

Immunoexpression of p53 and ki-67 correlated with clinicopathological parameters in predicting recurrence of oral squamous cell carcinoma



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ABSTRACT

Background: Prognosis of oral squamous cell carcinoma (OSCC) can be predicted by evaluating both clinicopathological parameters and molecular markers. Understanding the reasons of recurrence is important to increase the morbidity and mortality of the patient. **Aims and Objectives:** The objectives are to study the immunoexpression of p53 and Ki-67 in recurrent and non recurrent OSCC. And correlate the clinicopathological parameters among non-recurrent and recurrent OSCC to predict the prognosis of OSCC. **Materials and Methods:** Clinicopathological and immunoexpression of p53 and Ki-67 at the histologically negative margin (HNM) and invasive tumor front (ITF) were assessed in 50 cases of non-recurrent and recurrent OSCC. Overall survival and disease free survival was estimated. Statistical tests like Mann Whitney U test, t Test, Pearson correlation coefficient, Kaplan Meier curve and Log Rank test were used. **Results:** The Labelling Index (LI) of p53 and Ki-67 was marginally higher in recurrent than in non-recurrent OSCC cases. When compared with clinicopathologic parameters, non-recurrent OSCC cases showed no significance with p53 LI in margin and ITF except for site, muscle invasion, pattern of invasion (POI) and muscle invasion, whereas in recurrent OSCC cases p53 LI in HNM and ITF did not show any statistical significance except for duration. In both groups, OS and DFS was higher in patients with lesser LI while higher in the greater LI but more number of deaths occurred in this category respectively. **Conclusion:** To conclude p53 and Ki-67 positivity in the HNM and ITF of non-recurrent and recurrent OSCC was seen and thus can be an important marker for identification of high and low risk individuals.

Key words: Disease free survival; Histologically negative margin; Invasive tumor front; Non-recurrent; Overall survival; Recurrent; Survival rate

INTRODUCTION

Oral squamous cell carcinoma (OSCC) is the most common malignant tumor in Head & Neck region. Standard treatment for OSCC is surgery, radiation or both, and is sometimes combined with chemotherapy in advanced diseases. Despite the advent of newer treatment, its 5-year survival rate is around 50-60% due to locoregional recurrences.¹ The invasive tumor front (ITF) has been studied widely which shows a lower degree of differentiation and a greater grade

of cellular dissociation in comparison with other parts of the tumor. It is believed that the essential prognostic information about the tumor's invasive and metastatic ability can be deduced from ITF, where the deepest and apparently aggressive cells reside.² Apart from ITF, the presence of tumor in the adjacent margin is related with the high rate of OSCC relapses and shorter overall survival rate.³

p53 tumor suppressor gene has been found mutated in more than 50% of human cancers, and can be useful

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indicator to predict the recurrence of OSCC.⁴ Presence of mutated p53 in tumor adjacent epithelium and invasive tumor front of recurrent patients represent a risk marker of neoplastic recurrence of OSCC as reported in a study.⁵ Ki-67 is the proliferative human nuclear antigen which is expressed during G1, S, G2, M phases of cell cycle but absent in quiescent G0. Several studies have shown that it's a good surrogate marker for the loss of heterozygosity when applied to the non-neoplastic mucosa surrounding a primary OSCC.⁶ Thus, expression of p53 and Ki-67 in both ITF and HNM could act as a sensitive marker for detection of molecular alterations along with the clinicopathological parameters in predicting recurrences of OSCC.

MATERIALS AND METHODS

Patient selection- A total of 50 patients with OSCC who underwent tumor resection were retrospectively studied. Out of 50 cases, 25 were NRec cases without locoregional recurrence in a span of 3 years post-surgical treatment while the Rec cases included the cases without recurrence. Both the ITF and HNM were studied. Postoperative follow-up information including tumor recurrence and survival was obtained from the clinical records. Overall survival (OS) time was defined as time the patient stayed alive from the date of OSCC diagnosis to the end of the study or to death. Disease-free survival (DFS) time was defined as time from the date of diagnosis to the date of local recurrence or newly diagnosed metastasis.

Clinicopathological analyses- The clinical parameters included age, gender, habit, site, duration of recurrence and treatment. TNM staging was confirmed based on the American Joint Committee on Cancer staging system.⁷ Histological parameters included the grading, tumor thickness (TT), pattern of invasion (POI), lymph node metastasis (LNM), margin positivity and submucosal infiltration in the form of perivascular, perineural, muscle were documented.

