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Understanding molecular markers in recurrent oral squamous cell carcinoma treated with chemoradiation

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Abstract

Introduction: Oral cancer accounts for approximately 2.1% of all cancers worldwide. In India, oral squamous cell carcinoma (OSCC) is the most common cancer with half a million new cases diagnosed every year. More than 50% of patients eventually develop local recurrence or metastasis usually within the first 2-years following completion of treatment. It is beneficial to analyze the prognostic significance of Cyclin D1, p53 and EGFR which are critical mediators in the pathogenesis of OSCC. The objective of this study was to assess the association of expression of these markers with recurrence and pattern of recurrence in OSCC patients undergoing chemoradiation.

Materials and Methods: A Total 290 OSCC cases of locally advanced stage (III, IV) oral cancer with World Health Organization (W.H.O.) performance status of grade 0/1 in the year 2009–2012 were enrolled in the study. Treatment response was assessed according to W.H.O. criteria. Cyclin D1, EGFR and p53 expression in tumor tissue was estimated by immunohistochemical (IHC) method and quantified as percentage positive nuclei.

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Results: During the 2-years follow up, 56 (19.3%) patients recurred, out of which, 47 (83.9%) were locoregional and 9 (16.1%) distant sites. On correlating, χ^2 test showed significant ($P < 0.05$ or $P < 0.01$ or $P < 0.001$) association of marker expressions (Cyclin D1, EGFR and p53) with recurrence. The strong positive expressions of all three markers showed significant association with early time of recurrence. The multivariate logistic regression analysis showed significant ($P < 0.05$ or $P < 0.01$ or $P < 0.001$) association of recurrence with primary site, differentiation, Cyclin D1 and p53 expressions indicating these as an independent predictors of recurrence in OSCC. The Cyclin D1, EGFR and p53 expressions also showed significant ($P < 0.001$) poor survivals (OS, DFS and RFS) in patients with positive/strong positive expressions than negative expression suggesting their prognosis in OSCC.

Conclusion: Our results signifies that tumors over expressing Cyclin D1, EGFR and p53 are resistant to chemoradiation and are associated with increased risk of locoregional recurrence and metastasis in OSCC patients undergoing chemoradiation.

Keywords: Medicine, Cancer Research, Oncology

1. Introduction

Oral cancer accounts for approximately 2.1% of all cancers worldwide, with an estimated 300,000 new cases and 145,000 deaths in 2012 [1]. Moreover highest mortality rates (77%) occurred in the developing countries. In India, oral squamous cell carcinoma (OSCC) is the most common cancer with half a million new cases diagnosed every year [2]. It is more common in males with a large fraction of cases typically diagnosed in late stages and are usually treated by chemoradiation [3]. Prognosis is still relatively poor and has shown only slow progress with 5-year actuarial survival rates between 30% and 40% in most of the studies [4]. Patients in advance stage usually presents with multiple cervical lymph node metastasis, which further decreases the 5-years cancer specific survival by 50% when compared with node negative patients [5]. Despite of significant improvement achieved during the last decades in its detection, prevention and treatment; outcome and prognosis related to cure and survival have still been poorer due to tragic event of treatment resistance and tumor recurrence. More than 50% of patients eventually develop local recurrence or metastasis usually within the first 2 years following completion of treatment [6].

Prognosis and treatment outcome is certainly influenced by stage, tumor grade, site, depth of invasion, lymphovascular spread, patient's age, and performance; however it has been found that in same stage disease, response to treatment varies with individual, might be due to variation in tumor biology related to cell morphology or genetic phenotype of the tumor [7]. Therefore heterogeneity of

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tumor cells introduces significant threat in the management of OSCC. Recent advances in molecular biology field and improved conception of the pathogenesis of OSCC have provided access to many new orientations to the research in this direction. The molecular markers of concern are those involved in cell cycle regulation and cell signaling pathway. Oncogenes which promote cell and tumor growth includes growth factor receptors (hst-1, int-2, EGFR/erbB,c-erbB-2/Her-2, sis), intracellular signal transducer (ras,raf,stat-3), transcription factors (myc, fos, jun, c-myc), cell-cycle regulators (Cyclin D1), apoptosis regulatory protein (bcl-2, bax) & tumor suppressor gene (p53, Rb) which encodes proteins that typically transduce negative growth regulatory signals; these have been identified as genetic alterations in each of the pathological stages of OSCC [6]. Out of these, Cyclin D1, EGFR and p53 gene have emerged as exquisitely critical mediators in the pathogenesis of OSCC. Activation of Cyclin D1, p53, EGFR are known to be inhibitors of apoptosis and play crucial role in initiation of intracellular signaling pathways which regulate the activation of cell proliferation, invasion, angiogenesis and metastasis and thereby influence treatment outcome Expression of these proteins have also been correlated with a more aggressive phenotype and worse prognosis. Therefore, quantification of the activation status of these markers would be a potential parameter for predicting treatment outcome [8, 9, 10]. Association of these markers with treatment response and survival have been examined in many studies, but very few studies have correlated the expression of these markers with tumor recurrence, which is a major concern in OSCC [11, 12]. Most of the work has been done in other malignancies like breast, lung, colorectal, bladder and Gastrointestinal tract [13, 14, 15, 16, 17]. Owing to most feared malignancy due to its aggressive nature and high mortality rate, there has been a constant search for new prognostic and predictive markers so as to understand the tumor behaviour, treatment outcome and tendency to recur. Search for the new markers might be considered as an emerging approach to individualize and facilitate treatment planning by categorizing patients according to risk factors detected, so that high risk patients might be kept under aggressive surveillance for the assessment of disease relapse.

We have therefore conducted this study with the objective to assess the association of expression of Cyclin D1, EGFR and p53 with recurrence and pattern of recurrence in OSCC patients undergoing chemoradiation.

2. Materials and methods

Total 290 histologically proven new cases of locally advanced stage (III, IV, M0) oral cancer with World Health Organization (W.H.O.) performance status (PS) of grade 0/1 attending radiotherapy O.P.D, in the year 2009–2012 were enrolled in the study. These cases were assessed thoroughly (history, clinical examination and investigations). The study was approved by the ethics committee of the Institution,

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and written informed consent was obtained from all patients before enrollment. All the patients were given 2-cycles of induction taxol (175 mg/m^2 day 1) and cisplatin (50 mg/m^2 day 2) chemotherapy and were subjected for radiation along with concurrent cisplatin (35 mg/m^2) 4-weeks from completion of induction chemotherapy. Radiotherapy was given by External beam Conventional Method (200CGy/fraction to a total dose of 70 Gy in 35 fractions in 7-weeks by $cobalt^{60}$ to primary tumor site and neck. The protocol plan was continued despite mucositis or dermatitis. However, the dose of cisplatin was reduced to 50% if the calculated creatinine clearance level was 30–50 ml/min. No cisplatin was given if the creatinine clearance level was less than 30 ml/min. In presence of myelosupression (WBC count $<$ 4000/mm^3 or platelets count less than 100,000/mm^3), persistent fever that exceeded 38 °C or other clinically apparent infections, chemoradiation was postponed for 1-week or interrupted.

For histopathological and immunohistochemical studies, tumor samples from the lesion site was fixed in 10% buffered formalin and then embedded in paraffin. Paraffin embedded formalin fixed tissues were processed routine H & E stained sections evaluated to confirm the diagnosis of squamous cell carcinoma and to grade the lesion. Further sections were processed for Cyclin D1, EGFR and p53 biomarkers by immunohistochemistry (IHC) using primary monoclonal antibodies and a polymer based secondary antibody detection kit from Dakopatts, Denmark. Standard Immunohistochemistry protocol was used. In short deparaffinized rehydrated sections were blocked for endogenous peroxidases in 0.3% hydrogen peroxide in methanol, followed by a rinse in distill water. Antigen retrieval was achieved at 121 °C in 10 mM citrate buffer (pH 6.0) for 10 minutes using Pascal retrieval system from Dakopatts, Denmark. Slides cooled to room temperature were washed thrice with TBS and thereafter incubated overnight at 4 °C with Primary Antibdies to Cyclin D1 (Dakopatts Denmark), p53 (DO7, Leica Microsystems, Germany) and EGFR (BioGenex, USA). After washing with Tris-buffered saline, the sections were incubated for 30 minutes with secondary antibody. The Cyclin D1, EGFR and p53 were visualized with DAKO Liquid Diaminobenzidine substrate chromogen and counterstained with diluted Mayer's haematoxylin. Sections mounted with DPX were inspected under a Zeiss Z2 imager and photographed at 40X magnification. The immunohistochemical evaluation was carried out in tumor hotspots including the invasion front, which was regarded as most indicative of the biological activity of the tumor, in 10 high power fields. About 1500–2000 tumor cells were observed in all tumors at a magnification of 40X in 10 selected fields. For Cyclin D1 and EGFR tumors were labeled as negative if $<$10%, moderate positive between 10–50% and strongly positive if $>$50%, tumor cells expressed the antigen [18, 19]. The p53 expression was evaluated as negative if $<$10%, moderate positive between 10–25% and strongly positive if $>$25% [20].

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Assessment of tumor response was done by clinical examination, radiological investigations (CT-scan) 4–6 weeks after completion of treatment. Biopsy or fine needle aspiration cytology to determine pathological response was not performed routinely; it was done only in case of recurrent disease/partial response of the clinically and radiologically suspected lesion to confirm the presence of disease. The definitions of treatment response viz. complete response (CR), partial response (PR) and no response (NR) [stable disease (SD) + progressive disease (PD)] were based on the standard definitions established by W.H.O. (1979). After chemoradiation, patients were followed up to 2-years and were assessed for the recurrence and survival.

The primary end measures of the study was to assess the association of expression of Cyclin D1, EGFR and p53 with recurrence, time to recurrence and type of recurrence in OSCC patients undergoing chemoradiation. The secondary end point of the study was 2-years survivals (overall, disease free and recurrence free). Survival time was defined as the interval between the date of initial treatment and the date of the last follow up examination.

2.1. Sample size determination

The sample size and power the test statistics of present study is based on complete response followed by recurrence within 2 years in OSCC patients undergoing chemoradiation. Expecting at least 25% recurrence within two year after complete response in OSCC patients undergoing chemoradiation and considering 5% margin of error (type I error: $\alpha = 0.05$) and 80% power (type II error: $1\text{-}\beta = 0.80$), minimum 290 patients will be required for the study is evaluated [21] as below:

$$\begin{aligned} n &= \frac{t \times t \times p(1-p)}{e^2} \\ &= \frac{1.96 \times 1.96 \times 0.25(1-0.25)}{0.05^2} \\ &= 288.12 \\ &\approx 290 \end{aligned}$$

where,

n = sample size

t = confidence level of t statistic at 95%, standard value = 1.96

p = effect size = 25%

e = margin of error = 0.05%

Thus, a minimum 290 patients will required to get at least 25% recurrence in OSCC patients undergoing chemoradiation.

