

Original Research Article

Expression of proliferation markers in laryngeal squamous cell carcinoma: an association with clinico-pathological factors and treatment outcomes

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ABSTRACT

Background: The study was done with the objective to study the expression of epidermal growth factor receptor (EGFR), cyclin D1 and Ki-67 in laryngeal squamous cell carcinoma and to assess the correlation of all three proliferation markers with various clinic-pathological parameters, the treatment outcomes as well as survival.

Methods: We prospectively evaluated the surgical specimens of 72 laryngeal squamous cell carcinoma (LSCC) patients, treated with primary surgery and post-operative adjuvant therapy. Tumor tissue samples were analysed for the expression of EGFR, cyclin D1 and Ki-67 markers and analysis were done by immune-histochemistry and western blot test.

Results: EGFR showed significant expression in 67.6% and was insignificant in 31.9% patients in our analysis of 72 tumor samples. Cyclin D1 showed intense expression in 43%, and was insignificant in 57% patients. Ki-67 was intensely expressed in 43% patients. There was no correlation between expression of these markers with age, T-stage and N-stage. However, all the three markers showed significantly intense expression in tumours with extra capsular disease as well as perineural invasion (PNI) both of which are features of invasiveness of the tumor.

Conclusions: Estimation of biomarkers such as EGFR, cyclin D1, and Ki-67 could be beneficial in predicting tumor aggressiveness, prognosis and survival in LSCC patients. Thus, all the three proliferation markers can be categorized as markers of invasiveness. Combination of proliferation markers-EGFR, cyclin D1 and Ki-67 is useful pre-operatively in planning surgical strategies so as to decide a more radical approach for the resection of the primary as well as neck dissection.

Keywords: Cyclin D1, Epidermal growth factor receptor, Ki-67, Laryngeal squamous cell carcinoma, Proliferative markers

INTRODUCTION

Laryngeal squamous cell carcinoma (LSCC) is the most common malignant neoplasm of the upper aero-digestive tract in adults. LSCC accounts for about 1.9% of all cancers in males and 0.3% of all cancers in females, with

male to female ratio of 3.6:0.5.¹ More than 90% of laryngeal malignancies are squamous cell cancers and among these, more than 60 % tumours originate from glottis and supra-glottis while in less than 5% of cases originate from sub-glottis.¹

The increasing use of chemo and radiotherapy and conservative surgery to preserve organs and their functions has probably led to a better quality of life in patients with laryngeal cancer, but has definitely failed to improve survival, which consistently remain as the primary objective. These might be due to lack of prognostic stratification of LSCC patients as the combination of clinical and histological parameters has not been sufficiently reliable. Molecular characterization, by the study of molecular prognostic and predictive factors, is a more recent method of defining homogeneous sub-groups for clinical aggressiveness. In this context, tumor markers play a promising role.^{2,3}

Tumorigenesis in LSCC is multi-factorial and a multi-step process which involve number of tumor markers. A huge number of tumor markers have been studied individually and in combination for their role in both tumorigenesis and prognosis of LSCC.²⁻⁷ This includes various apoptotic markers, proliferative markers, inflammatory markers, viral markers, etc. Systematic study of biological markers can be integrated into clinical practice in the phases of prevention, diagnosis, prognostic assessment, treatment selection and synthesis of new drugs.

Here, in the current study the expression of three different proliferation markers {epidermal growth factor receptor (EGFR), Cyclin D1 and Ki-67} were studied in LSCC patients. These markers were selected based on commercial availability and encouraging literature. Numerous studies in the literature have stated the role of all these markers in tumorigenesis, diagnosis, prognosis as well as therapeutic management of the LSCC and other types of cancer.⁸⁻¹³

The present study was postulated to study the expression of three proliferation markers in LSCC. The study also aimed to establish the correlation of all three proliferation markers with various clinical and histo-pathological parameters, treatment outcomes as well as survival in patients with LSCC. It also aimed to identify the role of these markers with some of the known adverse prognostic factors in LSCC patients and also in prediction of poor prognostic groups that need additional treatment approaches.