Immunohistochemical analyses- The tissue sections of 4 μ m was deparaffinised, rehydrated and blocked with 3% hydrogen peroxide. Heat induced antigen retrieval was performed in Tris-EDTA Buffer (10mM), pH=9.0 using pressure cooker. The sections were incubated with mouse monoclonal antibody p53 (BP-53-12, PM 101-6ML) and Ki-67 (GM 001-PM 096-6ML) Gene Pulse Scientific, Bangalore, India. Sections were then incubated super sensitive polymer – HRP detection system. Immunoreactivity was revealed by adding diaminobenzidine (DAB). Sections were counterstained with hematoxylin, dehydrated and mounted.

Quantification methods- A total of 500 cells were counted in 40 X magnification in different representative field using an eyepiece graticule with positive staining cells at the ITF and HNM. (Figures 1, 2) The labelling index (LI) was calculated by dividing the number of positive cells by the total number of cells and multiplying by 100.

Statistical analysis- The data were analyzed by means of IBM SPSS Statistics 20.0 software. Clinicopathological parameters were compared using Mann Whitney U Test. The comparison of expression of p53 and Ki-67 in HNM and ITF was done using t Test. The clinicopathological parameters were compared with the LI of p53 and Ki-67 in HNM and ITF using Pearson correlation coefficient. Inter observer variability was assessed using Kappa Statistics. The treatment and LI of p53 and Ki-67 were compared with overall and disease free survival using Log Rank Test and Kaplan Meier Curve. $p < 0.05$ was considered significant.

RESULTS

Clinicopathological parameters

Clinicopathological parameters between both the groups are described in Table 1. Occurrence of OSCC showed a statistical significant correlation between sites in both the groups ($p=0.033$). Tumor thickness and muscle invasion showed a statistical association when compared between the groups ($p=0.010$). Muscle invasion had a significant correlation ($p=0.016$). A statistically highly significant association was observed with respect to treatment in both the groups ($p=0.001$).

p53 and Ki-67 expression in HNM and ITF of NRec and Rec OSCC

The LI of p53 and Ki-67 in HNM and ITF were higher in Rec OSCC in comparison to NRec OSCC. However, a statistical association of LI wasn't found between the 2 groups. (Table 2) The LI of p53 and Ki-67 was demonstrated to be higher in the ITF than in HNM in both groups and was statistically significant ($p=0.001$) as listed in Table 3. Two pathologists examined the LI of p53 and Ki-67 in margin and ITF independently and kappa (k) statistics was considered to avoid interobserver bias. (Table 4).

Comparison of LI of p53 and Ki-67 in HNM and ITF with clinicopathological parameters

Pearson correlation coefficient showed relation between the clinicopathological parameters with LI of p53 and Ki-67 in margin and ITF, out of the parameters only habits, site, staging, muscle invasion, pattern of invasion and duration of recurrence showed a statistical significance as given in Table 5.

