



MCM 2 a novel marker in predicting recurrence and prognosis at negative surgical margins of oral squamous cell carcinoma

Kiran Kumar^{a,*}, Kaveri Hallikeri^a, Venkatesh S Anehosur^b, Niranjana Kumar^b, Anil Kumar Desai^b

^a Department of Oral and Maxillofacial Pathology and Microbiology, SDM College of Dental Sciences and Hospital, Sattur, Dharwad, 580009, India

^b SDM Craniofacial Surgery and Research Centre, Sattur, Dharwad, 580009, India



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ABSTRACT

Objectives: The treatment failure in Oral carcinoma patients is mainly due to recurrence leading to a poor prognosis. Genetically transformed cells in the adjacent mucosal area thought to be the reason for local recurrences and an invasive tumour front area is known to host the aggressive tumour cells. Hence, the study conducted to quantify and compare proliferative markers Ki-67 and MCM 2 antigens at the margins and invasive tumour front to predict recurrence and prognosis in Oral squamous cell carcinomas.

Materials and method: The study involved paraffin tissue sections of 30 cases of recurrent, and 30 cases of non recurrent Oral squamous cell carcinomas were subjected to Immunohistochemical analysis at negative surgical margins and invasive tumour front (ITF). The mean labelling index (Li) of MCM 2 (Minichromosome Maintenance Protein 2) and Ki-67 was compared among the groups with 't' test to predict the recurrence and overall survival by Kaplan-meier curve and Log-rank test survival estimate.

Results: The Li of Ki-67 and MCM 2 were higher at negative margin and ITF of recurrent group compared to non recurrent OSCC group with statistically significant difference ($P < 0.05$) only with MCM 2. Li of MCM 2 at margin and ITF was a better predictor of overall survival than Ki-67. The overall survival was significantly lower in 43.95 months with Li of MCM 2 more than 48.2 at margin.

Conclusion: MCM 2 is a novel marker at negative margins in predicting the recurrence and survival of Oral squamous cell carcinoma.

1. Introduction

Head and neck squamous cell carcinomas (HNSCC) are heterogeneous but mostly preventable disease with complex molecular abnormalities. HNSCC are the sixth most common cancer in the world. In India, 30–40% of cancers involves oral cavity. In spite of various therapeutic strategies, the five year survival rate of patients with oral squamous cell carcinomas (OSCC) is still low mainly due to recurrence [1].

Intramucosal migration of cancer cells and genetically altered epithelial cells having abnormal proliferation in the mucosa adjacent to surgical margins is thought to be the main reason for local recurrences leading to the development of the second primary tumour [2]. Invasive tumour front (ITF) region known to have a high incidence of neoplastic cells with increased proliferation potential representing the aggressiveness of the tumour [3]. Hence, the molecular assessment of negative surgical margins and ITF area could constitute an effective approach to

predict the recurrence and the prognosis of OSCC.

Several molecular markers have been employed to get additional information about prognosis of OSCC [4]. Proliferative markers can give a simple, quick yet accurate measure of tumour growth and prognosis [5].

Ki-67 is the proliferative human nuclear antigen and is expressed during G1, S, G2, M phases of the cell cycle but absent in quiescent G0. Minichromosome maintenance proteins (MCM 2–7) are the prerequisite for cell-cycle initiation and DNA replication and are expressed throughout the whole cell cycle including cells leaving G0 to enter into the early G1 phase. This specific characteristic is not found in Ki-67, which is a widely used to assess proliferation [6].

The Ki-67 is a frequently used proliferative marker and good prognostic indicator of OSCC patient, even at normal oral mucosa distant from primary tumour [7,8]. Recently, MCM 2 has been identified as more sensitive marker than Ki-67 for tumour growth and prognosis in OSCC [9]. Although the MCM 2 is analysed at the tumour center

* Corresponding author.

E-mail addresses: kirankumarop@gmail.com (K. Kumar), drcauveri2005@gmail.com (K. Hallikeri), venkyrao12@yahoo.co.in (V.S. Anehosur), drniranjana108@gmail.com (N. Kumar), anildes2006@yahoo.co.in (A.K. Desai).

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[10,11] and at ITF [9], its expression at surgically negative margins has never been analysed. Furthermore, MCM2/Ki-67 ratio estimates the proportion of cells entering into the cell cycle and has been proposed to have prognostic relevance [6].