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2.2. Statistical analysis

Continuous data were summarized as Mean ± SE (standard error of the mean) while discrete (categorical) in number and percentage. Continuous groups were compared by Student's t-test or one way analysis of variance (ANOVA) followed by Tukey's post hoc test whereas applicable. Categorical groups were compared by chi-square (χ^2) test. Pearson correlation analysis was also done to asses association between the variables. Binary logistic regression analysis was done to assess association and risk predictors of recurrence. Cox-regression analysis was done to find out independent predictors of overall survival and recurrence. Survival between groups was done by Kaplan-Meier method followed by Log rank (Mantel-Cox: χ^2) test. A two-tailed ($\alpha = 2$) $P < 0.05$ was considered statistically significant.

3. Results

3.1. Clinico-pathological characteristics

The clinico-pathological characteristics of 290 OSCC patients are summarized in Table 1. The age of patients ranged from 20–67 years with mean (± SE) 50.49 ± 0.69 years and median 53 years. Most of the patients were ≤60 yrs (75.9%), mostly males (79.7%) and mostly belongs to poor socio-economic status (59.3%). Out of total, 66.2% patients had tobacco chewing habit, 62.4% had betel nut chewing habit and 55.9% had smoking habit. At initial presentation, the PS of 43.1% patients was poor. Further, in patients, buccal mucosa was the most common primary site (32.1%) followed by tongue (16.6%), alveolus (15.5%), retromolar trigone (RMT) (13.4%), hard palate (13.1%) and lip (9.3%). Moreover, histology of most of the patients was invasive squamous cell carcinoma (ISCC) (64.8%), grade well differentiated (67.2%), tumor size T4 (75.5%) and stage IV (78.6%). The radiological response of 44.1% patients was complete (CR), 46.6% was partial (PR) and 9.3% had no response (NR) accounting 90.7% responders (CR + PR).

3.2. Marker expressions

The Cyclin D1, EGFR and p53 protein expressions of OSCC patients were done using IHC (Fig. 1) and quantified in percentage(%) and sub grouped into three groups (negative, positive and strong positive) (Table 2). The Cyclin D1, EGFR and p53 expression of patients ranged from 0–90%, 0–90% and 0–86% respectively with mean (± SE) 40.69 ± 1.29%, 51.33 ± 1.43% and 43.35 ± 1.38% respectively and median 40.0%, 50.0% and 48.0% respectively. According to Cyclin D1 expression, 31 (10.7%) patients were negative, 197 (67.9%) were positive and 62 (21.4%) were strong positive. The EGFR expression showed 13 (4.5%), 135 (46.6%) and 142 (49.0%) respectively and p53 expression showed 35 (12.1%), 46 (15.9%) and 209 (72.1%) respectively. On correlating the quantitative expressions, the Pearson correlation analysis showed significant and positive correlation

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Table 1. Clinico-pathological characteristics of OSCC patients.

Variables	(n = 290) (%)
Age (yrs)	
≤60	220 (75.9)
>60	70 (24.1)
Sex	
Female	59 (20.3)
Male	231 (79.7)
Economic status	
Upper	7 (2.4)
Middle	111 (38.3)
Poor	172 (59.3)
Tobacco chewing	
No	98 (33.8)
Yes	192 (66.2)
Betel nut chewing	
No	109 (37.6)
Yes	181 (62.4)
Smoking	
No	128 (44.1)
Yes	162 (55.9)
Performance status	
Good	165 (56.9)
Poor	125 (43.1)
Primary site	
Buccal mucosa	93 (32.1)
Alveolus	45 (15.5)
Hard palate	38 (13.1)
Lip	27 (9.3)
RMT	39 (13.4)
Tongue	48 (16.6)
Histology	
SCC	102 (35.2)
ISCC	188 (64.8)

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Table 1. (*Continued*)

Variables	(n = 290) (%)
Grade	
WD	195 (67.2)
MD	77 (26.6)
PD	18 (6.2)
Tumor size	
T2	6 (2.1)
T3	65 (22.4)
T4	219 (75.5)
Node status	
N0	89 (30.7)
N1	96 (33.1)
N2	105 (36.2)
Stage	
III	62 (21.4)
IV	228 (78.6)
Response	
CR	128 (44.1)
PR	135 (46.6)
NR	27 (9.3)

SCC: squamous cell carcinoma, ISCC: invasive squamous cell carcinoma, WD: well differentiated, MD: moderately differentiated, PD: poorly differentiated, CR: complete response, PR: partial response, NR: no response.

between the marker expressions (Cyclin D1 vs. EGFR: r = 0.71, $P < 0.001$; Cyclin D1 vs. p53: r = 0.73, $P < 0.001$; EGFR vs. p53: r = 0.86, $P < 0.001$).

3.3. Diagnostics of markers

3.3.1. Association of marker expressions with clinico-pathological characteristics

To find out diagnostic significance of markers, the association between marker expressions (negative/positive/strong positive) and clinico-pathological characteristics was done using χ^2 test (Table 3). The χ^2 test showed significant ($P < 0.05$ or $P < 0.01$ or $P < 0.001$) association of Cyclin D1 expressions with performance status, histological grade, tumor size, node status, stage and radiological response. In contrast, EGFR expressions showed significant ($P < 0.05$ or $P < 0.01$ or $P < 0.001$) association with socio-economic status, histological grade, node status,

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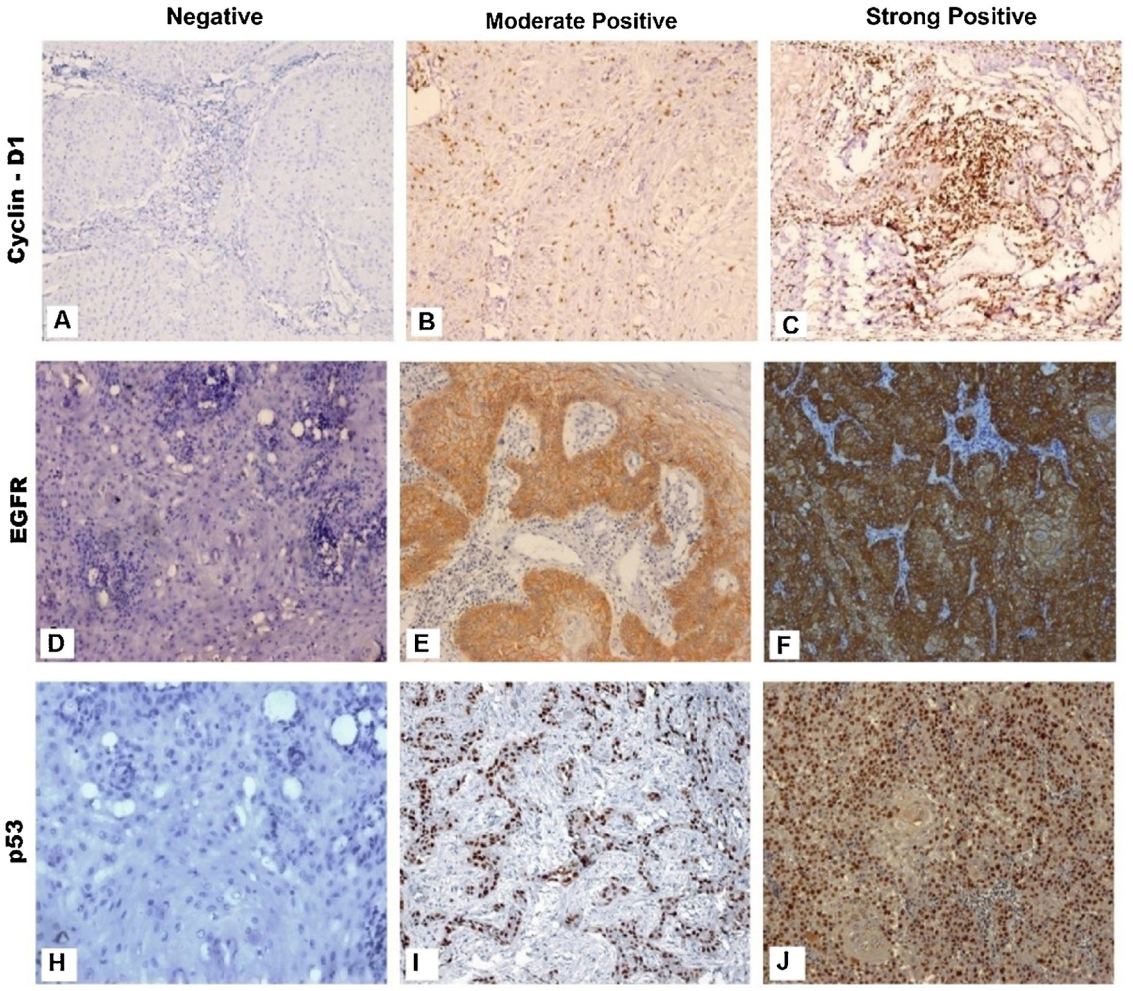

Fig. 1. Microphotograph showing Immunohistochemical expression of Cyclin D1 (A) showing negative nuclei (B) showing moderate positive stained nuclei (C) showing strongly positive stained nuclei (DAB × 125 × digital magnification); EGFR (D) showing negative cytoplasmic and membranous staining (E) showing moderate positive cytoplasmic and membranous staining (F) showing strongly positive cytoplasmic and membranous staining (DAB x 125 x digital magnification); p53 (H) showing negative nuclei (I) showing moderate positive stained nuclei (J) showing strongly positive stained nuclei (DAB x 125 x digital magnification) in OSCC.

stage and radiological response. Conversely, p53 expressions showed significant ($P < 0.05$ or $P < 0.01$ or $P < 0.001$) association with age, performance status, primary site, histological grade, tumor size, node status, stage and radiological response. Further, multivariate logistic regression analysis showed that the tobacco chewing, histological grade, tumor size, node status and stage were significant ($P < 0.05$ or $P < 0.001$) and an independent predictors of Cyclin D1 expressions (Table 4). In contrast, histological grade and node status were found significant ($P < 0.01$) and an independent predictors of EGFR expressions (Table 4).

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Table 2. Marker expressions of OSCC patients.

Marker expression	(n = 290) (%)
Cyclin D1	
Negative	31 (10.7)
Positive	197 (67.9)
Strong positive	62 (21.4)
EGFR	
Negative	13 (4.5)
Positive	135 (46.6)
Strong positive	142 (49.0)
p53	
Negative	35 (12.1)
Positive	46 (15.9)
Strong positive	209 (72.1)

Conversely, age, tobacco chewing, betel nut chewing, primary site, histological grade, tumor size, node status and stage were found to be the significant ($P < 0.05$ or $P < 0.01$) and independent predictors of p53 expressions (Table 4).