METHODS

Patient selection

This was a prospective cohort study. Patients with laryngeal squamous cell carcinoma who were treated with surgery and postoperative adjuvant treatment in the period 2001 to 2011 in the department of surgical oncology were selected for the study. The surgical specimens of 72 patients with LSCC who were treated with primary surgery and post-operative adjuvant therapy were evaluated. The primary site of disease was identified at three different sub-sites (glottis, supra-glottis

and sub-glottis) in the larynx. Clinical staging was done prior to surgery based on AJCC classification.¹⁴ All patients with either T3 or T4 disease with/without cervical lymphadenopathy based on clinical, radiological and endoscopic evaluation were considered suitable to undergo primary surgery. Pathological staging of the disease based on the histo-pathological features of the resected specimen was done based on the above staging system and grouped accordingly for the study. All patients received radical post-operative radiation or concurrent chemo-radiation. All 72 patients completed the intended treatment and were followed up till December 2016.

Inclusion criteria

Patients with advanced laryngeal squamous cell carcinoma who underwent primary laryngectomy with post-operative adjuvant treatment were included.

Exclusion criteria

Patients with recurrent laryngeal squamous cell carcinoma and patients with early laryngeal carcinoma were excluded.

Sample collection

Tumor tissue samples were analyzed for the expression of proliferation markers (EGFR, cyclin D1 and Ki-67). The informed consent was obtained from all patients prior to study conduct. After surgery, tumor tissue from the surgical specimen was collected and tissue was snap frozen in liquid nitrogen until analysis.

Experimental methodology

Analysis of cellular markers such as EGFR, cyclin D1 and Ki-67 was done by immuno-histochemistry and western-blot test. Immuno-histochemistry of the tissue was carried out using the standard streptavidin-biotin method. In brief, tissue sections were treated with hydrogen peroxide to block endogenous peroxidase activity followed by treatment with bovine serum albumin to reduce non-specific binding. Antigen retrieval was done using microwave method. This was followed by incubation of the sections with specific primary antibodies in appropriate dilutions. The sections were then incubated with biotinylated secondary antibody followed by streptavidin-peroxidase conjugate. The final reaction product was visualized using diaminobenzidine. The sections were counterstained with hematoxylin and mounted in DPX mountant.

The intensity of staining of the markers was scored as 0 (<10%), 1+(10-25%), 2+(25-75%) and 3+(>75%) for no staining, mild, moderate and intense staining, respectively. To make the data more compact and homogenous, nil and mildly stained tumors were analyzed as one single category (mild/insignificant) and;

moderately and severely stained tumors were analyzed as another category (intense/significant).

Statistical analysis

Statistical analysis was done using SPSS statistical software. Frequency tables were tested for association using chi-square test. Differences between observed values and clinical parameters were done using analysis of variance (ANOVA). Linear and multiple regression analysis was followed by a discriminative and multivariate analysis. Actuarial survival curves along with Mantle-cox statistics was used to evaluate significance of clinical and survival difference.

Data were analyzed using STATA IC/11.2 software package. Chi squared test or exact test were used to test the association between the various clinicopathological variables and molecular marker expression. Logistic regression analysis was used to estimate the odds ratios and 95 % confidence intervals. Log rank test was used to

test the equality of survivor functions. The survival (%) was derived by using life table method. Log rank test is used to compare the survival experience between groups.

RESULTS

We evaluated specimens of total 72 patients with LSCC who underwent primary surgery and received post-operative adjuvant therapy. The expression of EGFR, cyclin D1 and Ki-67 was established in each patient. The correlation of all three proliferation markers was recognized with different clinico-pathological variables and also with survival rate. The expression of all three proliferation markers (EGFR, cyclin D1 and Ki-67) was significant in older patients with age >60 years compared to <60 years patients ($p=0.04$). Table 1, delineates the expression (mild or intense) of EGFR, cyclin D1 and Ki-67 and; their correlation with various clinic-pathological variables. Median follow-up duration for the whole included cohort was 2.54 years (follow-up ranges from 5 months to 72 months).

Table 1: Expression of EGFR, cyclin D1 and Ki-67 and their correlation with clinico-pathological variables.