So, the present study was undertaken to quantify and compare Ki-67 and MCM 2 expression at histologically negative surgical margins and ITF to predict recurrence and prognosis in OSCC.

2. Materials and method

2.1. Study design

The present retrospective case-control type of study carried out during December 2016 to March 2018 involved archival tissue specimens of 30 cases of recurrent and 30 cases of non recurrent OSCC. Additional 10 normal buccal mucosa samples were collected as control from healthy individuals without any oral habits during extraction of impacted third molars and pre prosthetic surgery after obtaining consent. The present study was approved by the institutional ethical committee, SDM college and dental sciences and Hospital (IRB no:2016/S/OP/48).

2.2. Selection criteria

2.2.1. Inclusion criteria

1) Patients who have undergone Radical neck Dissection of lesion at buccal and tongue with or without adjunct therapy and are disease free for at least 3 years were considered non recurrent cases of OSCC. 2) Patients who have undergone Radical neck Dissection of lesion with or without adjunct radiotherapy reported back to the hospital with loco-regional recurrences of tumour were considered as recurrent cases of OSCC.

2.2.2. Exclusion criteria

1) Patients who have undergone surgery in any other hospital, but have come with a recurrence to our institution. 2) Patients suffering from known systemic nutritional deficiencies (Vitamin A, D and Zinc) 3) OSCC other than buccal mucosa and tongue. 3) Patients suffering from infectious diseases like HIV/AIDS, salivary gland neoplasm, blood cancer, lung cancer, breast cancer and other malignancies. 5) Patients with close/ positive margin (< 5 mm) and distant metastasis.

2.3. Specimen collection

The demographic details, clinicopathological details and archived tissue blocks of recurrent and non-recurrent cases of OSCC were retrieved. Tissue sections from negative surgical margins (> 0.5 mm from the tumour without dysplasia) and invasive tumour front of non recurrent and recurrent (primary) OSCC cases were collected and subjected for immunohistochemical (IHC) analysis.

2.4. Immunohistochemical (IHC) procedure

µm sections of paraffin embedded tissues were mounted on positively charged slides and were dried for 24 h at 37 °C. The sections were deparaffinised in xylene and rehydrated in descending grades of ethanol. Antigen retrieval was carried out by using tris-EDTA buffer (at pH 6.0). The slides were treated with 3% hydrogen peroxide to block the endogenous peroxidase activity for 15 min, followed by incubation with primary antibodies Ki-67 and MCM 2 (Biogenex, San Ramon, USA) for 1 h at room temperature. Further incubation with super-enhancer and secondary antibody (30 min) was done followed by visualization by chromogen DAB (Diamino benzidine) for 10 min. Phosphate buffer saline (PBS) was used to wash after each step. Finally, sections were counter stained by Harris Haematoxylin. Brown nuclear staining was considered as positive for Ki-67 and MCM 2.

2.5. IHC analysis (labelling Index)

The IHC stained sections were first examined at an ocular magnification of 10X objective and then a representative field (with even staining) was chosen randomly. The counting was performed with a binocular light microscope under high magnification of (X40). The microscope was fitted with an eyepiece (x10) having an oculometer grid with 100 blocks (10 × 10) to count the cells proficiently and without bias. In each high power field, the cells in each block of the grid were counted as the number of positive cells. A cell with nuclear staining without cytoplasmic staining was accounted for nuclear staining. Two individual observers were carried out all the observations to eliminate the inter observer bias. Up to 1000 cells (500 from negative margins, 500 cells from invasive front) were analysed for each case. The percentage of positive cells or labelling Index (Li) for each case of negative margins and ITF and for control was calculated.

2.6. Statistical analysis

The statistical analysis was done using the SPSS software package (version 21.0. Armonk, NY). Contingency tables and Chi-square test were used to compare the clinicopathological parameters between recurrent and non recurrent groups of OSCC. Parametric data were expressed as mean and standard deviation [M (SD)] and P value < 0.05 considered to indicate statistical significance. The ANOVA followed by POST HOC Tukey's test was used to compare the mean Li of Ki-67 and MCM 2 between normal, margin and ITF in non recurrent and recurrent OSCC. The mean Li of Ki-67 and MCM 2 was compared between recurrent and non recurrent groups of OSCC at the margin and ITF by student 't' test. Survival analysis (Overall survival) was done by constructing receiver operating characteristic (ROC) curve, Kaplan Meier curve followed by log rank test (Mantel-Cox test) to compare the difference in Ki-67 and MCM 2 expression with survival. Multivariate survival analysis was done to compare the clinicopathological factors and IHC markers by Cox proportional hazards model.