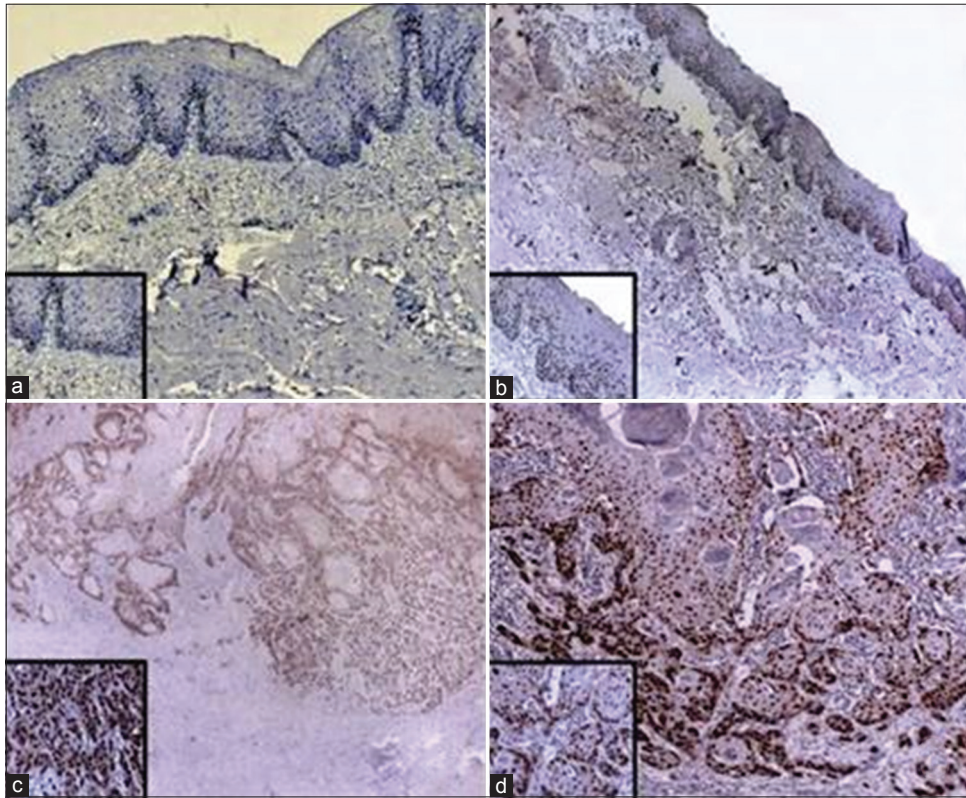


Figure 1: p53 (a,c) and Ki-67 (b,d) expression in HNM and ITF respectively in Recurrent OSCC (x10, x5) (Immunostaining: DAB chromogen, p53 and Ki-67 monoclonal antibody).

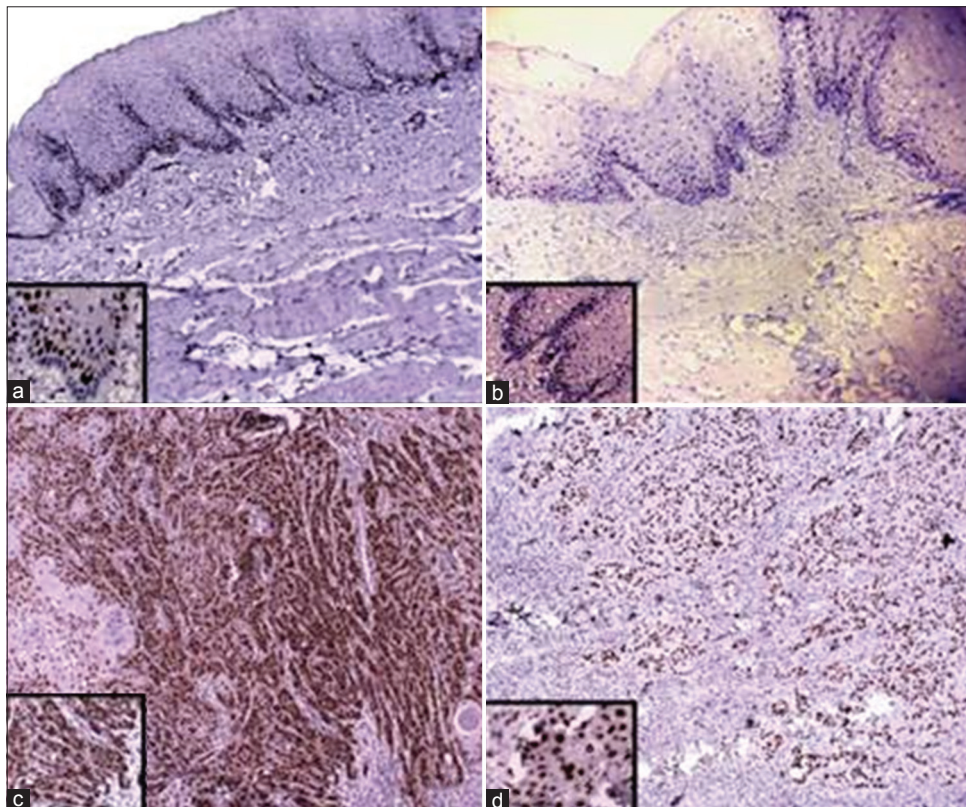


Figure 2: p53 (a,c) and Ki-67 (b,d) expression in HNM and ITF respectively in Non-recurrent OSCC (x10, x5) (Immunostaining: DAB chromogen, p53 and Ki-67 monoclonal antibody).