Variables	Total	EGFR		P	Cyclin D1			P	Ki-67		
	N (%)	Mild	Intense		Mild	Intense	Mild		Intense	P	
	N (%)	N (%)	N (%)		N (%)	N (%)	N (%)		N (%)	N (%)	N (%)
Age group (in years)	72 (100)	23 (31.9)	49 (68.05)	--	41 (56.9)	31 (43)	--	41 (56.9)	31 (43)	--	
<60	43 (59.7)	17 (39.5)	26 (60.5)	0.09	25 (58.1)	18 (41.9)	0.803	27 (62.8)	14 (48.3)	0.222	
>60	29 (40.3)	6 (20.7)	23 (79.3)		16 (55.2)	13 (44.8)		16 (37.2)	15 (51.7)		
Sub-site											
Supra-glottis	19 (51.4)	9 (47.4)	10 (52.6)	0.09	7 (36.2)	12 (63.8)	0.52	8 (42.9)	11 (57.1)	0.92	
Glottis	47 (65.3)	14 (29.3)	33 (70.2)	0.59	21 (44.7)	26 (55.3)	0.74	21 (44.7)	26 (55.3)	0.703	
Sub-glottis	6 (15.3)	00	6 (100)	0.05	4 (66.7)	2 (33.3)	0.392	3 (50)	3 (50)	1	
T-stage											
T3-stage	29 (40.3)	8 (27.6)	15 (34.9)	0.515	16 (55.2)	13 (44.8)	0.803	18 (62)	11 (37.9)	0.471	
T4-stage	43 (59.7)	21 (72.4)	28 (65.1)		25 (58.1)	18 (41.9)		23 (53.5)	20 (46.5)		
Nodal stage											
Node negative	35 (48.6)	9 (25.7)	26 (74.3)	0.27	18 (51.4)	17 (48.6)	0.358	18 (51.4)	17 (48.6)	0.368	
Node positive	37 (51.4)	14 (37.8)	23 (62.2)		23 (62.2)	14 (37.8)		23 (62.2)	14 (37.8)		
Grade											
Well differentiated	18 (25)	6 (33.3)	12 (66.7)	1	8 (44.4)	10 (55.6)	0.457	9 (50)	9 (50)	0.824	
Moderately differentiated	51 (70.8)	16 (31.4)	35 (68.6)		31 (60.8)	20 (39.2)		30 (58.8)	21 (41.2)		
Poorly differentiated	3 (4.2)	1 (33.3)	2 (66.7)		2 (66.7)	1 (33.3)		2 (66.7)	1 (33.3)		
Extra capsular spread											
Absent	59 (81.9)	23 (39)	36 (61)	0.006	41 (69.5)	18 (30.5)	0.000	41 (69.5)	18 (30.5)	<0.00	
Present	13 (18)	00	13 (100)		00	13 (100)		1	00		13 (100)
Perineral invasion											
Absent	53 (73.6)	23 (43.4)	30 (56.6)	0.001	41 (77.4)	12 (56.6)	0.000	41 (77.4)	12 (22.6)	<0.00	
Present	19 (26.4)	00	19 (100)		00	19 (100)		1	00		19 (100)
Treatment failure											
Absent	51 (70.8)	18 (35.3)	33 (64.7)	0.342	31 (60.8)	20 (39.2)	0.305	32 (62.8)	19 (37.2)	0.121	
Present	21 (29.2)	5 (23.8)	16 (76.2)		10 (47.6)	11 (52.4)		9 (42.9)	12 (57.1)		
Mortality											
Alive	57 (79.2)	19 (33.4)	38 (66.7)	0.622	32 (56.1)	25 (43.8)	0.788	32 (56.1)	25 (43.8)	0.788	
Dead	15 (20.8)	4 (26.7)	11 (73.4)		9 (60)	6 (40)		9 (60)	6 (40)		

ECs: extra capsular spread, EGFR: epidermal growth factor receptor, PNI: perineral invasion.

Table 2: Correlation of EGFR, cyclin D1 and Ki-67 expression with clinic-pathological variables and treatment outcome.