3. Result

The clinicopathological parameters of recurrent and non recurrent OSCC groups are compared in Table 1. The parameters like patients age below 40 years, tongue lesions, higher TNM staging, presence of perineural invasion and treatment with surgery alone were significantly associated (P < 0.05) with the recurrent OSCC group. The parameters like higher histopathological grade was only significantly associated (P < 0.05) with the non recurrent OSCC group. The mean recurrence period of recurrence OSCC group was 16 months (1.4 years).

Nuclear immunopositivity of Ki-67 & MCM 2 was mainly in basal and suprabasal compartments of normal (Figs. 4 and 7) and negative margins (Figs. 5 and 8). In OSCC areas, the immunopositivity was along the periphery of tumour islands (Fig. 6) and in invasive tumour fronts (Fig. 9). Since there was no statistically significant (P > 0.05, paired 't' test) inter observer variability in mean Li of Ki-67 and MCM 2 in normal mucosa, negative margins and ITF of both the groups, only observer 1 values were considered for analysis.

There was a gradual increase in mean Li values of Ki-67 and MCM 2 from normal to margin through ITF in both the groups (ANOVA, p < 0.05) (Table 2). The Pair wise comparison showed no significant difference (P > 0.05, POST HOC Tukey test) in Ki-67 values between normal and margins of both groups and MCM 2 values between normal and non recurrence margins (Table 2A).

The mean Li of Ki-67 and MCM 2 at margin and ITF was higher in recurrent than non recurrent group of OSCC. However, only MCM 2 was statistically significant ('t' test, p < 0.05) (Table 3). Ki-67/MCM 2 Ratio at margin and ITF [2.33(0.6) and 1.87(0.4)] in recurrent group of OSCC were slightly higher than non recurrent group of OSCC [2.13(0.6) and 1.66(0.5)]. But the difference was not statistically significant

Table 1

Comparison of clinicopathological parameters between recurrent and non recurrent groups of OSCC.

Parameters	category	Non recurrent OSCC	Recurrent OSCC	P value ^a
Age	< 40 years	4	11	0.036
	> 40 years	26	19	
Gender	Male	25	28	0.227
	female	5	2	
Habits	Chew tobacco	12	16	0.704
	Chew + Smoke	16	19	
	No habits	2	5	
Site (Primary)	Buccal mucosa	29	24	0.044
	tongue	1	6	
TNM staging (UICC TNM classification, 1997)	Stage I	0	2	0.001
	Stage II	1	3	
	Stage III	29	16	
	Stage IV	0	9	
Histopathology grading (Broder's)	Well	15	25	0.006
	Moderate	15	5	
Tumour thickness	1-5 mm	11	10	0.055
	6-10mm	17	11	
	> 10mm	2	9	
Perineural invasion (PNI)	present	4	11	0.036
	Absent	26	19	
Perivascular invasion (PVI)	present	1	2	0.548
	Absent	29	18	
Lymphnode metastasis	present	12	10	0.520
	Absent	18	20	
Bryne's grading of of invasive tumour front (ITF) (1992)	Grade1	19	15	0.529
	Grade2	8	12	
	Grade3	3	3	
Treatment	Surgery only	5	7	0.013
	Surgery + Radiotherapy	6	15	
	Surgert + Radiotherapy + Chemotherapy	19	8	

SD = Standard deviation.

Bold values represent statistically significant (p value less than 0.05)

^a P < 0.05 is significant (Chi-square test).**Table 2**

Comparison of Mean Li of Ki-67 and MCM 2 between normal, negative margins and ITF among recurrent and Non-recurrent groups of OSCC.

Markers and site	Groups	Sample (N)	Mean(SD)	P Value ^a
Mean Li Ki-67, Margin	Non	30	21.80(6.9)	0.041
	Recurrence	30	24.73(5.5)	
	Normal	10	19.66(3.1)	
	Total	70	22.75(6.1)	
Mean Li Ki-67, ITF	Non	30	41.64(15.4)	< 0.001
	Recurrence	30	45.50(10.9)	
	Normal	10	19.66(3.1)	
	Total	70	40.15(15)	
Mean Li MCM 2, Margin	Non	30	43.27(9)	< 0.001
	Recurrence	30	54.74(9.3)	
	Normal	10	38.38(4.6)	
	Total	70	47.48(10.7)	
Mean Li MCM 2 ITF	Non	30	61.82(12.8)	< 0.001
	Recurrence	30	81.09(8.7)	
	Normal	10	38.38(4.6)	
	Total	70	66.73(17.9)	

Bold values represent statistically significant (p value less than 0.05)

^a P < 0.05 is significant (ANOVA), SD = Standard deviation.