3.3.2. Association of marker expressions with recurrence, site and type of recurrence

The patients were followed-up for 2 years (24 month). The survival duration of patients ranged from 1–24 month with mean (± SE) 10.84 ± 0.39 months and median 9 months. During the period, 210 (72.4%) patients were alive, 32 (11.0%) lost to follow-up (LTF) and 48 (16.6%) died due to disease accounting total 242 (83.4%) patients live (alive + LTF). Further, at final evaluation, 128 (44.1%) patients were disease free (i.e. CR) of which 56 developed recurrence during the two-years follow-up and thus the prevalence of recurrence being 43.8% or in other words 234 (80.7%) patients were recurrence free. Of total recurrence, 47 (83.9%) had locoregional and 9 (16.1%) had distant recurrence. Further, 3 (5.4%) had recurred in bone, 1 (1.8%) had in brain, 5 (8.9%) had in lung, 31 (55.4%) had in nodal and 16 (28.6%) had in primary. The association of marker expressions with recurrence, site and type of recurrence is also analyzed and summarized in Table 5. On correlating, χ^2 test showed significant ($P < 0.05$ or $P < 0.01$ or $P < 0.001$) association of marker expressions (Cyclin D1, EGFR and p53) with recurrence. The Cyclin D1 and EGFR expressions also showed significant ($P < 0.05$ or $P < 0.001$) association with site and type of recurrence.

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Table 3. Association of marker expressions with clinico-pathological characteristics in OSCC patients (n = 290).

Variables	n(%)	Cyclin D1 expression				EGFR expression				p53 expression			
		Negative	Positive	Strong positive	P value	Negative	Positive	Strong positive	P value	Negative	Positive	Strong positive	P value
Age (yrs)													
≤60	220	25 (11.4)	142 (64.5)	53 (24.1)	0.080	12 (5.5)	96 (43.6)	112 (50.9)	0.117	30 (13.6)	27 (12.3)	163 (74.1)	**0.008**
>60	70	6 (8.6)	55 (78.6)	9 (12.9)		1 (1.4)	39 (55.7)	30 (42.9)		5 (7.1)	19 (27.1)	46 (65.7)	
Sex													
Female	59	8 (13.6)	45 (76.3)	6 (10.2)	0.058	5 (8.5)	30 (50.8)	24 (40.7)	0.138	10 (16.9)	10 (16.9)	39 (66.1)	0.390
Male	231	23 (10.0)	152 (65.8)	56 (24.2)		8 (3.5)	105 (45.5)	118 (51.1)		25 (10.8)	36 (15.6)	170 (73.6)	
Economic status													
Upper/Middle	118	12 (10.2)	79 (66.9)	27 (22.9)	0.866	1 (0.8)	63 (53.4)	54 (45.8)	**0.016**	15 (12.7)	19 (16.1)	84 (71.2)	0.953
Poor	172	19 (11.0)	118 (68.6)	35 (20.3)		12 (7.0)	72 (41.9)	88 (51.2)		20 (11.6)	27 (15.7)	125 (72.7)	
Tobacco chewing													
No	98	7 (7.1)	66 (67.3)	25 (25.5)	0.231	7 (7.1)	37 (37.8)	54 (55.1)	0.051	9 (9.2)	10 (10.2)	79 (80.6)	0.064
Yes	192	24 (12.5)	131 (68.2)	37 (19.3)		6 (3.1)	98 (51.0)	88 (45.8)		26 (13.5)	36 (18.8)	130 (67.7)	
Betel nut chewing													
No	109	11 (10.1)	73 (67.0)	25 (22.9)	0.869	7 (6.4)	43 (39.4)	59 (54.1)	0.116	12 (11.0)	14 (12.8)	83 (76.1)	0.457
Yes	181	20 (11.0)	124 (68.5)	37 (20.4)		6 (3.3)	92 (50.8)	83 (45.9)		23 (12.7)	32 (17.7)	126 (69.6)	
Smoking													
No	128	13 (10.2)	95 (74.2)	20 (15.6)	0.084	7 (5.5)	58 (45.3)	63 (49.2)	0.750	21 (16.4)	16 (12.5)	91 (71.1)	0.073
Yes	162	18 (11.1)	102 (63.0)	42 (25.9)		6 (3.7)	77 (47.5)	79 (48.8)		14 (8.6)	30 (18.5)	118 (72.8)	

(*Continued*)

Table 3. (*Continued*)

Variables	n(%)	Cyclin D1 expression				EGFR expression				p53 expression			
		Negative	Positive	Strong positive	P value	Negative	Positive	Strong positive	P value	Negative	Positive	Strong positive	P value
PS													
Good	165	21 (12.7)	117 (70.9)	27 (16.4)	**0.039**	8 (4.8)	86 (52.1)	71 (43.0)	0.067	26 (15.8)	29 (17.6)	110 (66.7)	0.037
Poor	125	10 (8.0)	80 (64.0)	35 (28.0)		5 (4.0)	49 (39.2)	71 (56.8)		9 (7.2)	17 (13.6)	99 (79.2)	
Primary site													
Buccal mucosa	93	8 (8.6)	67 (72.0)	18 (19.4)	0.560	5 (5.4)	34 (36.6)	54 (58.1)	0.064	3 (3.2)	8 (8.6)	82 (88.2)	<0.001
Other	197	23 (11.7)	130 (66.0)	44 (22.3)		8 (4.1)	101 (51.3)	88 (44.7)		32 (16.2)	38 (19.3)	127 (64.5)	
Histology													
SCC	102	6 (5.9)	76 (74.5)	20 (19.6)	0.099	2 (2.0)	53 (52.0)	47 (46.1)	0.175	13 (12.7)	20 (19.6)	69 (67.6)	0.391
ISCC	188	25 (13.3)	121 (64.4)	42 (22.3)		11 (5.9)	82 (43.6)	95 (50.5)		22 (11.7)	26 (13.8)	140 (74.5)	
Grade													
WD	195	31 (15.9)	149 (76.4)	15 (7.7)	<**0.001**	13 (6.7)	106 (54.4)	76 (39.0)	<**0.001**	34 (17.4)	46 (23.6)	115 (59.0)	<**0.001**
MD/PD	95	0 (0.0)	48 (50.5)	47 (49.5)		0 (0.0)	29 (30.5)	66 (69.5)		1 (1.1)	0 (0.0)	94 (98.9)	
Tumor size													
T2/T3	71	14 (19.7)	48 (67.6)	9 (12.7)	**0.005**	4 (5.6)	41 (57.7)	26 (36.6)	0.057	17 (23.9)	19 (26.8)	35 (49.3)	<**0.001**
T4	219	17 (7.8)	149 (68.0)	53 (24.2)		9 (4.1)	94 (42.9)	116 (53.0)		18 (8.2)	27 (12.3)	174 (79.5)	
Node status													
N0	89	16 (18.0)	69 (77.5)	4 (4.5)	<**0.001**	8 (9.0)	58 (65.2)	23 (25.8)	<**0.001**	18 (20.2)	25 (28.1)	46 (51.7)	<**0.001**
N1/N2	201	15 (7.5)	128 (63.7)	58 (28.9)		5 (2.5)	77 (38.3)	119 (59.2)		17 (8.5)	21 (10.4)	163 (81.1)	

(*Continued*)

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Table 3. (*Continued*)

Variables	n(%)	Cyclin D1 expression				EGFR expression				p53 expression			
		Negative	Positive	Strong positive	P value	Negative	Positive	Strong positive	P value	Negative	Positive	Strong positive	P value
Stage													
III	62	14 (22.6)	45 (72.6)	3 (4.8)	<**0.001**	4 (6.5)	39 (62.9)	19 (30.6)	**0.005**	17 (27.4)	19 (30.6)	26 (41.9)	<**0.001**
IV	228	17 (7.5)	152 (66.7)	59 (25.9)		9 (3.9)	96 (42.1)	123 (53.9)		18 (7.9)	27 (11.8)	183 (80.3)	
Response													
CR/PR	263	31 (11.8)	197 (74.9)	25 (13.3)	<**0.001**	13 (4.9)	135 (51.3)	115 (43.7)	<0.001	35 (13.3)	46 (17.5)	182 (69.2)	**0.003**
NR	27	0 (0.0)	0 (0.0)	27 (100.0)		0 (0.0)	0 (0.0)	27 (100.0)		0 (0.0)	0 (0.0)	27 (100.0)	

Bold values are highly significant.

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Table 4. Association of marker expressions with clinico-pathological characteristics in OSCC patients using multivariate logistic regression analysis (n = 290).

Variables	Cyclin D1 expression		EGFR expression		p53 expression	
	OR (95% CI)	*P* value	OR (95% CI)	*P* value	OR (95% CI)	*P* value
Age (yrs)						
≤60	Ref		Ref		Ref	
>60	1.09 (0.30–3.99)	0.902	1.03 (0.54–1.97)	0.928	0.16 (0.48–2.34)	**0.014**
Sex						
Female	Ref		Ref		Ref	
Male	0.27 (0.04–1.73)	0.168	0.59 (0.25–1.40)	0.234	1.24 (0.38–4.01)	0.718
Economic status						
Upper/Middle	Ref		Ref		Ref	
Poor	1.09 (0.34–3.45)	0.887	0.98 (0.55–1.74)	0.932	1.57 (0.73–3.35)	0.247
Tobacco chewing						
No	Ref		Ref		Ref	
Yes	4.96 (0.91–22.84)	**0.039**	1.22 (0.40–3.72)	0.724	8.92 (2.32–34.28)	**0.001**
Betel nut chewing						
No	Ref		Ref		Ref	
Yes	0.03 (0.00–1.51)	0.078	0.97 (0.31–3.04)	0.955	0.21 (0.05–0.86)	**0.031**
Smoking						
No	Ref		Ref		Ref	
Yes	3.00 (0.57–15.82)	0.195	1.59 (0.73–3.48)	0.243	1.47 (0.54–3.74)	0.470
PS						
Good	Ref		Ref		Ref	
Poor	0.94 (0.31–2.82)	0.912	0.70 (0.40–1.23)	0.217	0.70 (0.32–1.50)	0.355
Primary site						
Buccal mucosa	Ref		Ref		Ref	
Other	0.34 (0.09–1.34)	0.122	1.57 (0.88–2.80)	0.126	4.96 (1.90–13.04)	**0.001**
Histology						
SCC	Ref		Ref		Ref	
ISCC	2.82 (0.67–11.95)	0.159	0.88 (0.48–1.61)	0.675	1.09 (0.51–2.31)	0.833
Grade						
WD	Ref		Ref		Ref	
MD/PD	0.02 (0.00–0.15)	<**0.001**	0.42 (0.22–0.81)	**0.009**	0.03 (0.00–0.24)	**0.001**

(*Continued*)

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Table 4. (*Continued*)

Variables	Cyclin D1 expression		EGFR expression		p53 expression	
	OR (95% CI)	*P* value	OR (95% CI)	*P* value	OR (95% CI)	*P* value
Tumor size						
T2/T3	Ref		Ref		Ref	
T4	3.50 (0.69–17.41)	**0.043**	1.37 (0.22–8.62)	0.735	4.84 (0.69–12.17)	**0.046**
Node status						
N0	Ref		Ref		Ref	
N1/N2	0.29 (0.04–1.93)	**0.020**	0.33 (0.17–0.64)	**0.001**	0.31 (0.14–0.69)	**0.004**
Stage						
III	Ref	**0.031**	Ref	0.275	Ref	**0.040**
IV	0.01 (0.00–0.87)		0.35 (0.05–2.30)		0.02 (0.00–0.25)	
Response						
CR/PR	Ref		Ref		Ref	
NR	NA	–	NA	–	NA	–

NA: not applicable.