Variables			EGFR				Cyclin D1				Ki-67			
			Treatment failure	P value	Death	P value	Treatment failure	P value	Death	P value	Treatment failure	P value	Death	P value
Age (in years)	<60	Mild	5.8	1	11.8	1	8.0	1	16	0.12	7.4	1	14.8	0.28
		Intense	1		7.7		5.6		00		6.25		00	
	>60	Mild	67	1	33.3	1	50.0	0.24	31.2	0.46	50.0	0.26	35.7	1
		Intense	60		39.13		76.9		46.1		73.0		40	
T-stage	T3	Mild	12.5	1	25	1	6.25	0.29	31.3	0.41	5.6	0.14	27.8	0.67
		Intense	14.3		23.8		23.0		15.4		27.3		18.2	
	T4	Mild	26.7	0.32	13.3	0.69	36.0	0.75	16.0	0.702	34.8	0.54	17.4	1
		Intense	46.4		21.4		44.4		22.2		45.0		20	
N-stage	N -ve	Mild	11.1	0.23	11.1	0.64	11.1	0.02	16.7	0.44	11.1	0.027	16.7	0.44
		Intense	34.6		26.9		47		29.4		30.4		29.4	
	N +ve	Mild	28.6	1	21.4	1	34.8	0.47	26.0	0.21	30.4	1	26.0	0.22
		Intense	30.4		17.4		21.4		7.14		28.6		7.1	
PNI	No	Mild	79.2	0.53	22.2	0.34	24.2	1	21.9	0.15	21.9	0.07	21.9	0.45
		Intense	28.3		25.7		22.2		5.6		50.0		33.3	
	Yes	Mild	0	0.631	0	0.53	00	0.09	00	0.08	00	0.165	00	0.302
		Intense	80.7		56.7		53.8		38.5		31.6		10.5	
ESC	No	Mild	21.7	1	17.4	1	16.7	1	21.9	0.45	21.9	0.74	21.9	0.15
		Intense	25		16		31.6		33.3		27.8		5.6	
	Yes	Mild	00	0.093	00	0.224	24.4	0.49	00	0.30	00	0.087	00	0.081
		Intense	53.8		38.5		41.7		10.5		53.9		38.4	

ECS: extra capsular spread, EGFR: epidermal growth factor receptor, PNI: perineural invasion.

Table 3: Correlation of EGFR, cyclin D1 and Ki-67 expression and various clinic-pathological variables with over-all and disease-free survival at 2 and 5 years.