Table 1: Comparison between the clinicopathologic parameters between NRec and Rec groups

Parameters	Category	Non-recurrent OSCC	Recurrent OSCC	p value
Age	<40 yrs	4 (16.0)	6 (24.0)	0.484
	>40 yrs	21 (84.0)	19 (76.0)	
Gender	Male	22(88.0)	23(92.0)	0.641
	Female	3 (12.0)	2 (8.0)	
Habits	Nil	3 (12.0)	5 (20.0)	0.253
	Tobacco Combination	19 (76.0)	19 (76.0)	
Site	Buccal mucosa	3 (12.0)	1 (4.0)	0.033
	Tongue Others	19 (76.0)	11 (44.0)	
Staging	Stage I	2 (8.0)	6 (24.0)	0.904
	Stage II	4 (16.0)	8 (32.0)	
	Stage III	6 (24.0)	8 (32.0)	
	Stage III	17 (68.0)	12 (48.0)	
H/P Grading	Well	2 (8.0)	5 (20.0)	0.750
	Moderate	18 (72.0)	19 (76.0)	
TT	0-5 mm	7 (28.0)	6 (24.0)	0.010
	6-10 mm	11 (44.0)	4 (16.0)	
	11-15 mm	12 (48.0)	13 (52.0)	
Muscle invasion	No	2 (8.0)	8 (32.0)	0.016
	Yes	4 (16.0)	12 (48.0)	
PNI	No	21 (84.0)	13 (52.0)	0.068
	Yes	23(92.0)	18 (72.0)	
PVI	No	2 (8.0)	7 (28.0)	0.556
	Yes	24 (96.0)	23(92.0)	
LNM	Positive	1 (4.0)	2 (8.0)	1.000
	Negative	10 (40.0)	10 (40.0)	
POI	1	15 (60.0)	15 (60.0)	0.215
	2	4 (16.0)	3 (12.0)	
	3	8 (32.0)	6 (24.0)	
	4	11 (44.0)	10 (40.0)	
Treatment	Surgery without adjunct therapy	2 (8.0)	6 (24.0)	<0.001
	Surgery with adjunct therapy	1 (4.0)	8 (32.0)	
Margin +ve	Clear	24 (96.0)	17 (68.0)	0.111
	Involved	9 (36.0)	9 (36.0)	
Duration	<1 year	16 (64.0)	16 (64.0)	-
	1-2 yrs	-	18 (72.0)	
	>2 yrs	-	5 (20.0)	
			2 (8.0)	

P<0.05

Comparison of survival analysis with treatment modality and labelling index using Kaplan Meier & Log rank test

The treatment modalities used for treating conventional OSCC were either Surgery or a combination of Surgery and Radiotherapy or chemotherapy was used to predict OS and DFS in 2 groups showed similar OS with more number of deaths occurred in a shorter span of time in Rec OSCC whereas a greater DFS was seen in NRec OSCC as compared to Rec OSCC. (Figure 3).

To predict the survival outcome in patients between the 2 groups, the 3 year OS and DFS were compared to the LI

Table 2: Comparison of LI of p53 and Ki-67 between NRec and Rec OSCC cases in histologically negative margin (HNM) and the invasive tumor front (ITF)

Markers	Groups	Mean	SD	t value	Significance
p53ITF	Non-recurrent OSCC	44.25	29.65	0.063	0.950
	Recurrent OSCC	44.78	29.81		
p53 margin	Non-recurrent OSCC	19.61	13.00	0.003	0.998
	Recurrent OSCC	19.63	14.48		
Ki-67 ITF	Non-recurrent OSCC	63.26	9.99	0.934	0.355
	Recurrent OSCC	66.17	11.94		
Ki-67 margin	Non-recurrent OSCC	21.51	7.85	0.608	0.546
	Recurrent OSCC	22.85	7.70		

p<0.05

Table 3: Comparison of LI of both the markers within NRec and Rec OSCC

Group	Markers	Mean	SD	t value	Significance
Non-recurrent OSCC	p53 LI (ITF)	44.25	29.65	3.805	<0.001
	P53 LI (margin)	19.61	13.00		
Non-recurrent OSCC	Ki-67 LI (ITF)	63.26	9.99	16.419	<0.001
	Ki-67 LI (margin)	21.51	7.85		
Recurrent OSCC	p53 LI (ITF)	44.78	29.81	3.795	<0.001
	p53 LI (margin)	19.63	14.48		
Recurrent OSCC	Ki-67 LI (ITF)	66.17	11.94	15.237	<0.001
	Ki-67 LI (margin)	22.85	7.70		