Bold values are highly significant.

3.3.3. Association of marker expressions with time of recurrence

The association of marker expressions with time of recurrence of 56 recurrent patients is summarized in Table 6. The recurrence time of patients ranged from 2–21 months with mean (± SE) 8.59 ± 0.60 months and median 7-months. The expressions of all three markers showed significant association with time of recurrence. On comparing, Student's t test showed significant and early recurrence time in patients with strong positive Cyclin D1 (10.37 ± 0.88 vs. 6.54 ± 0.63, $P = 0.001$) and EGFR (11.32 ± 1.19 vs. 7.19 ± 0.56, $P = 0.001$) expressions as compared to respective positive expressions. Further, ANOVA followed by Tukey post hoc test also showed significant and early mean recurrence time in patients with p53 positive (19.25 ± 0.85 vs. 12.67 ± 0.88, $P = 0.028$) and strong positive (19.25 ± 0.85 vs. 7.47 ± 0.48, $P < 0.001$) expressions as compared to negative expression. Moreover, the mean recurrence time was also found earlier in patients with p53 strong positive expression as compared to positive expression (12.67 ± 0.88 vs. 7.47 ± 0.48, $P = 0.025$).

3.4. Prognosis of markers

The follow up (survival) duration of patients ranged from 1–24 month with mean (± SE) 10.84 ± 0.39 months and median 9-months. During the period, 210 (72.4%) patients were alive, 32 (11.0%) lost to follow-up (LTF) and 48 (16.6%) died due to

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Table 5. Association of marker expressions with recurrence, site of recurrence and type of recurrence in OSCC patients.

Variables	n	Cyclin D1 expression				EGFR expression				p53 expression			
	(%)	Negative	Positive	Strong positive	*P* value	Negative	Positive	Strong positive	*P* value	Negative	Positive	Strong positive	*P* value
Recurrence													
No	234	31 (13.2)	167 (71.4)	36 (15.4)	<**0.001**	13 (5.6)	116 (49.6)	105 (44.9)	**0.008**	31 (13.2)	43 (18.4)	160 (68.4)	0.014
Yes	56	0 (0.0)	30 (53.6)	26 (46.4)		0 (0.0)	19 (33.9)	37 (66.1)		4 (7.1)	3 (5.4)	49 (87.5)	
Site of recurrence													
Bone	3	0 (0.0)	0 (0.0)	3 (100.0)	**0.007**	0 (0.0)	0 (0.0)	3 (100.0)	**0.032**	0 (0.0)	0 (0.0)	3 (100.0)	0.904
Brain	1	0 (0.0)	0 (0.0)	1 (100.0)		0 (0.0)	0 (0.0)	1 (100.0)		0 (0.0)	0 (0.0)	1 (100.0)	
Lung	5	0 (0.0)	0 (0.0)	5 (100.0)		0 (0.0)	0 (0.0)	5 (100.0)		0 (0.0)	0 (0.0)	5 (100.0)	
Nodal	31	0 (0.0)	22 (71.0)	9 (29.0)		0 (0.0)	16 (51.6)	15 (48.4)		3 (9.7)	1 (3.2)	27 (87.1)	
Primary	16	0 (0.0)	8 (50.0)	8 (50.0)		0 (0.0)	3 (18.8)	13 (81.3)		1 (6.3)	2 (12.5)	13 (81.3)	
Type of recurrence													
Locoregional	47	0 (0.0)	30 (63.8)	17 (36.2)	<**0.001**	0 (0.0)	19 (40.4)	28 (59.6)	**0.019**	4 (8.5)	3 (6.4)	40 (85.1)	0.465
Distant	9	0 (0.0)	0 (0.0)	9 (100.0)		0 (0.0)	0 (0.0)	9 (100.0)		0 (0.0)	0 (0.0)	9 (100.0)	

Bold values are highly significant.

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Table 6. Correlation between marker expression and time of recurrence (month) of OSCC patients (n = 56).

Marker expression	n	Mean ± SE	t/F value	*P* value
Cyclin D1				
Negative	0	–		
Positive	30	10.37 ± 0.88	3.46	**0.001**
Strong positive	26	6.54 ± 0.62		
EGFR				
Negative	0	–		
Positive	19	11.32 ± 1.19	3.58	**0.001**
Strong positive	37	7.19 ± 0.56		
p53				
Negative	4	19.25 ± 0.85	26.90	**<0.001**
Positive	3	12.67 ± 0.88		
Strong positive	49	7.47 ± 0.48		

Bold values are highly significant.

disease accounting total 242 (83.4%) patients live (alive + LTF). Further, at final evaluation, 128 (44.1%) patients were disease free (i.e. CR) and 234 (80.7%) were recurrence free.

To find out prognosis of markers, the associations of markers expressions with overall survival (OS), disease free survival (DFS) and recurrence free survival (RFS) were done using Kaplan-Meier method followed by Log rank test and summarized in Table 7, Table 8, Table 9 respectively and Fig. 2, Fig. 3, Fig. 4 respectively. The Cyclin D1, EGFR and p53 expressions showed significant ($P < 0.001$) associations with OS, DFS and RFS and showed poor OS, DFS and RFS survivals in patients with positive/strong positive expressions than negative expression.

3.5. Predictors of overall survival

To find out independent predictors of overall survival, univariate (unadjusted) and multivariate (adjusted) Cox regression analysis was done between survival (survival duration and event) and clinico-pathological characteristics and markers expressions and summarized in Table 10. The univariate Cox regression analysis showed significant ($P < 0.05$ or $P < 0.01$ or $P < 0.001$) association of overall survival with performance status, primary site, histological grade, tumor size, node status, stage, radiological response and marker expressions (Cyclin D1, EGFR and p53) suggesting these as predictors of overall survival. The multivariate Cox regression analysis further showed significant ($P < 0.05$ or $P < 0.001$) association

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Table 7. Association of marker expression with overall survival of OSCC patients using Kaplan-Meier survival analysis (n = 290).

Marker expression	n	Mean			Median			χ^2 value	*P* value
		Estimate	SE	95% CI	Estimate	SE	95% CI		
Cyclin D1									
Negative	31	13.87	1.40	11.12–16.62	15.00	3.96	7.24–22.76	49.80	<**0.001**
Positive	197	11.86	0.46	10.95–12.77	9.00	0.41	8.19–9.81		
Strong positive	62	6.10	0.50	5.12–7.08	6.00	1.03	3.99–8.01		
EGFR									
Negative	13	14.69	2.15	10.47–18.91	18.00	2.50	13.11–22.89	59.95	<**0.001**
Positive	135	13.82	0.60	12.64–15.00	12.00	2.05	7.98–16.02		
Strong positive	142	7.66	0.38	6.92–8.41	8.00	0.37	7.27–8.73		
p53									
Negative	35	17.49	1.15	15.24–19.74	19.00	0.98	17.08–20.93	84.84	<**0.001**
Positive	46	17.37	0.89	15.62–19.12	18.00	0.68	16.67–19.33		
Strong positive	209	8.30	0.34	7.64–8.96	7.00	0.31	6.40–7.60		

Bold values are highly significant.

Table 8. Association of marker expression with disease free survival of OSCC patients using Kaplan-Meier survival analysis (n = 128).

Marker expression	n	Mean			Median			χ^2 value	*P* value
		Estimate	SE	95% CI	Estimate	SE	95% CI		
Cyclin D1									
Negative	18	18.94	1.45	16.10–21.79	21.00	0.69	19.65–22.35	68.00	<**0.001**
Positive	84	17.64	0.62	16.43–18.86	18.00	0.42	17.19–18.81		
Strong positive	26	9.96	0.37	9.23–10.70	10.00	0.63	8.77–11.23		
EGFR									
Negative	11	16.55	2.06	12.52–20.57	18.00	1.06	15.92–20.09	29.50	<**0.001**
Positive	80	18.24	0.63	17.01–19.46	19.00	0.56	17.91–20.09		
Strong positive	37	11.92	0.77	10.40–13.44	11.00	0.53	9.97–12.04		
p53									
Negative	33	18.30	1.05	16.24–20.37	19.00	0.96	17.12–20.88	19.69	<**0.001**
Positive	39	18.77	0.84	17.11–20.42	19.00	0.94	17.17–20.84		
Strong positive	56	13.32	0.73	11.89–14.76	11.00	0.62	9.78–12.22		

Bold values are highly significant.

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Table 9. Association of marker expression with recurrence free survival of OSCC patients using Kaplan-Meier survival analysis (n = 234).

Marker expression	n	Mean			Median			χ^2 value	*P* value
		Estimate	SE	95% CI	Estimate	SE	95% CI		
Cyclin D1									
Negative	31	13.87	1.40	11.12–16.62	15.00	3.96	7.24–22.76	124.84	<**0.001**
Positive	167	11.05	0.49	10.09–12.02	8.00	0.37	7.28–8.72		
Strong positive	36	3.31	0.38	2.56–4.05	3.00	0.22	2.58–3.43		
EGFR									
Negative	13	14.69	2.15	10.47–18.91	18.00	2.50	13.11–22.89	77.04	<**0.001**
Positive	116	13.42	0.66	12.12–14.72	10.00	1.61	6.84–13.16		
Strong positive	105	6.16	0.33	5.52–6.81	6.00	0.32	5.38–6.63		
p53									
Negative	31	17.58	1.26	15.11–20.05	19.00	1.11	16.82–21.18	106.33	<**0.001**
Positive	43	17.21	0.95	15.35–19.07	18.00	0.73	16.57–19.43		
Strong positive	160	6.94	0.30	6.34–7.53	7.00	0.20	6.61–7.39		

Bold values are highly significant.

of overall survival with primary site, radiological response and p53 expression indicating these as significant and an independent predictors of overall survival in OSCC patients.