Variables			EGFR						Cyclin D1						Ki-67					
			OS			DFS			OS			DFS			OS			DFS		
			2 yr (%)	5 yr (%)	P value	2 yr (%)	5 yr (%)	P value	2 yr (%)	5 yr (%)	P value	2 yr (%)	5 yr (%)	P value	2 yr (%)	5 yr (%)	P value	2 yr (%)	5 yr (%)	P value
Age	<60	Mild	92.5	92.5	1	86.9	86.9	1	87	65.2	0.12	87	65.2	1	95.6	89.6	0.28	87.9	65.9	1
		Intense	88.5	88.5		95.8	78.4		95.2	88.6		100	100		90.5	90.5		100	100	
	>60	Mild	83.3	33.3	1	83.3	62.5	1	80	40.3	0.46	86.2	43	0.24	76.9	38	1	84	29.7	0.26
		Intense	76.4	22.4		95.6	23.8		75.5	9.2		83.5	33.4		78.9	15.8		85.9	42.9	
T-stage	T3	Mild	100	75	1	87.5	65.6	1	100	88.2	0.4	86.3	38	0.2	100	88.9	0.67	87.6	38.9	0.14
		Intense	100	73		94.3	48.1		75	60		86.6	69.3		73.3	57		85.7	66.7	
	T4	Mild	85.2	68.2	0.6	85.2	85.2	0.32	82.9	53	0.7	86.9	76.7	0.75	81.8	54.7	1	86	75.3	0.54
		Intense	81.8	37.4		88.9	69.1		83	39.6		88.5	73		58.5	38.9		89.6	74.6	
N-stage	N -ve	Mild	100	80	0.64	88.9	88.9	0.23	93.1	83.8	0.4	87.8	58.5	0.02	93.3	84.8	0.44	88	58.7	0.02
		Intense	87.5	56		91.5	57.5		61.8	46.3		72	62.5		87.5	43.6		93.5	60.1	
	N +ve	Mild	84	65.3	1	84	74.1	1	86	53	0.2	86	53	0.4	85.4	55.9	0.21	83.4	46	1
		Intense	90.7	41.3		90.8	24.8		92.8	47		92.8	92.8		92.8	46.4		92.9	92.9	
ECS	No	Mild	89.7	70.5	1	85.8	79.2	1	88.9	64.2	0.15	86.7	54.9	1	88.7	66.9	0.15	86.5	51.2	0.7
		Intense	90.7	62.9		93.8	57.5		78.8	68.3		91.6	91.6		93.9	62.6		91.7	91.7	
	Yes	Mild	0	0	0.2	0	0	0.09	0	0	0.8	0	0	0.09	0	0	0.08	0	0	0.08
		Intense	83.9	16.7		83.9	35.9		83.9	16.8		83.9	35.9		83.9	16.7		59.9	35.9	
PNI	No	Mild	89.7	70.5	0.3	85.8	79.2	0.53	88.9	64.2	0.45	86.6	54.9	0.1	88.7	66.9	0.45	86.5	51.2	0.07
		Intense	85.5	47.2		85.5	28.3		82	27.3		82	54.6		82.5	22.2		82.5	55	
	Yes	Mild	0	0	0.53	0	0	0.63	0	0	0.3	0	0	0.45	0	0	0.30	0	0	0.16
		Intense	94.3	56.7		92.3	80.7		74	56.6		92.3	80.7		94.1	56.6		92.3	80	

DFS: disease free survival, ECS: extra capsular spread, EGFR: epidermal growth factor receptor, OS: over-all survival, PNI: perineural invasion.

EGFR showed intense expression in 49/72 (68.05%), and mild/insignificant expression in 23/72 (31.9%). This showed intense expression in all cases with perineural invasion (PNI) 19/19 ($p=0.0001$) and also in tumours with extracapsular extension (13/13). There were twenty-one cases of treatment failure and 16/21 (76.2 %) showed intense expression of EGFR compared to 33/51 (64.7 %) successfully treated patients ($p=0.342$).

Cyclin D1 showed intense expression in 31/72 (43%) patients and mild in 41/72 (57%) patients. Cyclin D1 as per our analysis showed no significant difference in the intensity as the T-stage, N stage or grade of tumor advances, it showed statistically significant intense expression in tumors with ECS (92.3%) as well as PNI (94.7%) ($p<0.0001$).

This marker showed intense expression in 8/13 (51.5%) tumors with pyriform fossa (PFS) extension compared to 23/59 (38.9%) without PFS extension ($p=0.057$). When we studied the Cyclin D1 expression in node negative patients, those with intensely expressed cyclin D1 showed worse disease-free survival compared to Cyclin D1 insignificant expression (at 5 years - DFS 83.8% and 43.6%, respectively; $p=0.02$). Out of 15 patients who died, 6 (20.5%) patients showed intense expression of cyclin D1 and 9 (11.5%) patients showed only minimal expression ($p=0.788$).

Ki-67 was intensely expressed in 31/72 (43.1%) and mildly expressed in 41/72 (56.9%) patients and absent in 6 (8.3%) patients. It was expressed in 6/13 (46.1%) and 25/59 (42.1%) patients with and without PFS extension, respectively ($p=0.039$). Our study displayed a positive correlation of Ki-67 expression with ECS ($p=0.0001$) and PNI ($p=0.0001$). The treatment was unsuccessful in 21 patients and of these, 12 (57.1%) showed intense expression of Ki-67 as compared to 19/51 (37.3%) who were successfully treated ($p=0.121$).

The percentages of treatment failure and death in patients with mildly or intensely expressed EGFR, cyclin D1 and Ki-67 in different clinic-pathological variables are individually mentioned in Table 2. Furthermore, Table 3 demonstrate the survival analysis (overall and disease-free survival) based on EGFR, cyclin D1 and Ki-67 expression and its correlation with different clinico-pathological variables.