p<0.05

of p53 and Ki-67. (Figure 4) The cut off value for p53 was taken estimate OS and DFS in accordance to various studies i.e <10, >10 and median of Ki-67 were evaluated in both HNM and ITF. In NRec OSCC, a statistical significance was observed between p53 LI in ITF and OS, p53 margin and DFS, Ki-67 LI in ITF and OS, Ki-67 ITF and DFS. In Rec OSCC, a statistical significance was obtained between Ki-67 in margin and OS and since none of the recurrent cases were disease free from the time of primary treatment to the 3 year span of post-operative follow up, no statistics could be computed. (Table 6).

DISCUSSION

In OSCC, anatomical site is considered as one of the important parameter to predict prognosis and survival rate. In the present study, a statistical significance was observed in relation to site among the 2 groups. The occurrence of OSCC in buccal mucosa can be attributable to the use of smokeless tobacco prevalent in the local area. The second predominant site associated was tongue. Cancers of tongue have high recurrences due to the rich lymphatic network, higher chemical carcinogen metabolization and high permeability due to thin non-keratinized epithelium.⁷ Other sites affected in the present study included retromolar trigone, floor of mouth and alveolus. Anatomical limits and difficult access reduce the possibility for wide resection margins in sites such as oropharynx and retromolar trigone thereby causing incomplete removal of tumor.

Table 4: Measurement of agreement between the two observers for p53 and Ki-67 in margin and ITF

	Kappa value	Approx sig
p53 ITF	0.624	<0.001
Ki-67 ITF	0.409	0.003
p53 margin	0.722	<0.001
Ki-67 margin	0.602	<0.001

TT may exert influence on the survival rates.⁸ In our study, TT was seen to be greater in the recurrent group and a statistical significance was observed. Brandwein-Gensler et al showed PNI (of nerves >1 mm in diameter) was significantly associated with increased local recurrence.⁹ In our study PNI didn't show a statistical significance to predict recurrence but 7 cases out of total 9 showed recurrences. A greater number of recurrent cases had undergone only surgery with no adjunct therapy than the patients who underwent adjunct radiotherapy. A high statistical significance was noted between the 2 groups.

The positivity for p53 in OSCC varies in different studies from 0-100%.¹⁰ In NRec and Rec OSCC, p53 LI in ITF was [(44.25±29.65), (44.78±29.51)] whereas in HNM it was [(19.61±13), (19.63±14.48)] respectively whereby no statistical association was found between them. In this study, both NRec and Rec OSCC showed higher p53 LI in ITF in comparison to HNM which is indicative of noticeable accumulation of p53 positive cells at the ITF of tumors due to the presence of amplified and uninhibited cell proliferation at ITF accumulating essential genetic alterations for invasion and metastasis.

Oliveira et al., didn't find any correlation between p53 and Ki-67 in ITF and HNM whereas in our study the recurrent

Table 5: Comparison of p53 and Ki-67 LI in margin and ITF between NRec and Rec OSCC clinicopathological parameters

Groups	Region	Habits	Site	Staging	Muscle invasion	POI	Duration of recurrence
Non-Recurrent OSCC	P53 Margin	0.850	0.045	0.514	0.14	0.036	
	P53 ITF	0.957	0.092	0.606	0.034	0.004	
	Ki-67 Margin	0.517	0.918	0.634	0.516	0.695	
	Ki-67 ITF	0.851	0.066	0.223	0.201	0.926	
Recurrent OSCC	P53 Margin	0.256	0.772	0.738	0.078	0.791	0.034
	P53 ITF	0.662	0.782	0.753	0.068	0.614	0.046
	Ki-67 Margin	0.014	0.202	0.056	0.597	0.041	0.449
	Ki-67 ITF	0.977	0.074	0.003	0.525	0.550	0.268

p<0.05

Table 6: Comparison of p53 and Ki-67 LI with OS and DFS

Groups	Marker	Cut off limit	Confidence Interval (CI)		Log rank (Mantel Cox)	
			Upper	Lower	Chi square	Sig
Non-recurrent OSCC	p53 margin&DFS	1=<10	39.07	45.58	6.228	0.013
		2=<10	35.40	38.53		
	p53 ITF & OS	1=<65.2	39.43	45.36	6.039	0.014
		2=>65.2	35.01	38.66		
	Ki-67 ITF & OS	1=<65.2	34.44	38.72	5.284	0.022
		2=>65.2	36.72	42.10		
Ki-67ITF & DFS	1=<65.2	34.52	38.79	4.697	0.030	
	2=>65.2	38.13	43.42			
Recurrent OSCC	Ki-67 margin & OS	1=<24.2	37.76	44.90	3.638	0.050
		2=>24.2	27.45	38.96		

