (69)	40 (66)		
Present	44 (33)	23 (31)	21 (34)		
Tumor stage				-0.049	0.576
Early stage	65 (48)	34 (46)	31 (51)		
Advanced stage	70 (52)	40 (54)	30 (49)		
Histological grade				+0.324	**0.0001**
I*	57 (42)	42 (57)	15 (25)		
II** + III***	78 (58)	32 (43)	46 (75)		
Keratin formation				-0.002	0.985
Absent	11 (08)	06 (08)	05 (08)		
Present	124 (92)	68 (92)	56 (92)		
Lymphatic permeation				-0.035	0.687
Absent	95 (70)	51 (69)	44 (72)		
Present	40 (30)	23 (31)	17 (28)		
Vascular permeation				+0.084	0.332
Absent	125 (93)	70 (95)	55 (90)		
Present	10 (07)	04 (05)	06 (10)		

*Well differentiated tumors, **Moderately differentiated tumors, ***poorly differentiated tumors

In tongue cancer patients, univariate analysis for OS demonstrated that besides tumor size (HR=5.63, 95% CI=2.17-14.61, p=0.0001), nodal status (HR=3.07, 95% CI=1.18-7.99, p=0.021), tumor stage (HR=7.09, 95% CI=2.61-19.28, p=0.0001), lymphatic permeation (HR=3.90, 95% CI=1.52-9.99, p=0.004) and vascular permeation (HR=4.86, 95% CI=1.69-13.95, p=0.003), the significant biomarkers were Stat3 (HR=4.39, 95% CI=1.43-13.40, p=0.009), c-myc (HR=5.35, 95% CI=1.22-23.31, p=0.026), p53 (HR=4.25, 95% CI=1.39-12.98, p=0.001) and Bcl-2 (HR=2.67, 95% CI=1.03-6.91, p=0.043) (Table 5). In these patients, Cox multivariate analysis showed that Stat3 (HR=5.51, 95% CI=1.23-25.31, p=0.028), Bcl-2 (HR=4.19, 95% CI=1.17-14.97, p=0.027) and p53 (HR=3.69, 95% CI=1.01-13.43, p=0.048) emerged as significant independent prognosticators for OS after adjusting for tumor size, tumor stage, nodal status, lymphatic and vascular permeation (Table 5).

Kaplan and Meier survival analysis using combination of markers in tongue carcinoma. Cox proportional multivariate survival analysis demonstrated that Stat3 and c-myc remained most significant risk predictors for reduced RFS in tongue cancer patients. The Kaplan and Meier survival analysis was carried out using combination of these two markers in this group of patients (Table 6).

Table 3. Incidence of biomarkers and their correlation with buccal mucosa and tongue carcinoma tumors

Markers	Total No (%)	Buccal mucosa No (%)	Tongue No (%)	Correlation r	p value
Total	135	74	61		
EGFR	64 (47)	41 (55)	30 (49)	+0.062	0.475
Stat3	59 (44)	32 (43)	27 (44)	+0.010	0.906
H-ras	57 (42)	33 (45)	24 (39)	-0.053	0.542
c-myc	86 (64)	45 (61)	41 (67)	+0.066	0.445
p53	64 (47)	34 (46)	30 (49)	+0.032	0.711
cyclin D1	43 (32)	18 (24)	25 (41)	+0.177	**0.039**
p16	80 (59)	47 (64)	33 (54)	-0.095	0.271
Rb	94 (70)	48 (65)	46 (75)	+0.114	0.188
Bcl-2	68 (50)	20 (44)	36 (43)	-0.051	0.554

Eighty-two percent of tongue cancer patients whose tumors were positive for Stat3 and c-myc had a significantly reduced RFS as compared with 47% of patients with any one marker positive and 18% of patients with both the markers negative. (Log rank =15.16, df=2, p=0.0005; Table 6; Figure 1).