DISCUSSION

This study evaluated the expression of three proliferative markers such as EGFR, cyclin D1 and Ki-67 in the specimens of total 72 patients of LSCC who underwent primary surgery. The correlation of expression all three proliferative markers with various clinic-pathological parameters was also established in addition to the survival analysis. The expression of all three proliferation markers (EGFR, cyclin D1 and Ki-67) was significant in older patients with age >60 years compared to <60 years

patients ($p=0.04$). This stressed the importance of evaluating these markers in the older age group which may help in their prognostication.

EGFR

EGFR is a cell surface membrane receptor protein which gets activated when specific ligands such as epidermal growth factor and tumour growth factor binds with it. It initiates a cascade of reactions which ultimately lead to DNA synthesis. EGFR mutation was known to be associated with LSCC. EGFR showed significant expression in 49 (67.6%) patients in the present analysis of 72 tumor samples. Various previous studies have also demonstrated over-expression of EGFR in more than 50 % of cases which depicted its association with carcinoma of larynx.^{8,9,15} EGFR over-expression/amplification may be considered as a valuable predictor of tumor aggressiveness/invasiveness and metastatic potential for a cost-effective treatment.

In the present study, EGFR showed significantly intense expression in all the tumours with extra capsular spread (ECS) (13/13) as well as with PNI (19/19) ($p<0.0001$) and both of these have been deliberated as features of invasiveness of tumor. Wei et al.⁹ also showed that EGFR over-expression did not correlate with the tumor behaviour, even though its expression was significantly higher in malignant tissues than in non-malignant tissues.

In previous studies, EGFR expression has been linked with a higher probability of relapse, poor prognosis and relative resistance to chemotherapy and radiotherapy.¹⁵⁻¹⁹ In the present study, 16/21 (76.2%) treatment failure patients and 33/51 (64.7%) successfully treated patients showed intense EGFR expression which was not statistically significant ($p=0.34$). Major published studies presented contradictory verdicts regarding the role of EGFR in the survival of carcinoma patients. In a study by Almadori et al.¹⁵ EGFR status appeared as an independent prognostic factor for disease free survival in laryngeal cancer patients. At 5 years follow-up in this study, recurrence free survival was 66% for patients with EGFR-negative tumors compared with 15% for patients with EGFR-positive tumors. Number of previous studies proved that over-expression of EGFR has been associated with an increased risk of death from disease.^{3,16,18} However, our study didn't reveal any association between EGFR and survival.

Cyclin D1

Cyclin D1 is a member of the cyclin protein family that regulate cell cycle progression. The synthesis of cyclin D1 has been initiated during G1-phase of cell cycle and drives the G1/S phase transition. Defects in cyclin D1 regulation proposed to be responsible for absence of growth control in cancer cells. Cyclin D1 has been implicated in the development and progression of several cancers including breast, oesophagus, bladder and lungs.

Cyclin D1 intense expression is seen in 40-70 % of LSCC.^{10,11,20} In present study, cyclin D1 showed intense/significant expression in 31/72 (43%) patients. Simsek et al showed cyclin D1 expression in 70 % of LSCC.¹⁰ Similarly, a study by Kapral et al also showed significantly higher expression of cyclin D1 in laryngeal tumor tissue compared to surrounding non-neoplastic tissues.¹¹

The over-expression of cyclin D1 and its association with clinico-pathological features in LSCC has been analysed in various studies.²⁰⁻²³ Although cyclin D1 as per our analysis showed no significant difference in the intensity as the T-stage, N-stage or grade of tumor advances, it showed statistically significant intense expression in tumors with ECS (92.3%) as well as PNI (94.7%) ($p < 0.0001$).