p<0.05

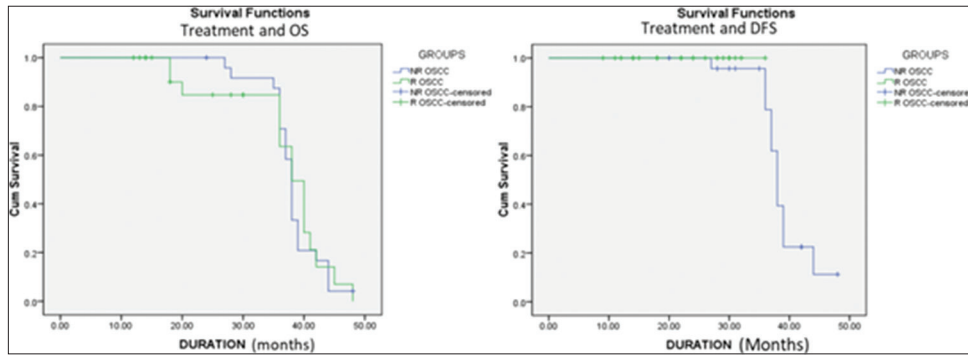


Figure 3: Kaplan- Meier curve showing comparison of treatment with OS and DFS between NRec and Rec OSCC.

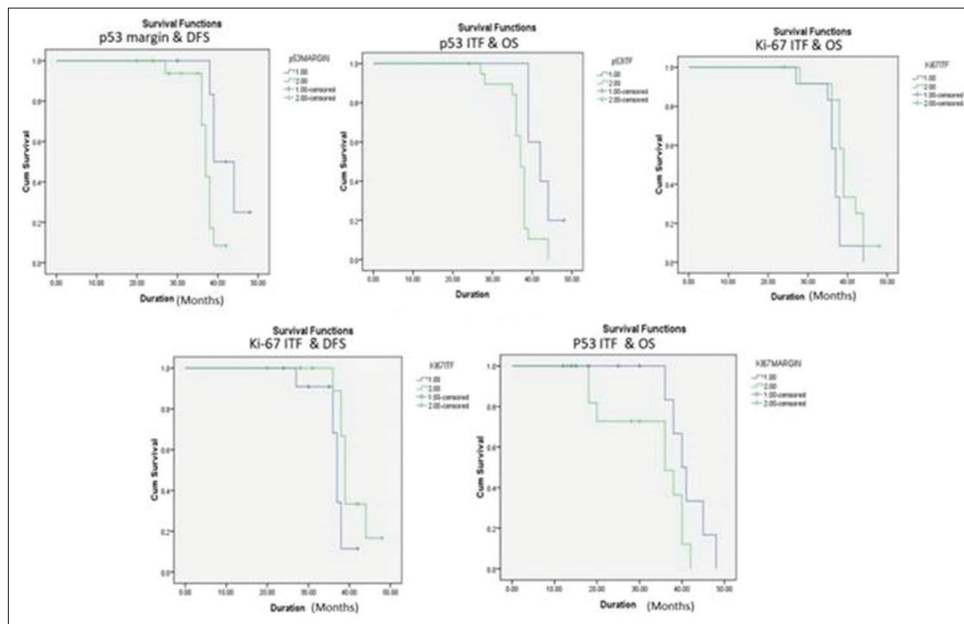


Figure 4: Kaplan- Meier curve showing survival in NRec cases (a,b,c,d) and Rec OSCC.

category had a marginal increase in expression.⁵ Kato et al., suggested that highly invasive tumors exhibit p53 mutation and PCNA activity, thereby increasing the proliferative capacity at the ITF of OSCC with increased chances of recurrence and therefore a worse prognosis.¹¹ In, addition investigators must set an arbitrary threshold of the percentage of stained cells that will be used to characterise sections as positive or negative.¹² Recent studies have set this threshold at 10% stained cells.¹³ Hence, in NRec OSCC, p53 overexpression in ITF was seen in (20/25) cases while in recurrent OSCC it was (18/25). Similar range of p53 overexpression was observed by Gonzalez-Moles.¹⁴ Whereas median value of proliferative index was used to classify Ki-67 positive and Ki-67 negative.¹⁵ In NRec OSCC, the median value of LI in margin was 21.2 and ITF was 65.2 while in Rec group the median value of LI in margin was 24.2 and ITF was 69.56. In NRec OSCC, margin and ITF showed overexpression of Ki-67 in (13/25) and (14/25) cases whereas in Rec OSCC overexpression was seen in (13/25) and (15/25) cases respectively.