Table 4. Univariate and multivariate survival analysis for relapse-free survival in buccal mucosa and tongue carcinoma patients (Cox proportional hazard model)

	Buccal Mucosa (n=46)			Tongue (n=43)		
Variables	HR	(95% CI)	p value	HR	(95% CI)	p value
Univariate analysis						
Age	3.51	(1.18-10.48)	**0.024**	1.47	(0.64-3.34)	0.357
Sex	0.85	(0.31-2.34)	0.766	0.78	(0.30-1.98)	0.603
Habit	0.47	(0.15-1.41)	0.180	1.73	(0.59-5.11)	0.316
Tumor size	2.32	(1.26-7.21)	0.057	3.39	(1.46-7.85)	**0.004**
Nodal status	3.01	(1.26-7.21)	**0.013**	1.85	(0.76-4.53)	0.712
Tumor stage	3.83	(1.53-9.57)	**0.004**	3.73	(1.62-8.58)	**0.002**
Histological grade	2.38	(1.00-5.66)	**0.050**	1.12	(0.41-3.02)	0.819
Keratin formation	0.96	(0.22-4.16)	0.965	4.29	(0.57-31.94)	0.155
Lymphatic permeation	2.34	(0.96-5.67)	0.060	2.42	(1.05-5.55)	**0.036**
Vascular permeation	0.78	(0.10-5.82)	0.810	3.21	(1.18-8.76)	**0.022**
EGFR	1.09	(0.45-2.65)	0.834	1.68	(0.74-3.81)	0.214
Stat3	1.11	(0.46-2.63)	0.811	4.22	(1.64-10.86)	**0.003**
H-ras	2.13	(0.89-5.08)	0.087	1.10	(0.47-2.61)	0.815
c-myc	1.10	(0.45-2.65)	0.830	3.88	(1.31-11.48)	**0.014**
p53	1.99	(0.84-4.69)	0.116	3.66	(1.42-9.39)	**0.007**
cyclin D1	0.60	(0.20-1.80)	0.371	1.91	(0.84-4.38)	0.122
p16	3.06	(1.02-9.14)	**0.045**	1.47	(0.62-3.49)	0.372
Rb	1.45	(0.56-3.75)	0.436	0.63	(0.26-1.55)	0.322
Bcl-2	1.84	(0.78-4.36)	0.161	1.77	(0.78-4.04)	0.170
Multivariate analysis						
Stat3*	-		-	2.98	(1.05-8.41)	**0.039**
c-myc*	-		-	3.71	(1.12-12.27)	**0.031**

*After adjusted for tumor size, tumor stage, lymphatic and vascular permeation.

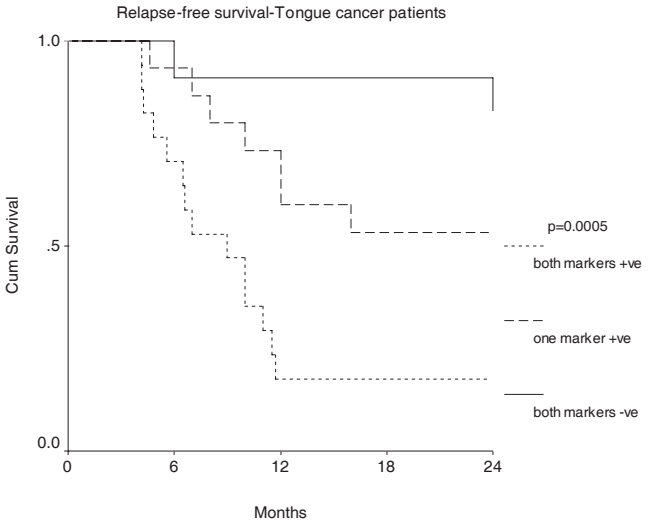

Fig. 1. Kaplan-Meier survival curves showed that tongue cancer patients with Stat3 and c-myc positive tumors had reduced relapse-free survival compared to patients with both the markers negative tumors.