(Table 4).

Sensitivity and specificity of mean Li of Ki-67 and MCM 2 at margin and ITF were calculated and receiver operating characteristic (ROC) curve was constructed to predict the survival of all cases of OSCC irrespective of their recurrence status. Mean follow up period was 46 months. MCM 2 was found to be a better survival predictor than Ki-67 with p value < 0.05 (Fig. 1). Based on this, Kaplan-Meier curves and

Log-rank test for survival analysis was carried out only for MCM 2.

Mean cut off value for MCM 2 at margin (48.2) and at ITF (71.5) was calculated. The overall survival period was significantly lower in 43.95months with P < 0.05 if the mean Li of margin more than 48.2 (Fig. 2). The mean survival was lower if the mean Li value higher than 71.5 but was not statistically significant (Fig. 3).

The Cox regression analysis showed parameters like gender, higher TNM stage (stage II and III), Perineural invasion (PNI), Bryne's grade 1 and treatment with surgery alone as poor prognostic indicators with high odds ratio but none were significant with a p value > 0.05 (Data not shown). The Multivariate analysis showed no significant association of clinicoopathological parameters with the molecular markers in predicting survival with value p > 0.05 (Data not shown).

4. Discussion

The studies have shown parameters like TNM staging, tumour thickness, depth of invasion, surgical margin status, tumour budding, vascular and perineural invasion, bone invasion, extracapsular lymph node spread and the presence of distant metastasis are significant in determining the recurrence and prognosis of OSCC [12]. Although these criteria's are useful, they do not explain why lesions diagnosed at an early stage with negative margins have loco regional recurrence and poor prognosis [13]. In the present study, all recurrent cases had clear margins (> 5 mm) but had loco regional recurrence with an average period of 16 months. The parameters like TNM staging, perineural invasion and treatment only with surgery were significant in predicting the recurrence. Higher histopathological grade was only significantly higher in non recurrent group in our study (Table 1). Although, the correlation of histopathological grade with recurrence of OSCC is debatable, Other parameters like lymph node metastasis, invasive tumour front grading are considered to be the important prognostic indicators

Table 2A
Pair-wise comparison of Mean Li of Ki-67 and MCM 2 between normal, negative margins and ITF among recurrent and Non-recurrent groups of OSCC.

Dependent Variable	(I) group	(J) group	Mean Difference (I-J)	Std. Error	P Value ^a
Mean Li Ki-67, Margin	NON RECURRENCE	RECURRENCE	− 2.9	1.541914	0.148
		NORMAL	2.1	2.180595	0.589
Mean Li Ki-67, ITF	RECURRENCE	NORMAL	5.07	2.180595	0.059
	NON RECURRENCE	RECURRENCE	− 3.86	3.236111	0.461
Mean Li MCM 2, Margin		NORMAL	21.98	4.576552	< 0.001
	RECURRENCE	NORMAL	25.84	4.576552	< 0.001
Mean Li MCM2, Margin	NON RECURRENCE	RECURRENCE	− 11.46	2.249955	< 0.001
		NORMAL	4.89	3.181916	0.28
Mean Li MCM2, ITF	RECURRENCE	NORMAL	16.36	3.181916	< 0.001
	NON RECURRENCE	RECURRENCE	− 19.26	2.678871	< 0.001
		NORMAL	23.44	3.788496	< 0.001
	RECURRENCE	NORMAL	42.71	3.788496	< 0.001

Bold values represent statistically significant (p value less than 0.05)
^a P < 0.05 is significant (POSTHOC TUKEY TEST).

Table 3
Comparison of Mean Li of Ki-67 and MCM 2 between recurrent and Non-recurrent groups of OSCC at negative margins and ITF among.