3.6. Predictors of recurrence

The association of recurrence with clinico-pathological characteristics and marker expressions was done using univariate (unadjusted) and multivariate (adjusted) logistic regression analysis and summarized in Table 11. The univariate logistic regression analysis showed significant association ($P < 0.05$ or $P < 0.01$ or $P < 0.001$) of primary site, differentiation, node status, response and marker expressions (Cyclin D1, EGFR and p53) with the recurrence. The multivariate logistic regression analysis further showed significant ($P < 0.05$ or $P < 0.01$ or $P < 0.001$) association of recurrence with primary site, differentiation, Cyclin D1 and p53 expressions indicating these significant and an independent risk predictors of recurrence in OSCC patients. Further, the univariate Cox regression analysis showed significant ($P < 0.05$ or $P < 0.01$ or $P < 0.001$) association of recurrence with economic status, histology, histological grade, tumor size, node status, stage and marker expressions (Cyclin D1, EGFR and p53) suggesting these as predictors of recurrence (Table 12). The multivariate Cox regression analysis further showed significant ($P < 0.05$ or $P < 0.01$) association of recurrence with node status,

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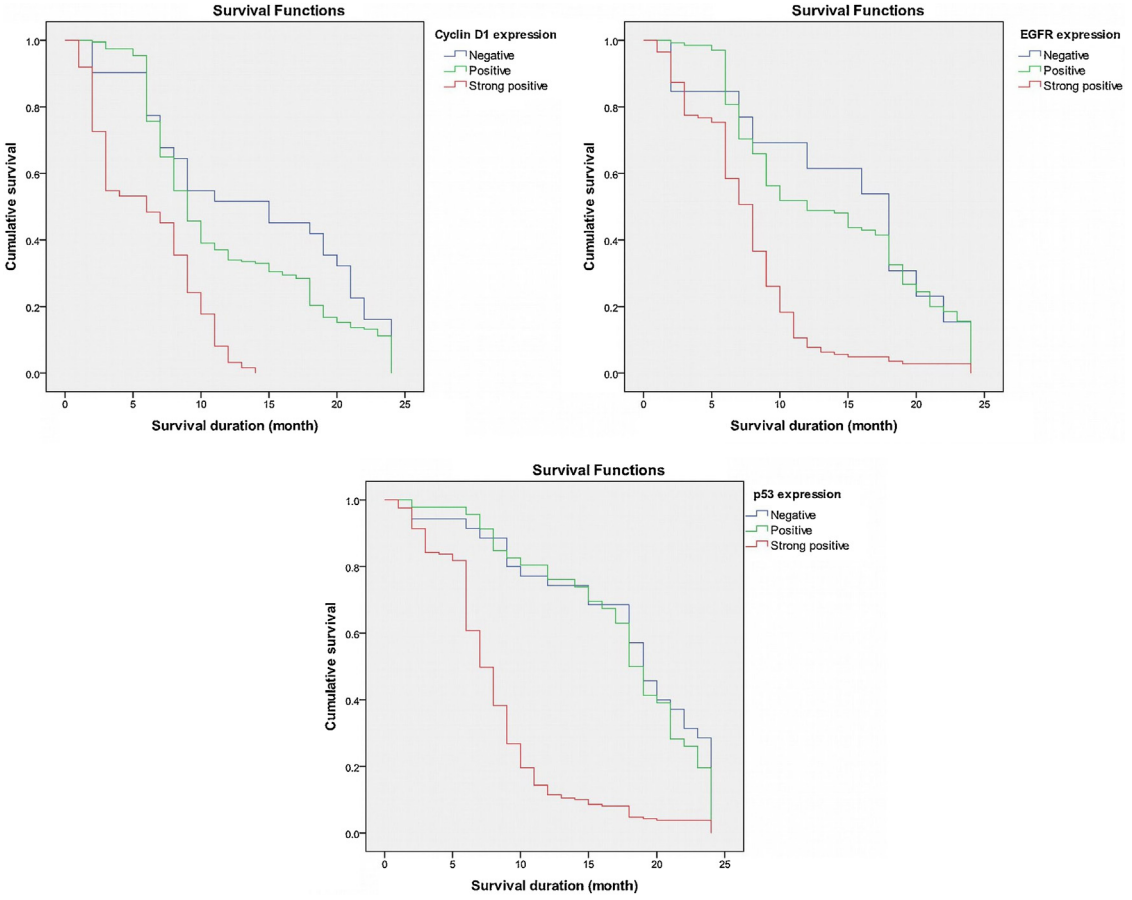

Fig. 2. Overall survival according to marker expressions were compared by Kaplan-Meier method followed by Log rank (Mantel-Cox: χ^2) test. Marker expressions showed significant association with overall survival in OSCC patients (Cyclin D1: $\chi^2 = 49.80$, df = 2, $P<0.001$, EGFR: $\chi^2 = 59.95$, df = 2, $P < 0.001$, and p53: $\chi^2 = 84.84$, df = 2, $P < 0.001$).

Cyclin D1 and p53 expressions indicating these as significant and an independent predictors of recurrence in OSCC patients undergoing chemoradiation (Table 12).

3.7. Survival of recurrent patients

The time to recurrence survival of recurrent patients according to site and type of recurrence were also evaluated and summarized in Table 13 and Fig. 5. There was no significant difference in the median survival in patients according to site of recurrence ($\chi^2 = 7.69$, $P = 0.104$). In contrast, the median survival in patients with distant recurrence was found significantly lower as compared to locoregional ($\chi^2 = 4.86$, $P = 0.027$).

4. Discussion

Squamous cell carcinoma is by far the most common cancer type of the oral cavity, representing more than 90% of all oral cancer [22]. The overall survival rates have

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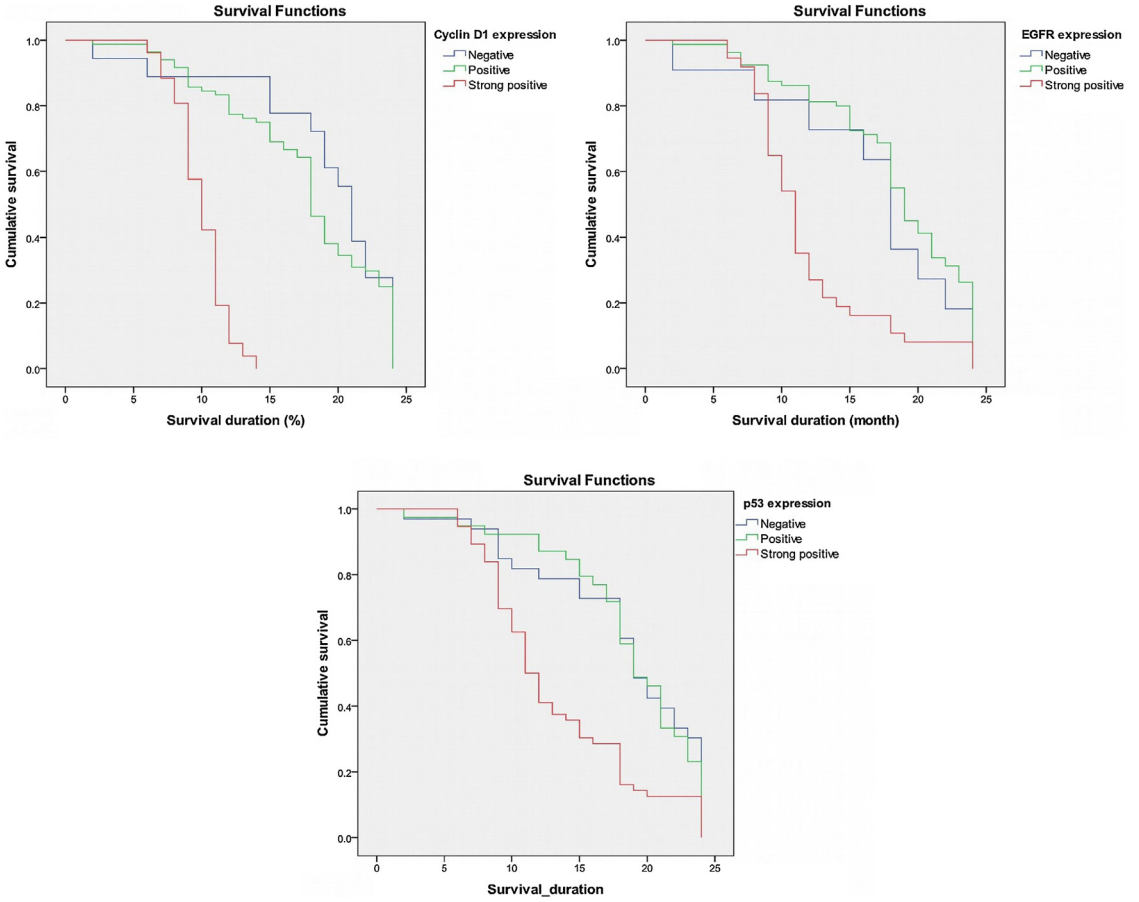

Fig. 3. Disease free survival according to marker expressions were compared by Kaplan-Meier method followed by Log rank (Mantel-Cox: χ^2) test. Marker expressions showed significant association with overall survival in OSCC patients (Cyclin D1: $\chi^2 = 68.00$, df = 2, $P < 0.001$, EGFR: $\chi^2 = 29.50$, df = 2, $P < 0.001$, and p53: $\chi^2 = 19.69$, df = 2, $P < 0.001$).

not improved for more than decades even in major treatment centers inspite of all possible definitive treatment approaches; dreadful and alarming event of locoregional relapse is customary and elucidates for major cause of morbidity and mortality in these patients. This has engendered and entrenched notion to identify molecular biomarkers that accurately predict patients at risk for disease recurrence and treatment resistance. Furthermore identification of biomarkers that would signal increased risk of treatment failure in OSCC would have a major impact on treatment decisions and its outcome [23]. Despite of tremendous search for prognostic markers, no single molecular study till date has been shown to identify and focus at high risk for recurrences which is the most distressing component of treatment failure in the patients of OSCC [12]. OSCC pathogenesis is a complex process and thought to arise as a result of multiple molecular events developing from the combined effect of an individual's genetic predisposition and exposure to environmental carcinogens. These genetic alterations includes

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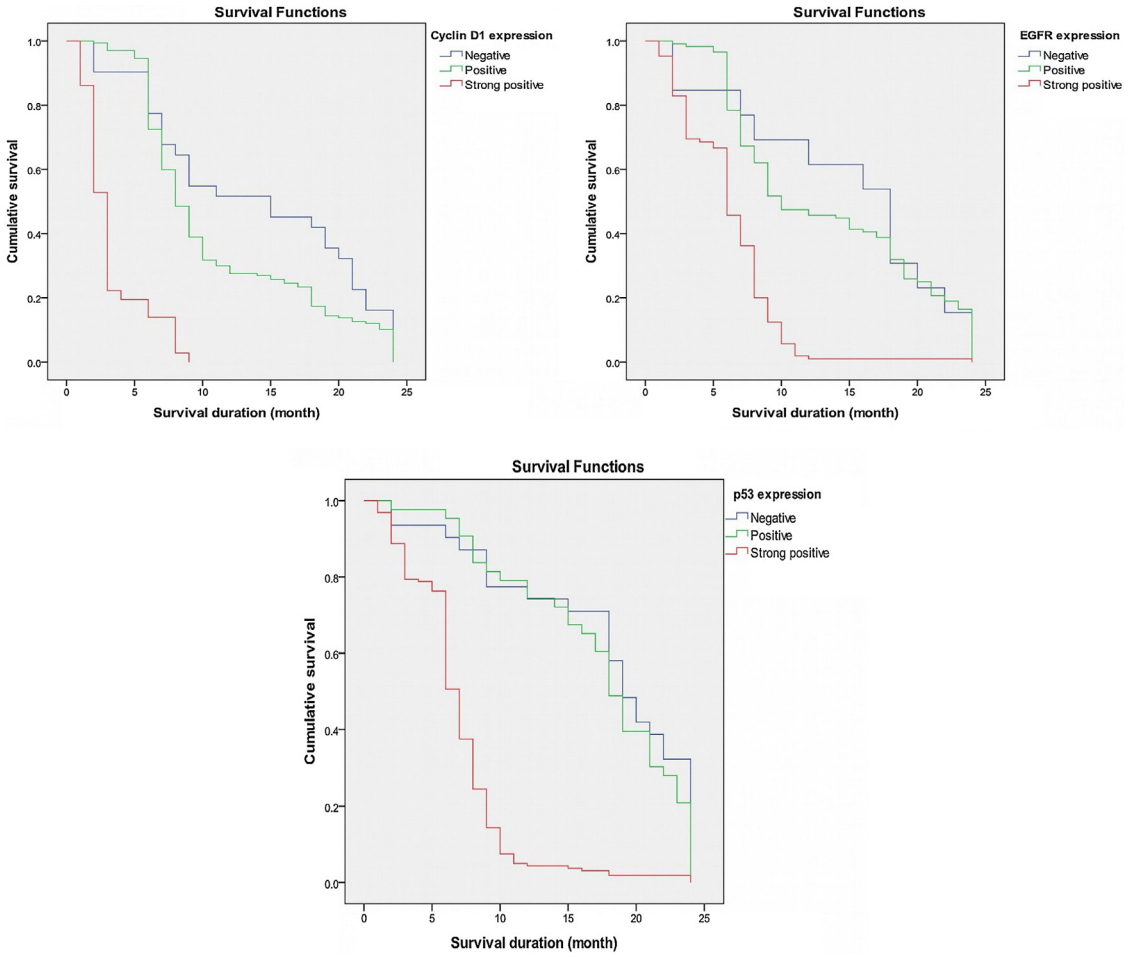