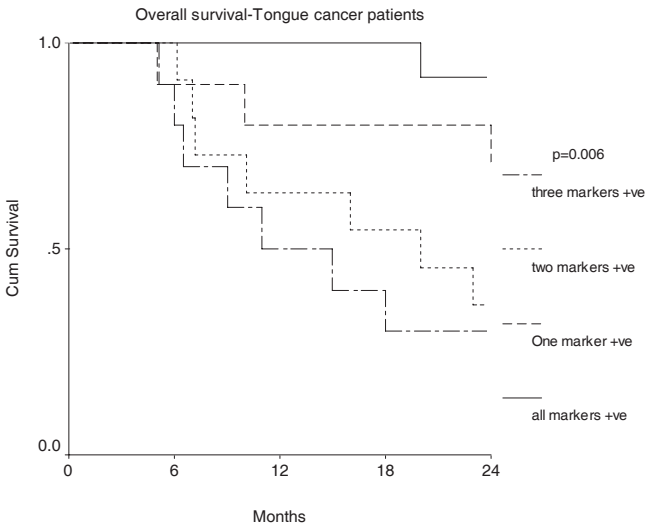

Fig. 2. Kaplan-Meier survival curves showed that tongue cancer patients with Stat3, p53 and Bcl-2 positive tumors had poor overall survival than patients with all three markers negative tumors.

For overall survival, combination of Stat3, Bcl-2 and p53 was carried out to performed Kaplan and Meier analysis. The prognosis of tongue cancer patients was deteriorated gradually with the increase in the co-expression of these markers, and the patient group with tumors positive for all three markers had significantly worse survival than the other groups. Accordingly, 70% of tongue cancer patients whose tumors were positive for Stat3, Bcl-2 and p53 had a significantly inferior OS as compared with 64% patients with any two markers positive, 30% patients with any one marker positive and 8% of patients with all three markers negative. (Log rank =12.45, df=3, p=0.006; Table 6; Figure 2).

Discussion

In the present study, we comapred the protein expression of EGFR, Stat3, c-myc, H-ras, p53, cyclin D1, Rb, p16 and Bcl-2 between buccal mucosa and tongue carcinoma tumors, and assessed for their prognostic value in a site-specific manner. While comparing marker expressions, significant difference was observed in the incidence of cyclin D1 with higher expression in tongue carcinoma compared to buccal mucosa. Moreover, Cox multivariate survival analysis demonstrated that in tongue cancer patients Stat3 and its downstream target molecules, such as c-myc, p53 and Bcl-2 emerged as independent prognosticators. On the other hand, in buccal mucosa patients, none of the studied biomarkers emerged as independent significant prognosticator in multivariate analysis. Although, univariate analysis for RFS and OS showed that loss of p16 expression and overexpression of H-ras were significant prognostic biomarkers in buccal carcinoma patients, they lost their significance after adjusting for significant co-founding variables in Cox multivariate analysis. Thus, our resluts indicated that OSCC, although a histological entity, the biological behavior of buccal mucosa and tongue carcinoma tumors is different. The heterogeniety of molecular mechanism with tumor anatomic site of the oral cavity supports such differences.

Overexpression of cyclin D1 was noted in 41% of tongue carcinoma patients compared to 24% of patients with buccal cancer. In our earlier study, Vora et al. [19] also observed overexpression of cyclin D1 in tongue carcinoma. Similar to our results, Carlos de et al. [20] and Bova et al. [13] have reported higher accumulation of cyclin D1 in tongue cancer. Overexpression of cyclin D1 has been associated with a more aggressive tumor phenotype and reduced survival in patients with operable HNSCC from different anatomical sites [21, 22, 23]. However, the mechanisms responsible for overexpression of cyclin D1 in tongue cancer, and how it confers a more aggressive malignant phenotype remain unclear. Although, the contribution of CCND1 gene amplification to overexpression has been well documented [24], other likely mechanisms include up-regulation of receptor and signaling pathways that converge on cyclin D1 gene expression [25]. Further, the role of cyclin D1 as a prognostic marker in HNSCC remains controversial. In our study, we found lack of correlation between overexpression of cyclin D1 and disease outcome. In contrast, Michalaides et al. [23] demonstrated that overexpression of cyclinD1 was an independent prognostic variable in HNSCC, however, this study comprised predominantly hypopharyngeal and laryngeal carcinomas. Several studies have described the expression of cyclin D1 in various anatomic sites of the oral cavity with expression being more often detected in sites like