In the present study, 12 tumors were negative for p53 which could be attributable to non-sense and frame-shift mutations of p53 gene leading to deletion or truncation of the protein, hence do not show detectable levels of p53 protein immunohistochemically or loss of p53 activity due to gross deletion that abolishes all p53 protein production and also, tumors that follow different carcinogenic pathways in which the p53 abrogation does not play a role can be a reason for negative expression of p53.¹⁶

In NRec and Rec OSCC, Ki-67 expression in ITF was [(63.26±9.99), (66.17±11.94)] whereas in HNM it was [(21.51±7.85), (22.85±7.70)] no statistical association was found between the groups respectively. A highly statistical significance was noted between p53 and Ki-67 in HNM and p53 in ITF within the NRec and Rec groups.

Montebugnoli et al, study showed Ki-67 protein was significantly correlated with loss of heterozygosity in the

oral mucosa surrounding OSCC and as advocated as a good predictor marker. Also showed a positive expression at ITF.¹⁷

Clinicopathological parameters were compared with the labelling index of p53 and Ki-67 in HNM and ITF. A study by Oliveira et al didn't demonstrate any statistically significant value in the evaluation of clinicopathological parameters between NRec and Rec OSCC in contrast to our study in which site, habit, staging, muscle invasion, POI, duration of recurrence showed a significant statistical association.⁵

Cooper et al have also reported that postoperative radiotherapy and chemotherapy can improve DFS and improve local and regional control rate in patients with OSCC.¹⁸ On comparison of survival rate between NRec and Rec OSCC, it was observed that OS in NRec OSCC was 48 months with overall good prognosis with 2 deaths whereas in Rec OSCC the 8 patients died between 10 months to 30 months.

The 3 year OS and DFS were compared to the LI of p53 and Ki-67 to assess their role in predicting the survival outcome. In NRec OSCC, it was observed that the cases with a higher p53 and Ki-67 index in margin and ITF had a reduced OS and DFS whereas the cases with a lesser nuclear expression had an increased OS. In the present study, LI of p53 and Ki-67 was associated with the survival of the patients whereas Koelbl et al & Bettendorf et al in their study have reported no association of proliferative markers like Ki-67, p53, PCNA and survival in OSCC.^{19,20} Since none of the recurrent cases were disease free from the time of primary treatment to the 3 year span of post-operative follow up, no statistics could be computed.

Our study included limitations like long term follow ups should be done for the cases showing tumor suppressor gene mutation and higher proliferative index. Single margin cannot be a representative of entire tumor, number of samples can be increased. Standardized procedure has to be adapted in obtaining margins according to site specificity and combination of other proliferative markers to predict recurrences such as MCM2, Geminin, PCNA etc.

CONCLUSION

The current study described p53 and Ki-67 positivity in histologically negative margin and invasive tumor front in both the groups. Few clinicopathological parameters showed association with p53 and Ki-67 positivity. Overexpression of these two markers was related to the prognosis and survival.

Thus, with the application of molecular methods in conjugation with routine H & E staining & IHC could lead to a better understanding of the biologic nature of OSCC, long term survival rate, and more selective preoperative management. Further investigations may provide more insights to this interesting field of study with long term follow ups. Only one negative margin was studied and standardized procedure has to be adapted in obtaining margins according to site specificity and combination of other proliferative markers to predict recurrences such as MCM2, Geminin, PCNA etc.

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Authors Contribution:

BB- Conceptualized study, literature search, statistically analyzed and interpreted, prepared first draft of manuscript and critical revision of the manuscript;
KH- Concept and design of the study, reviewed the literature, manuscript preparation, critical revision of the manuscript; statistically analyzed and interpreted;
KK- Concept and design of the study, revision of manuscript and review of study.

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