Fig. 4. Recurrence free survival according to marker expressions were compared by Kaplan-Meier method followed by Log rank (Mantel-Cox: χ^2) test. Marker expressions showed significant association with overall survival in OSCC patients (Cyclin D1: $\chi^2 = 124.84$, df = 2, $P < 0.001$, EGFR: $\chi^2 = 77.04$, df = 2, $P < 0.001$, and p53: $\chi^2 = 106.33$, df = 2, $P < 0.001$).

mutations or amplification of oncogenes as well as inactivation of tumor suppressor genes evident by expression of various molecular markers, leading to tumorogenesis which acquire self sufficient unexceptional growth and escape growth inhibitory signals, making the cells immortal resulting in uncontrolled tumor growth [23]. Over a period of time and with further exposure to carcinogens, the cancer cells accumulate further mutations and acquire more evil characteristics such as ability to invade and move into adjoining tissues, progress to lymphatics and blood vessels, tumor angiogenesis and may reside and grow to distant sites [24].

While on conventional treatment regimen or in the post treatment follow up period some cancer cells due to genetic instability may acquire further mutations and become resistant or refractory to the treatment delivered, leading to progression or

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Table 10. Predictors of overall survival of OSCC patients using Cox-regression analysis (n = 290).

Predictors	Univariate		Multivariate	
	OR (95% CI)	*P* value	OR (95% CI)	*P* value
Age (yrs)				
≤60	Ref		Ref	
>60	1.22 (0.94–1.60)	0.142	1.01 (0.74–1.36)	0.972
Sex				
Female	Ref		Ref	
Male	0.96 (0.72–1.28)	0.793	1.09 (0.75–1.60)	0.657
Economic status				
Upper/Middle	Ref		Ref	
Poor	0.91 (0.72–1.15)	0.409	1.19 (0.92–1.54)	0.194
Tobacco chewing				
No	Ref		Ref	
Yes	1.26 (0.99–1.61)	0.065	0.92 (0.59–1.43)	0.701
Betel nut chewing				
No	Ref		Ref	
Yes	1.24 (0.97–1.57)	0.085	1.22 (0.75–1.97)	0.426
Smoking				
No	Ref		Ref	
Yes	0.96 (0.76–1.21)	0.724	0.93 (0.65–1.32)	0.668
Performance status				
Good	Ref		Ref	
Poor	0.75 (0.60–0.95)	**0.017**	0.87 (0.67–1.12)	0.273
Primary site				
Buccal mucosa	Ref		Ref	
Other	1.59 (1.23–2.04)	<**0.001**	1.33 (1.01–1.75)	**0.044**
Histology				
SCC	Ref		Ref	
ISCC	0.82 (0.64–1.05)	0.123	0.90 (0.69–1.17)	0.437
Grade				
WD	Ref		Ref	
MD/PD	0.62 (0.48–0.80)	<**0.001**	1.15 (0.82–1.62)	0.415

(*Continued*)

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Table 10. (*Continued*)

Predictors	Univariate		Multivariate	
	OR (95% CI)	*P* value	OR (95% CI)	*P* value
Tumor size				
T2/T3	Ref		Ref	
T4	0.75 (0.57–0.98)	**0.037**	0.67 (0.31–1.43)	0.300
Node status				
N0	Ref		Ref	
N1/N2	0.62 (0.48–0.80)	<**0.001**	0.94 (0.69–1.29)	0.717
Stage				
III	Ref		Ref	
IV	0.68 (0.51–0.90)	**0.007**	1.48 (0.66–3.32)	0.340
Response				
CR/PR	Ref		Ref	
NR	0.02 (0.01–0.04)	<**0.001**	0.03 (0.01–0.07)	<**0.001**
Cyclin D1				
Negative	Ref		Ref	
Positive	0.31 (0.20–0.49)	<**0.001**	0.93 (0.49–1.76)	0.819
Strong positive	0.40 (0.29–0.54)	<**0.001**	0.86 (0.54–1.38)	0.536
EGFR				
Negative	Ref		Ref	
Positive	0.40 (0.22–0.70)	**0.002**	0.96 (0.49–1.87)	0.894
Strong positive	0.43 (0.33–0.55)	<**0.001**	0.86 (0.61–1.21)	0.384
p53				
Negative	Ref		Ref	
Positive	0.32 (0.22–0.46)	<**0.001**	0.36 (0.21–0.60)	<**0.001**
Strong positive	0.34 (0.25–0.48)	<**0.001**	0.39 (0.25–0.61)	<**0.001**

Bold values are highly significant.

recurrence of cancer [24, 25]. Moreover cytotoxic treatment regimen which itself is immunosuppressive and generates of lot of stress from cradle to grave might also enhance the odds of risk of tumor recurrence due to ineffective immunosuppressive treatment which is only enabling the immune system of the body to identify and destroy the newly formed tumor cells, when they are few in number [26]. Hence high mortality rate due to treatment resistance, tumor recurrence and aggressive disease course are the considerable frightening challenges mainly in the storyline of scarcity of resources available with the patients. So at a very critical moment molecular markers had actually mesmerized into conclusion of having significant

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Table 11. Association of recurrence with clinico-pathological characteristics in OSCC patients using logistic regression analysis (n = 290).

Predictors	Recurrence		Univariate		Multivariate	
	No (n = 234) (%)	Yes (n = 56) (%)	OR (95% CI)	*P* value	OR (95% CI)	*P* value
Age (yrs)						
≤60	176 (75.2)	44 (78.6)	Ref		Ref	
>60	58 (24.8)	12 (21.4)	1.21 (0.60–2.44)	0.598	0.95 (0.40–2.25)	0.899
Sex						
Female	45 (19.2)	14 (25.0)	Ref		Ref	
Male	189 (80.8)	42 (75.0)	1.40 (0.71–2.78)	0.337	2.18 (0.68–7.02)	0.191
Economic status						
Upper/Middle	97 (41.5)	21 (37.5)	Ref		Ref	
Poor	137 (58.5)	35 (62.5)	0.85 (0.47–1.55)	0.589	0.59 (0.28–1.23)	0.159
Tobacco chewing						
No	76 (32.5)	22 (39.3)	Ref		Ref	
Yes	158 (67.5)	34 (60.7)	1.35 (0.74–2.46)	0.334	0.66 (0.10–4.44)	0.673
Betel nut chewing						
No	85 (36.3)	24 (42.9)	Ref		Ref	
Yes	149 (63.7)	32 (57.1)	1.32 (0.73–2.38)	0.365	1.26 (0.18–8.94)	0.817
Smoking						
No	101 (43.2)	27 (48.2)	Ref		Ref	
Yes	133 (56.8)	29 (51.8)	1.23 (0.68–2.20)	0.494	1.29 (0.46–3.62)	0.629
Performance status						
Good	136 (58.1)	29 (51.8)	Ref		Ref	
Poor	98 (41.9)	27 (48.2)	0.77 (0.43–1.39)	0.391	1.51 (0.74–3.11)	0.262
Primary site						
Buccal mucosa	83 (35.5)	10 (17.9)	Ref		Ref	
Other	151 (64.5)	46 (82.1)	0.40 (0.19–0.82)	**0.013**	0.21 (0.09–0.54)	**0.001**
Histology						
SCC	84 (35.9)	18 (32.1)	Ref		Ref	
ISCC	150 (64.1)	38 (67.9)	0.85 (0.45–1.57)	0.597	1.31 (0.58–2.95)	0.523
Grade						
WD	178 (76.1)	17 (30.4)	Ref		Ref	
MD/PD	56 (23.9)	39 (69.6)	0.14 (0.07–0.26)	<**0.001**	0.15 (0.06–0.37)	<**0.001**

(*Continued*)

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Table 11. (*Continued*)

Predictors	Recurrence		Univariate		Multivariate	
	No (n = 234) (%)	Yes (n = 56) (%)	OR (95% CI)	*P* value	OR (95% CI)	*P* value
Tumor size						
T2/T3	61 (26.1)	10 (17.9)	Ref		Ref	
T4	173 (73.9)	46 (82.1)	0.62 (0.29–1.30)	0.202	3.01 (0.38–24.08)	0.300
Node status						
N0	83 (35.5)	6 (10.7)	Ref		Ref	
N1/N2	151 (64.5)	50 (89.3)	0.22 (0.09–0.53)	**0.001**	0.47 (0.17–1.32)	0.152
Stage						
III	55 (23.5)	7 (12.5)	Ref		Ref	
IV	179 (76.5)	49 (87.5)	0.47 (0.20–1.09)	0.077	0.30 (0.03–2.72)	0.287
Cyclin D1 expression						
Negative	31 (13.2)	0 (0.0)	Ref		Ref	
Positive	167 (71.4)	30 (53.6)	NA	–	NA	–
Strong positive	36 (15.4)	26 (46.4)	0.25 (0.13–0.47)	<**0.001**	0.34 (0.26–1.57)	**0.033**
EGFR expression						
Negative	13 (5.6)	0 (0.0)	Ref		Ref	
Positive	116 (49.6)	19 (33.9)	NA	–	NA	–
Strong positive	105 (44.9)	37 (66.1)	0.47 (0.25–0.86)	**0.014**	0.76 (0.30–1.94)	**0.567**
p53 expression						
Negative	31 (13.2)	4 (7.1)	Ref		Ref	
Positive	43 (18.4)	3 (5.4)	0.42 (0.14–1.25)	0.120	1.89 (0.39–9.20)	0.430
Strong positive	160 (68.4)	49 (87.5)	0.23 (0.07–0.77)	**0.007**	0.29 (0.15–3.71)	**0.019**

NA: not applicable.