Table 5. Univariate and multivariate survival analysis for overall survival in buccal mucosa and tongue carcinoma patients (Cox proportional hazard model)

	Buccal mucosa (n=46)		Tongue (n=43)	
Variables	HR (95% CI)	p value	HR (95% CI)	p value
Univariate analysis				
Age	0.41 (0.13-1.26)	0.121	1.05 (0.41-2.68)	0.905
Sex	0.85 (0.27-2.60)	0.776	0.92 (0.30-2.80)	0.884
Habit	0.53 (0.15-1.87)	0.329	1.59 (0.46-5.49)	0.464
Tumor size	3.51 (1.35-9.13)	**0.010**	5.63 (2.17-14.61)	**0.0001**
Nodal status	4.16 (1.58-10.90)	**0.004**	3.07 (1.18-7.99)	**0.021**
Tumor stage	8.63 (2.46-30.19)	**0.001**	7.09 (2.61-19.28)	**0.0001**
Histological grade	2.37 (0.90-6.25)	0.080	1.19 (0.39-3.63)	0.755
Lymphatic permeation	3.06 (1.18-7.97)	**0.021**	3.90 (1.52-9.99)	**0.004**
Vascular permeation	1.05 (0.14-7.94)	0.960	4.86 (1.69-13.95)	**0.003**
EGFR	1.61 (0.62-4.19)	0.323	2.08 (0.82-5.29)	0.122
Stat3	1.28 (0.49-3.33)	0.605	4.39 (1.43-13.40)	**0.009**
H-ras	3.06 (1.17-7.96)	**0.022**	1.05 (0.39-2.81)	0.915
c-myc	0.89 (0.34-2.34)	0.818	5.35 (1.22-23.31)	**0.026**
p53	2.49 (0.96-6.48)	0.060	4.25 (1.39-12.98)	**0.011**
cyclin D1	0.62 (0.18-2.17)	0.460	2.20 (0.85-5.70)	0.103
p16	3.15 (0.90-11.02)	0.072	1.59 (0.59-4.24)	0.354
Rb	1.30 (0.45-3.70)	0.619	0.62 (0.23-1.66)	0.344
Bcl-2	1.29 (0.50-3.36)	0.593	2.67 (1.03-6.91)	**0.043**
Multivariate analysis				
Stat3*	-	-	5.51 (1.23-25.31)	**0.028**
Bcl-2*	-	-	4.19 (1.17-14.97)	**0.027**
p53*	-	-	3.69 (1.01-13.43)	**0.048**

*After adjusted for tumor size, tumor stage, nodal status, lymphatic and vascular permeation.

Table 6. Kaplan-Meier survival analysis using combination of biomarkers in patients with tongue carcinoma

	Relapse-free survival				Overall Survival		
Combination of biomarkers	N	Patients relapsed No (%)	p value	Combination of markers	N	Patients died No (%)	p value
Stat3 & c-myc	43	23		Stat3, Bcl-2 & p53	43	18	
Both -ve	11	02 (18)	**0.0005**	All -ve	12	01 (08)	**0.006**
Any one +ve	15	07 (47)		One +ve	10	03 (30)	
Both +ve	17	14 (82)		Two +ve	11	07 (64)	
				Three +ve	10	07 (70)	

tongue [26, 27]. In contrast, Raju et al. [28] found cyclin D1 expression being most common in the gingiva and cheek. This variation in expression by sites in the oral cavity has been suggested to be related to racial differences and varying environmental risk factors.