Parameters	Group	Sample (N)	Mean(SD)	‘P’ Value ^a
Mean Li Ki-67, Margin	Non Recurrence	30	21.8(6.9)	0.078
	Recurrence	30	24.7(5.5)	
Mean Li Ki-67, ITF	Non Recurrence	30	22.2(6.6)	0.27
	Recurrence	30	25(5.7)	
Mean Li MCM 2, Margin	Non Recurrence	30	43.2(9)	< 0.001
	Recurrence	30	54.7(9.3)	
Mean Li MCM 2, ITF	Non Recurrence	30	61.8(12.8)	< 0.001
	Recurrence	30	81(8.7)	

SD = Standard deviation.
Bold values represent statistically significant (p value less than 0.05)
^a P < 0.05 is significant (‘t’ test).

Table 4
Comparison of mean MCM2/Ki67 ratio between Recurrent and nonrecurrent group.

Parameters	Group	N	Mean(SD)	‘P’ Value (‘t’ test)
MCM 2/Ki-67 ratio, margin	No Recurrence	30	2.13(0.6)	0.248
	Recurrence	30	2.33(0.6)	
MCM 2/Ki-67 ratio, ITF	Non Recurrence	30	1.66(0.5)	0.119
	Recurrence	30	1.87(0.4)	

^aP < 0.05 is significant (‘t’ test).
SD = Standard deviation.

for recurrence by many authors [13,14]. However, none of the clinicopathological parameters had a significant correlation with the survival (P > 0.05). In this regard, the identification of molecular markers considered a useful tool to identify a lesion’s aggressiveness. The study involved molecular analysis at two locations, i.e., histologically negative surgical margins and ITF areas of OSCC. It is believed that, loco regional recurrence may be due to minimal residual disease (MRD) or field cancerisation model proposed by Slaughter et al [15]. In MRD, small clusters of histopathologically undetectable tumour cells proliferate leading to local recurrence. The field cancerisation describes preneoplastic cells surrounding the OSCC which develop into second primary tumours following additional genetic hits [15]. ITF of OSCC known to reside more aggressive cells and play a vital role in many molecular interactions causing increased angiogenesis, alteration of adhesion molecules, overproduction of enzymes that degrade the extracellular matrix and an increase in the expression of proteins related to cell proliferation [16]. Hence, the molecular analysis was carried out at histologically negative margins and ITF to predict the recurrence and prognosis.

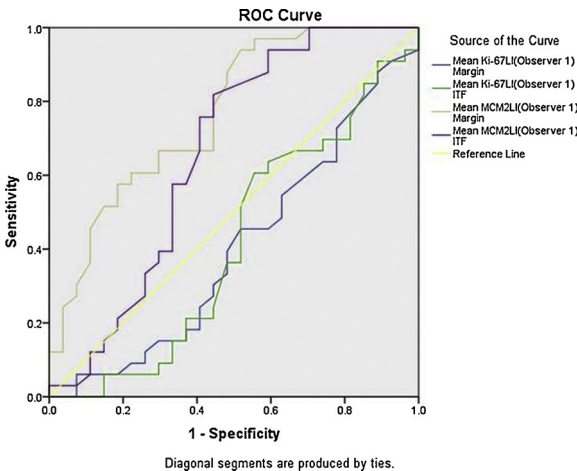


Fig. 1. Receiver operating characteristic (ROC) curve for Ki-67 and MCM 2 at margin and ITF of all cases of OSCC showing MCM 2 as a better predictor of survival.

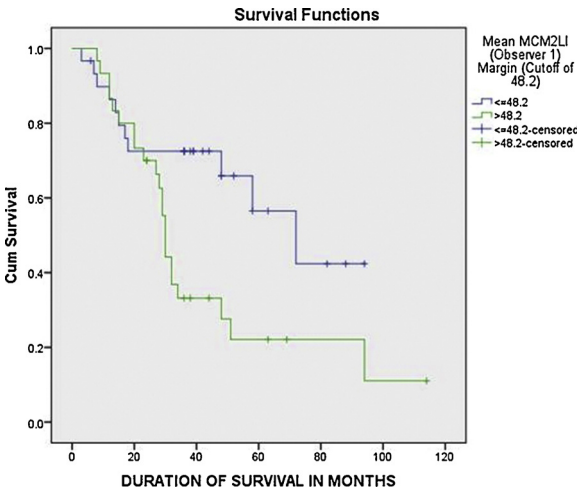


Fig. 2. Kaplan-Meier curve and Log-rank test survival estimate for Li MCM 2 at margin showing significantly lower overall survival period (P value 0.018) of 43.95 months with mean Li MCM 2 more than 48.2.