Bold values are highly significant.

role in prognosis, prediction of disease course and selection of treatment regimen. Although many molecular markers and tumor markers have been studied and have revolutionized the pathogenesis of head and neck squamous cell carcinoma (HNSCC) still they are not yet ready to be used in routine clinical, investigative and therapeutic procedures in patients with these tumors. Preponderantly tumor stage, patient's age and performance status still remains the basis for therapeutic decisions [22, 23, 27, 28]. These all have attributed to the urge of advancement and innovations in research approaches of molecular biology, genomics and proteomics with a tremendous hope at the end of tunnel [28]. Improvement and development of research procedures would assist to understand more specific and sensitive

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Table 12. Predictors of recurrence of OSCC patients using Cox regression analysis (n = 56).

Predictors	Univariate		Multivariate	
	OR (95% CI)	*P* value	OR (95% CI)	*P* value
Age (yrs)				
≤60	Ref		Ref	
>60	1.01 (0.53–1.95)	0.968	0.95 (0.44–2.05)	0.903
Sex				
Female	Ref		Ref	
Male	0.65 (0.33–1.27)	0.204	0.82 (0.24–2.82)	0.758
Economic status				
Upper/Middle	Ref		Ref	
Poor	1.65 (0.94–2.88)	**0.040**	1.59 (0.71–3.57)	0.263
Tobacco chewing				
No	Ref		Ref	
Yes	0.82 (0.46–1.45)	0.488	1.11 (0.18–6.92)	0.912
Betel nut chewing				
No	Ref		Ref	
Yes	0.75 (0.43–1.31)	0.309	0.98 (0.13–7.59)	0.984
Smoking				
No	Ref		Ref	
Yes	0.74 (0.42–1.29)	0.287	1.15 (0.42–3.18)	0.787
Performance status				
Good	Ref		Ref	
Poor	0.66 (0.38–1.14)	0.131	0.86 (0.39–1.89)	0.705
Primary site				
Buccal mucosa	Ref		Ref	
Other	1.29 (0.64–2.59)	0.475	0.83 (0.33–2.07)	0.689
Histology				
SCC	Ref		Ref	
ISCC	0.53 (0.29–0.95)	**0.033**	0.54 (0.23–1.27)	0.159
Grade				
WD	Ref		Ref	
MD/PD	0.43 (0.23–0.81)	**0.009**	1.61 (0.54–4.83)	0.392

(*Continued*)

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Table 12. (*Continued*)

Predictors	Univariate		Multivariate	
	OR (95% CI)	*P* value	OR (95% CI)	*P* value
Tumor size				
T2/T3	Ref		Ref	
T4	0.42 (0.19–0.94)	**0.035**	1.18 (0.25–5.61)	0.836
Node status				
N0	Ref		Ref	
N1/N2	0.16 (0.05–1.09)	<**0.001**	0.06 (0.02–3.67)	**0.003**
Stage:				
III	Ref		Ref	
IV	0.24 (0.09–0.68)	**0.007**	1.27 (0.14–11.62)	0.835
Cyclin D1 expression				
Negative/Positive	Ref		Ref	
Strong positive	0.41 (0.23–0.73)	**0.002**	0.34 (0.12–0.99)	**0.048**
EGFR expression				
Negative/Positive	Ref		Ref	
Strong positive	0.42 (0.23–0.77)	**0.005**	1.31 (0.50–3.45)	0.586
p53 expression				
Negative/Positive	Ref		Ref	
Strong positive	0.16 (0.06–0.47)	**0.001**	0.09 (0.02–0.49)	**0.006**

Bold values are highly significant.

markers aiding in tumor diagnosis, selection of treatment modality, monitoring of response to therapeutic interventions, early detection of tumor recurrence, prediction of the treatment outcome and identification of subsets of patients with unfavorable outcome during the therapeutic interventions and follow-up period, thereby facilitating to achieve beneficial outcome in terms of response and survival in the patients with these tumors.

The current study has hence being proposed to evaluate expressions and correlation of Cyclin D1, EGFR and p53 with stratification of recurrences in oral cancer patients. Plethora of studies suggesting prognostics of expression of Cyclin D1, EGFR and p53 have been documented in malignancies of head and neck, breast, lung, colorectal, bladder and Gastrointestinal tract, however there has been limited convincing studies on evaluation of risk and pattern of recurrences in oral cancer, wherein timeline evaluation, stratification of the risk and pattern of recurrence is warranted to achieve the substantial and sustainable benefit of delivered treatment [29, 30]. Although some of the studies have documented that p53 mutation can

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Table 13. Time to recurrence survival of OSCC patients according to site and type of recurrence using Kaplan-Meier survival analysis (n = 234).

Survival-Recurrent patients	n	Mean			Median			χ^2 value	p value
		Estimate	SE	95% CI	Estimate	SE	95% CI		
Site of recurrence									
Bone	3	7.33	0.67	6.03–8.64	8.00	0.00	NA	7.69	0.104
Brain	1	6.00	0.00	6.00–6.00	6.00	NA	NA		
Lung	5	5.20	1.07	3.11–7.29	5.00	1.10	2.85–7.15		
Nodal	31	8.58	0.76	7.09–10.07	7.00	0.80	5.44–8.56		
Primary	16	10.06	1.38	7.36–12.77	7.00	6.00	0.00–18.76		
Type of recurrence									
Locoregional	47	9.09	0.69	7.74–10.43	7.00	0.86	5.32–8.68	4.86	**0.027**
Distant	9	6.00	0.69	4.65–7.35	6.00	0.75	4.54–7.46		

NA: not applicable.

Bold values are highly significant.

estimates disease prognosis more accurately than clinical staging in HNSCC, and could stratify patients according to their risk of locoregional recurrence but have not reached to a significant conclusion [22, 29]. A prospective study in Chinese patient with laryngeal squamous cell carcinoma concluded that p53, p21 and Cdc2 may be involved in the occurrence, development and recurrence of tumor. It also

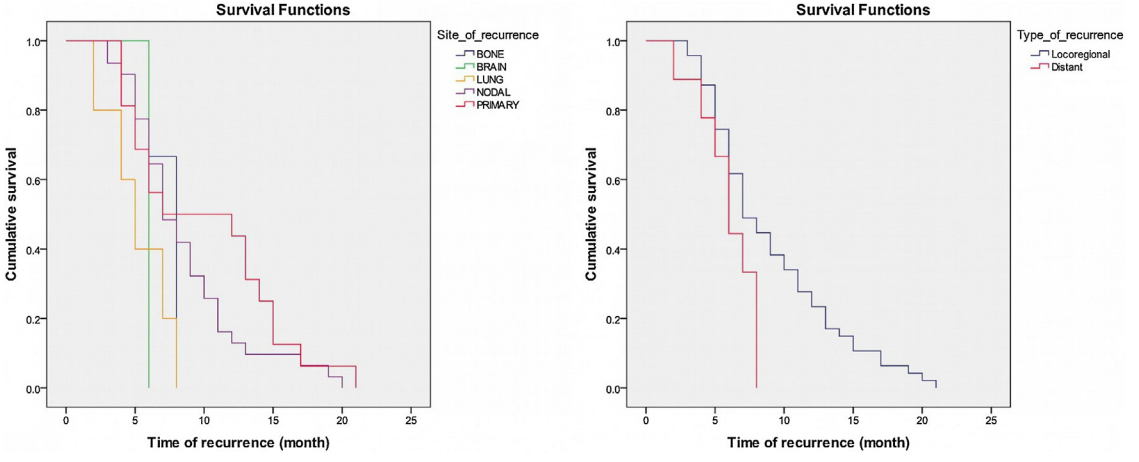

Fig. 5. Time to recurrence survival according to site and type of recurrence were compared by Kaplan-Meier method followed by Log rank (Mantel-Cox: χ^2) test. Site ($\chi^2 = 7.69$, df = 4, $P = 0.104$) showed insignificant association while type of recurrence ($\chi^2 = 4.86$, df = 1, $P = 0.027$) showed significant association with time to recurrence survival in OSCC patients undergoing chemoradiation.

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suggested that combined detection of these three proteins might be a useful parameter for screening patients at high risk of recurrence which is worthy of further molecular studies [28]. In our study also we found that patients with strong positive p53 expression were associated with early recurrence as compared to moderately positive or negative expression. Some more studies have documented that overexpression of p53 is a potential marker for recurrence of tumor although been proved in various other types of malignancies. In ER-positive breast cancer, p53 accumulation was found to be a strong predictor of both early and late recurrence [31]. Studies have suggested association of p53 overexpression with high risk for disease recurrence and poor survival in colorectal carcinoma and urothelal cancer [32, 33]. p53 overexpression, has also been found to be a good prognostic marker to predict the risk of local recurrence and distant metastasis in bone tumor [23, 32, 34]. p53 gene mutation frequently occurs in recurrent ovarian cancer and influences salvage chemotherapy thereby impacting upon the prognosis of recurrent disease [35]. Studies have cited correlation of EGFR expression with recurrence in rectal and penile cancer but with a limitation of long survival period [36, 37]. In advanced head and neck cancer studies have suggested EGFR as robust predictor of local relapse but failed to predict correlation with distant metastasis [11]. In the present study we have observed that overexpression of EGFR was related to early recurrence and to a greater extent to distant sites in OSCC patients treated by chemoradiation. Therefore understanding of EGFR expression has a significant implication and can contribute to the identification of patients with an increased risk of recurrences and in targeting treatment for better outcome. Cyclin D1 genetic alteration was found to be an early event in the carcinogenesis of transitional cell carcinoma of bladder and resulted in the rapid recurrence of a subset of superficial bladder cancer [38]. In esophageal squamous cell carcinoma, there was increased early recurrence of disease in Cyclin D1 overexpressed patients [39]. Furthermore, Cyclin D1 overexpression has also been found to be associated with tumor aggression, reduced survival and tumor recurrence in HNSCC although mostly studied in different anatomical sites [12]. In nasopharyngeal carcinoma, early local recurrence rate were significantly higher in patients with high levels of Cyclin D1 evaluated before initiation of radiation therapy when compared with patients with low or no expression [40]. In fact there are very few convincing studies documented correlating Cyclin D1 expression with the pattern of recurrence in oral cancer patients [41]. In our study we speculated that patients with overexpression of Cyclin D1 were significantly associated with increased recurrence. Patients with higher expression had more propensities to recur in distant sites along with early time to recurrence as compared to positive/ negative expression.

Therefore in view of immense bulk of oral cancer patients in productive years of life and excessively with greater tendency to recur, it would be beneficial to

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evaluate correlation of these markers with risk and pattern of recurrence so as to select the patients and customize the therapeutic intervention to maximize its benefit in oral cancer. Here we conclude that over expression of Cyclin D1, EGFR and p53 are associated with tumor recurrence, reduced time to recurrence at primary and distant sites. As evident in our study tumors over expressing Cyclin D1, EGFR and p53 are resistant to chemoradiation and expression of these markers might be an indicator of the risk of locoregional recurrence and metastasis in patients of OSCC treated by chemoradiation. Hence, comprehensive and collaborative interpretation of expression of Cyclin D1, EGFR and p53 would contribute to the identification of patients at increased risk of tumor recurrence and further these patients might be benefited from more intensive and targeted treatment regimen. The findings of the present study may be further validated on larger sample size, co expressions of the markers and by evaluation and exploration of respective gene locus and genetic stability which was the limitation of the study.