With regard to clinicopathological parameters, only histological grade of the tumor showed significant difference between buccal mucosa and tongue carcinoma tumors. Seventy-five percent of tongue carcinoma tumors showed higher histological grade compared to 43% of buccal mucosa tumors. Consistent findings reported by Costa et al. [29] with high histological score in tongue carcinoma. In addition, several investigators have demonstrated a close relationship between the degree of histological differentiation and incidence of lymph node metastasis in OSCC [30, 31]. In agreement to this, the present study demonstrated significant positive correlation between high histological grade of the tumors and tongue cancer indicating aggressive behavior of this anatomic site of the oral cavity.

Moreover, the most striking finding of the current study we noted when we assessed site-specific prognostic value of studied biomarkers. In buccal mucosa patients, multivariate

survival analysis demonstrated that amongst studied biomarkers, none of them emerged as independent significant prognosticator. Although, in univariate survival analysis, loss of p16 expression and overexpression of H-ras showed significant prognostic values for RFS and OS, respectively. They lost their significance when multivariate analysis was performed. This led us to suppose that for our series of buccal cancer patients other biomarkers may have relationship with the prevalence of this site of cancer. Therefore, for OSCC patients having buccal cancer, it is important to identify new prognostic marker that accurately reflect the biological aggressiveness of this site of the oral cavity.

On the other hand, in tongue carcinoma, Cox multivariate survival analysis demonstrated that overexpression of Stat3 and its downstream targets c-myc, p53 and Bcl-2 were independent prognosticators for poorer survival. Stat3 represents a critical regulatory switch that drives the transcription of various target genes including c-myc, cyclin D1, p53 and Bcl-2 and plays a critical role in cell oncogenesis through the combined inhibition of apoptosis and activation of cell cycle progression [32, 33, 34, 35]. In accordance to our results, Masuda et al. [36] demonstrated that increased phospho-Stat3 correlated with lower disease-free survival in tongue cancer patients. Contrary results were obtained in nasopharyngeal carcinoma, where Stat3 alone could not predict prognosis but along with Stat5 predicted better disease free and overall survival [37]. Even in breast cancer, Stat3 did not have any significant predictive value for predicting outcome [38]. Moreover, we also observed that tongue cancer patients whose tumor showed combined expression of Stat3 and c-myc was 4.70 times more likely to develop recurrence compared to tongue cancer patients with absence of these two markers together. Stat3 and c-myc transcription factors play an important role in tumorigenesis [39, 40], and it has been shown that for the transforming activity of Stat3, c-myc might be cooperating with the Stat3 oncogene [41]. Further, overexpression of c-myc actively participates in the p53 concert by accumulating different genetic lesions and maintaining the proliferative potential of cells [42]. Regarding p53, it is postulated that loss of p53 function renders the cell susceptible to further genetic alteration [12, 43, 44]. In addition, Lin et al [45] provided evidence that constitutive activation of Stat3 selectively occurs in cells harboring p53 mutation or deletion and cancer cells expressing constitutively active Stat3 may need to further mutate p53 to escape p53-dependent apoptosis and be able to continue tumor progression. They further speculated that other p53 downstream target(s) may be involved in the inhibition of Stat3 phosphorylation and activity. Stat3 is an important transcription factor for the regulation of Bcl-2 gene expression. Numerous studies have shown that Stat3 activity promotes tumor cell survival by up-regulating anti-apoptotic genes [46]. In this context, recently, Sepúlveda et al. [47] demonstrated that Stat3 activation provided an anti-apoptotic advantage in human $CD34^{+}$ cells, essentially owing to the overexpression of bcl-2. Thus, our results indicated that in patients with tongue carcinoma, expression of Stat3, c-myc, p53 and Bcl-2 were the most significant independent prognosticators that may influence clinical decision making and treatment strategies of this group of OSCC patients.

In conclusion, our study demonstrated that buccal mucosa and tongue carcinoma have distinct difference in biological behavior and clinical outcome. Therefore, our approach of identification of site-specific prognostic biomarkers might be helpful to precisely identify patients with an aggressive phenotype, and can be used at diagnosis to add prognostic information and thereby helpful in guiding therapeutic decisions.

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