Cyclin D1 over-expression has yielded contradictory results, i.e. most authors reported the correlation of overexpression with a poor outcome and it also enhanced radio-sensitivity in some studies. Pignatatro et al, found a significant association between cyclin D1 over-expression and tumour recurrence, however Ioachim et al, didn't find any correlation between cyclin D1 expression and treatment failure.^{24,25} Our present study displayed no association between cyclin D1 and survival. In support to the present finding various studies in literature also represented no correlation with survival and expression of cyclin D1.^{23,26,27} Literature stated that intense cyclin D1 expression has been associated with increased risk of death and its over-expression has been correlated very well with bad prognosis among laryngeal tumors.^{3,10,28-30} Cyclin D1 plays an important role in cell cycle and that might be the reason for worse prognosis of its over-expression.

In LSCC, patients with node positive disease have higher chances of disease recurrence. However, when we studied the cyclin D1 expression in node negative patients, those with intensely expressed cyclin D1 showed worse disease-free survival compared to cyclin D1 insignificant expression (at 5 years- DFS 83.8% and 43.6%, respectively; $p = 0.02$). This indicated that intense expression of cyclin D1 affects the disease-free survival even in relatively good prognostic group. This study suggested that, the patients with clinically node negative and intensely expressed cyclin D1 would do better with neck dissection or irradiation even though this might not be the standard treatment. However, this needs to be confirmed in randomised trials.

Ki-67

Ki-67 is a non-histone nuclear protein expressed in proliferating cells and used as cell proliferative marker. Its exact role in cell division has not been elucidated yet, however recently its role in various carcinomas including carcinoma of larynx has been established.^{12,31} This study revealed the intense expression of Ki-67 in 31 (43%)

patients. Similarly, in a study by Mondal et al, the mean Ki-67 labelling index in hyperplasia, dysplasia and carcinoma were 12.15%, 22.03% and 35.53%, respectively.¹² This signifies the role of Ki-67 in carcinogenesis of LSCC. In contrary to the present study, literature stated positive correlation of Ki-67 expression and tumor grades.^{12,32-36} However, the present study displayed a positive correlation of Ki-67 expression with ECS ($p = 0.0001$) and PNI ($p = 0.0001$) which has not been established previously.

No significant correlation was observed of Ki-67 expression with treatment failure and survival in the current analysis. Nichols et al, in a retrospective study on 75 patients with T1-T2 glottic squamous cell carcinoma treated with radiation therapy revealed a strong correlation between Ki-67 expression and recurrence following radiation. However, the relationship has not been proved yet as some authors demonstrated higher Ki-67 index to be a positive predictor, whereas other studies found the reverse.^{31,37,13} Thus, more studies in future are needed to establish its correlation with recurrence and survival after treatment.

An important finding was that the patients with node negative and intense expression of Ki-67 showed poor disease-free survival compared to those with insignificant expression (at 5 years - 43.6% and 84.8%, respectively; $p = 0.02$). This finding has been considered crucial as Ki-67 expression could be useful as a prognostic factor in node negative cases. Overall survival has not affected which suggested that Ki-67 predicted for recurrence and salvage treatment was effective as a prognostic factor in node negative cases. This would reduce the need for unnecessary treatment in patients who are not likely to relapse.

The present finding bridges the gap in knowledge as till date no study in the literature stated the association of EGFR, cyclin D1 and Ki-67 expression with the risk of ECS and PNI in LSCC. The results suggested that estimation of all these proliferation markers may be used in planning pre-operative surgical strategies and also guide towards more radical approach for the resection of the primary as well as neck dissection. High intensity EGFR was predictive of involvement of contralateral lymph nodes which may indicate a need for contralateral neck dissection. All the three proliferation markers EGFR, cyclin D1 and Ki-67 can be categorized as markers of invasiveness. No other studies till date has studied this combination using commercially available kits.

Study limitation

Despite of these positive results, the study carries some limitations such as limited number of enrolled patients thus analysed limited cases. There were limited number of tumour tissue for the marker analysis. Thus, in future

large-scale study should be performed which add on the significance to this study.

CONCLUSION

Overall, from the results of this study, it can be concluded that biomarkers can be considered as beneficial in predicting aggressiveness of tumor and poor prognosis of the treatment. It can be proposed that in advanced cases of LSCC, estimation of EGFR, cyclin D1, and Ki-67 may be advantageous in planning therapeutic strategy and can form the integral part in work-up of these cases.

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