Declarations

Author contribution statement

Seema Gupta: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Vandana Singh Kushwaha: Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data.

Sandeep Verma: Conceived and designed the experiments; Performed the experiments; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Huma Khan: Contributed reagents, materials, analysis tools or data.

M.L.B. Bhatt: Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Nuzhat Husain: Conceived and designed the experiments; Contributed reagents, materials, analysis tools or data.

Mahendra Pal Singh Negi: Analyzed and interpreted the data.

Vivek Vidyadhar Bhosale: Conceived and designed the experiments; Performed the experiments; Wrote the paper.

Ashim Ghatak: Conceived and designed the experiments; Performed the experiments.

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Competing interest statement

The authors declare no conflict of interest.

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Additional information

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References

[1] J. Ferlay, I. Soerjomataram, R. Dikshit, S. Eser, C. Mathers, M. Rebelo, et al., Cancer incidence and mortality worldwide: sources, methods and major patterns in GLOBOCAN 2012, Int. J. Cancer 136 (5) (2015) E359–86.

[2] D.M. Parkin, F. Bray, J. Ferlay, P. Pisani, Global cancer statistics, 2002, CA Cancer J. Clin. 55 (2005) 74–108.

[3] J.S. Cooper, T.F. Pajak, A.A. Forastiere, J. Jacobs, B.H. Campbell, S.B. Saxman, Postoperative concurrent radiotherapy and chemotherapy for high-risk squamous-cell carcinoma of the head and neck, N. Engl. J. Med. 350 (19) (2004) 1937–1944.

[4] M. Al-Sarraf, Treatment of locally advanced head and neck cancer: historical and critical review, Cancer Control 9 (2002) 387–399.

[5] A. Capote, V. Escorial, M.F. Muñoz-Guerra, F.J. Rodríguez-Campo, C. Gamallo, L. Naval, Elective neck dissection in early-stage oral squamous cell carcinoma-does it influence recurrence and survival? Head Neck 29 (2007) 3–11.

[6] V.S. Kushwaha, S. Gupta, N. Husain, et al., Gefitinib, methotrexate and methotrexate plus 5-fluorouracil as palliative treatment in recurrent head and neck squamous cell carcinoma, Cancer Biol. Ther. 16 (2) (2015) 346–351.

[7] K.A. Gold, E.S. Kim, Role of molecular markers and gene profiling in head and neck cancer, Curr. Opin. Oncol. 21 (2009) 206–211.

[8] S. Koontongkaew, The tumor microenvironment contribution to development, growth invasion and metastasis of head and neck squamous cell carcinomas, J. Cancer 4 (2013) 66–83.

 http://dx.doi.org/10.1016/j.heliyon.2016.e00206

[9] B. Kumar, K.G. Cordell, J.S. Lee, et al., EGFR, p16, HPV titer, Bcl-xL and p53, sex, and smoking as indicators of response to therapy and survival in oropharyngeal cancer, J. Clin. Oncol. 26 (2008) 3128–3137.

[10] M. Shiraki, T. Odajima, T. Ikeda, et al., Combined expression of p53, cyclin D1 and epidermal growth factor receptor improves estimation of prognosis in curatively resected oral cancer, Mod. Pathol. 18 (2005) 1482–1489.

[11] K.K. Ang, B.A. Berkey, X. Tu, et al., Impact of epidermal growth factor receptor expression on survival and pattern of relapse in patients with advanced head and neck carcinoma, Cancer Res. 62 (2002) 7350–7356.

[12] R. Michalides, N. van Veelen, A. Hart, B. Loftus, E. Wientjens, A. Balm, Overexpression of cyclin D1 correlates with recurrence in a group of forty-seven operable squamous cell carcinomas of the head and neck, Cancer Res. 55 (5) (1995) 975–978.

[13] I. Sestak, J. Cuzick, Markers for the identification of late breast cancer recurrence, Breast Cancer Res. 17 (2015) 10.

[14] M.V. Brock, C.M. Hooker, E. Ota-Machida, et al., DNA methylation markers and early recurrence in stage I lung cancer, N. Engl. J. Med. 358 (11) (2008) 1118–1128.

[15] Y. Wang, T. Jatkoe, Y. Zhang, et al., Gene expression profiles and molecular markers to predict recurrence of Dukes' B colon cancer, J. Clin. Oncol. 22 (9) (2004) 1564–1571.

[16] Y. Lotan, A. Bagrodia, N. Passoni, et al: Prospective evaluation of a molecular marker panel for prediction of recurrence and cancer-specific survival after radical cystectomy, Eur. Urol. 64 (3) (2013) 465–471.

[17] J.X. Jing, Y. Wang, X.Q. Xu, et al Tumor markers for diagnosis, monitoring of recurrence and prognosis in patients with upper gastrointestinal tract cancer, Asian Pac. J. Cancer Prev. 15 (23) (2014) 10267–10272.

[18] M. Shiraki, T. Odajima, T. Ikeda, et al., Combined expression of p53, cyclin D1 and epidermal growth factor receptor improves estimation of prognosis in curatively resected oral cancer, Mod. Pathol. 18 (11) (2005) 1482–1489.

[19] V. Pesutić-Pisac, A. Punda, I. Gluncić, V. Bedeković, A. Pranić-Kragić, N. Kunac, Cyclin D1 and P27 expression as prognostic factor in papillary carcinoma of the thyroid: association with clinicopathological parameters, Croat. Med. J. 49 (5) (2008) 643–649.

 http://dx.doi.org/10.1016/j.heliyon.2016.e00206

[20] I. Cruz, S.S. Napier, I. van der Waal, et al., Suprabasal p53 Immunoexpression is strongly assicated with high grade dysplasia and risk for maliganant transformation in potentioally malignant oral lesion Nothern Ireland, J. Clin. Pathol. 55 (2) (2002) 98–104.

[21] G.W. Snedecor, W.G. Cochran, Statistical Methods, 8th Ed., Iowa State Press, Ames, 1989.

[22] S. Gupta, H. Khan, V.S. Kushwaha, et al., Impact of EGFR and p53 expressions on survival and quality of life in locally advanced oral squamous cell carcinoma patients treated with chemoradiation, Cancer Biol. Ther. 16 (9) (2015) 1269–1280.

[23] N.M. Hassan, M. Tada, J. Hamada, et al., Presence of dominant negative mutation of TP53 is a risk of early recurrence in oral cancer, Cancer Lett. 270 (1) (2008) 108–119.

[24] A. Marusyk, K. Polyak, Tumor heterogeneity: causes and consequences, Biochim. Biophys. Acta 1805 (1) (2010) 105–117.

[25] M. Baumann, M. Krause, R. Hill, Exploring the role of cancer stem cells in radioresistance, Nat. Rev. Cancer 8 (7) (2008) 545–554.

[26] B.J. Braakhuis, R.H. Brakenhoff, C.R. Leemans, Treatment choice for locally advanced head and neck cancers on the basis of risk factors: biological risk factors, Ann. Oncol. 23 (Suppl. 10) (2012) x173–7.

[27] H. Khan, S. Gupta, N. Husain, et al., Correlation between expressions of Cyclin D1, EGFR and p53 with chemoradiation response in patients of locally advanced oral squamous cell carcinoma, BBA Clin. 3 (2014) 11–17.

[28] J.Q. Yang, H.X. Liu, Z. Liang, Y.M. Sun, M. Wu, Over-expression of p53, p21 and Cdc2 in histologically negative surgical margins is correlated with local recurrence of laryngeal squamous cell carcinoma, Int. J. Clin. Exp. Pathol. 7 (7) (2014) 4295–4302.

[29] M.M. Jalali, A. Heidarzadeh, M.J. Zavarei, Sarmast H.p53 overexpression impacts on the prognosis of laryngeal squamous cell carcinomas, Asian Pac. J. Cancer Prev. 12 (7) (2011) 1731–1734.

[30] H. Yasuda, K. Tanaka, S. Saigusa, et al., Elevated CD133, but not VEGF or EGFR, as a predictive marker of distant recurrence after preoperative chemoradiotherapy in rectal cancer, Oncol. Rep. 22 (4) (2009) 709–717.

[31] M. Payandeh, M. Sadeghi, E. Sadeghi, Differences in Prognostic factors between early and late recurrence breast cancers, Asian Pac. J. Cancer Prev. 16 (15) (2015) 6575–6579.

 http://dx.doi.org/10.1016/j.heliyon.2016.e00206

[32] Y. Wang, T. Jatkoe, Y. Zhang, et al., Gene expression profiles and molecular markers to predict recurrence of Dukes' B colon cancer, J. Clin. Oncol. 22 (9) (2004) 1564–1571.

[33] K. Kim, C.O. Sung, B.H. Park, et al., Immunoprofile-based subgrouping of urothelial bladder carcinomas for survival prediction, Hum. Pathol. 46 (10) (2015) 1464–1470.

[34] F.W. Ismail, A.M. Shamsudin, Z. Wan, S.M. Daud, M.S. Samarendra, Ki-67 immuno-histochemistry index in stage III giant cell tumor of the bone, J. Exp. Clin. Cancer Res. 29 (2010) 25.

[35] T. Irie, J. Kigawa, Y. Minagawa, et al., Alteration of a p53 gene status affects outcome of patients with recurrent ovarian cancer, Oncology 58 (3) (2000) 237–241.

[36] G. Di Lorenzo, S. Perdonà, C. Buonerba, et al., Cytosolic phosphorylated EGFR is predictive of recurrence in early stage penile cancer patients: a retropective study, J. Transl. Med. 11 (2013) 161.

[37] D. Azria, F. Bibeau, N. Barbier, et al., Prognostic impact of epidermal growth factor receptor (EGFR) expression on loco-regional recurrence after preoperative radiotherapy in rectal cancer, BMC Cancer 5 (2005) 62.

[38] K.Y. Shin, G. Kong, W.S. Kim, T.Y. Lee, Y.N. Woo, J.D. Lee, Overexpression of cyclin D1 correlates with early recurrence in superficial bladder cancers, Br. J. Cancer 75 (12) (1997) 1788–1792.

[39] R. Mathew, S. Arora, R. Khanna, N.K. Shukla, M. Mathur, R. Ralhan, Alterations in cyclin D1 expression in esophageal squamous cell carcinoma in the Indian population, J. Cancer Res. Clin. Oncol. 127 (4) (2001) 251–257.

[40] J.P. Lai, C.L. Tong, C. Hong, et al., Association between high initial tissue levels of cyclin d1 and recurrence of nasopharyngeal carcinoma, Laryngoscope 112 (2) (2002) 402–408.

[41] R.J. Bova, D.I. Quinn, J.S. Nankervis, I.E. Cole, B.F. Sheridan, M.J. Jensen, et al., Cyclin D1 and p16INK4A expression predict reduced survival in carcinoma of the anterior tongue, Clin Cancer Res. 5 (10) (1999) 2810–2819.

 http://dx.doi.org/10.1016/j.heliyon.2016.e00206