Molecular analysis, such as Polymerised chain reaction, In-situ hybridization and cytogenetic analysis have been employed to detect the genetically altered epithelial cells, but IHC method is simple and cost effective [17]. The present study employed two proliferative markers Ki-67 and

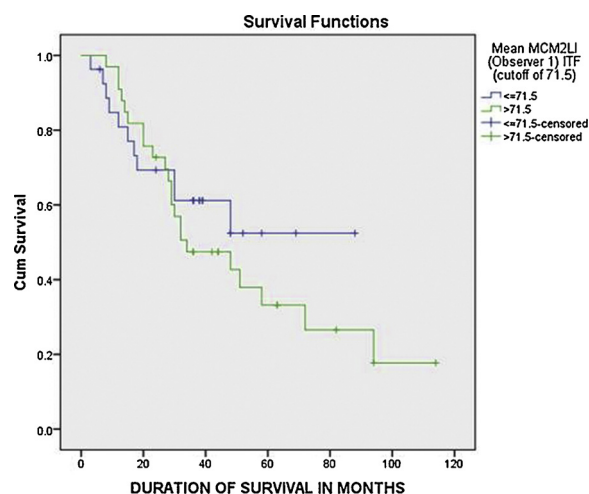


Fig. 3. Kaplan-Meier curve and Log-rank test survival estimate for Li MCM 2 at ITF showing lower overall survival period with mean Li MCM 2 more than 71.5 but not statistically significant (P value 0.412).

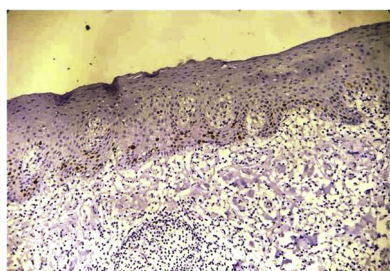


Fig. 4. Immunohistochemical expression of Ki-67 protein in Normal mucosa ($\times 100$ total magnification).

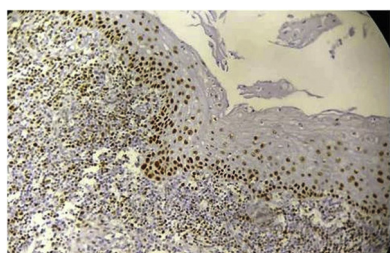


Fig. 5. Immunohistochemical expression of Ki-67 protein in negative margins ($\times 200$ total magnification).

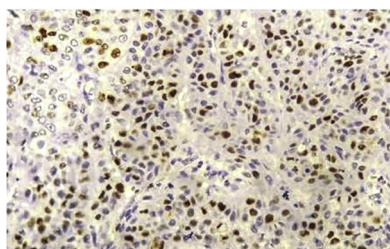


Fig. 6. Immunohistochemical expression of Ki-67 protein in invasive tumour front ($\times 400$ total magnification).

MCM 2. Ki-67 has high sensitivity and specificity in labelling proliferating cells in neoplastic tissues and gives the total number of proliferating cells. Additionally, its expression may also appear when DNA synthesis is blocked or in cells undergoing apoptosis. Ki-67 is considered as one of the best predictors of survival in patients with lung

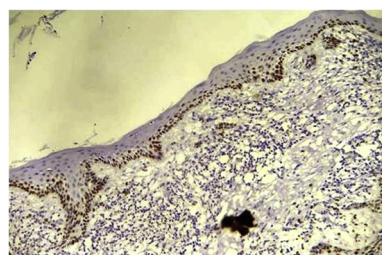


Fig. 7. Immunohistochemical expression of MCM 2 protein in Normal mucosa ($\times 100$ total magnification).

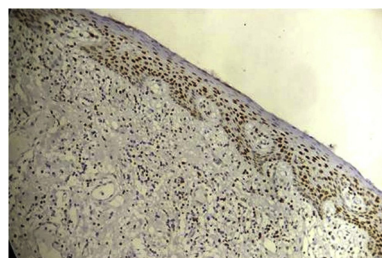


Fig. 8. Immunohistochemical expression of MCM 2 protein in negative margins ($\times 200$ total magnification).

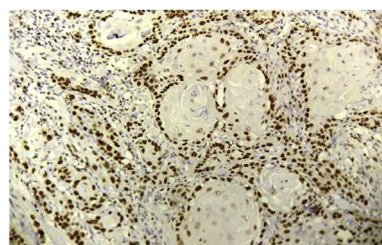


Fig. 9. Immunohistochemical expression of MCM 2 protein in invasive tumour front ($\times 400$ total magnification).

cancer, breast cancer and prostate cancer [18]. In OSCC, studies have shown conflicting results about the relationship between Ki-67 expression and survival [18]. The MCM family proteins have an elemental action in DNA replication and used as a marker to evaluate the cell proliferation. MCM family proteins mainly, MCM2, MCM5 and MCM7 have shown to have prognostic value in many cancers [19]. Studies have shown significant correlations of the MCM 2 expression with poor prognosis in OSCC and more sensitive than Ki-67 [6,9,10,11,19 and 20].

All studies of Ki-67 and MCM2 have mainly concentrated on tumour centre and ITF. Although the Ki-67 expression has been analysed at negative margins [20], there are no studies investigating the MCM 2 expression at the negative surgical margin predicting recurrence and survival. The present study investigated MCM 2 expression at negative margins and ITF.

The present study showed higher mean Li for Ki-67 and MCM 2 in histologically negative margins compared to normal mucosa (Table 2) suggesting a high cell turnover rate and might have potential to form second primary tumours [20]. However, only expression of MCM 2 at recurrent margin compared to normal was significant (Table 2A). The mean Li of Ki-67 and MCM 2 was higher at negative margin and ITF of recurrent cases compared to non recurrent OSCC cases with statistically significant difference with only MCM 2 (Table 3) suggesting MCM 2 a more sensitive and Novel marker in predicting the loco regional recurrence.

MCM 2/Ki-67 ratio estimates the proportion of cells licensed to proliferate (early G_1 phase) and higher the MCM2/Ki-67 ratio represent greater proportion of cells in a licensed non cycling state. The decrease

in the MCM2/Ki67 ratios reflects a shift in the tumour cell population from a predominantly non proliferating licensed state as in well differentiated tumours to an actively cycling state as in poorly differentiated tumours [17]. In the present study, the MCM2/Ki-67 ratio at the margin and ITF was marginally higher in the recurrent OSCC group than non recurrent group and was not statistically significant suggesting poor predictive parameter.

In the present study, Mean Li of MCM 2 at margin and ITF was a better predictor of overall survival than Ki-67 (Fig. 1). The mean survival was significantly lower in 43.95 months with MCM 2 index more than 48.2 at margin, suggesting MCM as novel marker predicting the survival in the OSCC (Fig. 2). Although, the mean survival was also lower with higher expression of MCM 2 at ITF, it was not statistically significant (Fig. 3). Some previous studies have shown the superiority of MCM 2 over Ki-67 and suggested as a sensitive marker [6,10] like our study, but our study highlights the MCM 2 expression at margins as a novel marker.

The present study did not find any significant correlation of Ki-67 and MCM 2 expression with clinicopathological parameters and overall survival. Szelachowska J et al [10] have also reported no significant correlation of MCM2 and Ki-67 with clinicopathological parameters similar to our study. Montebugnoli et al [8] have showed tumour differentiation, clinical stage and Ki-67 expression from distant mucosa were independent prognostic factors for disease free survival by multivariate analysis using the Cox proportional hazards model. Lopes VKM et al [18] have showed significant increase in Ki-67 expression in larger tumours (T3,T4) compared to smaller tumours (T1,T2) suggesting proliferative index interfere with survival rates. Da Silva et al [21] have reported higher expression of Ki-67 associated with lymph nodes metastasis but did not reveal any impact on survival. Gueiros et al [22] have shown significant association of higher Ki-67 expression with nodal metastasis and distant metastasis and MCM 2 with tumor size and advanced clinical staging. However, they failed to establish the any relationship with survival. These conflicting results of the studies may be related to number of sample analysed, heterogeneity of sample or method of IHC analysis. In the present study, MCM2 expression at non involved margins emerged as novel marker predicting the recurrence and overall survival but failed as an independent prognostic marker of survival.

5. Conclusion

Investigating cellular proliferation at histologically negative margins in OSCC by using immunobiomarkers gives better understanding of protein expression and helps us in predicting survival. Our study findings suggest that MCM 2 is a novel marker at negative margins in predicting the recurrence and survival of oral squamous cell carcinoma. MCM 2 marker may help surgeons to reconsider on resection extent and planning adjuvant therapy in high risk oral squamous cell carcinoma patients. Further study with large sample size and multiple negative margins is recommended to confirm the prognostic value of MCM 2.

Acknowledgments and disclosure statements

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