Original Article

Minichromosome maintenance protein 5 – a promising prognostic marker of oral epithelial dysplasias and oral squamous cell carcinomas

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Abstract

Background: Early diagnosis is the single most effective means of reducing the mortality rate of oral cancer.

Aim: This study was undertaken to assess the expression of minichromosome maintenance protein 5 (MCM5) in oral epithelial dysplasias and oral squamous cell carcinomas (OSCCs) and to evaluate their possible role as a biomarker for early diagnosis and prognosis of OSCC.

Design: A retrospective cross-sectional study.

Materials and Methods: The study was conducted to assess the expression of MCM5 immunohistochemically in the tissue samples of oral epithelial dysplasias ($n = 27$) and OSCCs ($n = 30$) diagnosed between 2014 and 2019.

Statistical Analysis: The difference in the mean nuclear labelling index (LI) between the groups and the subgroups was analysed statistically using the Kruskal–Wallis test and the *post hoc* test, and the Dunn–Bonferroni multiple comparison analysis was conducted for pairwise comparison between the four main groups and the subgroups. The association between mean MCM5 LI and clinicopathological parameters was analysed using Spearman's rank correlation coefficient.

Results: A progressive increase in the nuclear expression of MCM5 protein (P-value <0.001) was noticed from normal oral mucosa through oral epithelial hyperplasia and oral epithelial dysplasia to OSCC. A significant correlation was also observed between the mean nuclear MCM5 LI of OSCC and TNM staging ($R^2 = 0.268$, $P = 0.029$).

Conclusion: Our findings suggest that MCM5 may be of great value in assessing the malignant potential of dysplastic lesions and may serve as biomarker of utility in the early diagnosis and prognosis of OSCC.

Keywords: Epithelial dysplasia, MCM5, oral squamous cell carcinoma

INTRODUCTION

Oral squamous cell carcinoma (OSCC) is the sixth most common malignancy worldwide[1] and in the Indian subcontinent, and accounts for one-third of the world's burden of oral and oropharyngeal carcinomas.[2] The high mortality and morbidity rates in spite of the advances in

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the treatment modalities emphasize on the importance of detecting and treating OSCC appropriately, as early as possible. As most of the oral cancers are preceded by a clinically and histologically distinct premalignant stage, early detection of epithelial changes at the premalignant stage is imperative for prevention and better management of oral cancer patients. Though newer techniques are changing the face of oral cancer diagnosis, a promising biomarker, which might be helpful for assessing the potential of a dysplastic cell to undergo malignant transformation, is yet to be identified. As one of the features of epithelial dysplasia and malignancy is ectopic cell cycle entry, markers to detect and quantify proliferating cells will be having a great value in early detection of OSCC. Minichromosome maintenance proteins (MCMs), an essential component for the control of deoxyribonucleic acid (DNA) replication related to cell proliferation, had been suggested to be a promising proliferative cell cycle marker which may serve as a useful biomarker for cancer screening, surveillance and prognosis.[3] Though their potential role as a promising biomarker in the early diagnosis and prognosis of cancers of oesophagus,[4] cervix,[5] larynx,[6] thyroid[7] and salivary gland[8] had been well-documented, studies in epithelial dysplasias and squamous cell carcinomas arising from oral mucosa are limited in number. In OSCC, expressions of various MCM proteins had been analysed with MCM2 being the most studied protein. The increased expression of MCM5 in oral precancer stage and a significant association between higher expression of MCM5 protein and aggressive progression and poor prognosis of OSCC had been observed in the Taiwanese population[9] but no further studies had been conducted and no data in this regard are available from rest of the countries. This study undertaken to assess the expression of MCM5 in oral epithelial dysplasias and squamous cell carcinomas in the Indian population showed a progressive increase in MCM5 expression with increasing grades suggesting that MCM5 may play an important role in the progression of OSCC, the clinical relevance of which was further explored by analysing the association between MCM5 expression and TNM staging.

MATERIALS AND METHODS

After obtaining approval from the Institutional Ethics Committee, a retrospective cross-sectional study was conducted to assess the expression of MCM5 protein in the tissue sections from a study population which comprised four groups. A control group consisting of 10 tissue samples from normal oral mucosa formed group I. The sections of normal mucosae obtained from tissues were removed during third molar extractions; 10 cases of oral epithelial hyperplasia, 30 cases of oral epithelial dysplasias (10 cases each from mild, moderate and severe dysplasias) and 30 cases of OSCC (10 cases each from all the three grades) formed group II, group III and group IV, respectively. Three cases of severe dysplasias had to be discarded during evaluation due to poor quality of staining. All the tissue samples considered for the study in group IV were from patients who have not undergone any treatment for oral cancer. After obtaining informed consent from the subjects selected for the study, paraffin blocks were retrieved from the archives of the Department of Oral and Maxillofacial Pathology and the sections obtained from these blocks were stained using haematoxylin and eosin stains to confirm the diagnosis. For assessing the correlation between MCM5 expression and TNM staging, the details regarding TNM staging of OSCC cases were retrieved from medical records.

Immunohistochemistry

MCM5 expression was assessed by the immunohistochemical method using polymer–horseradish peroxidase (HRP) technique. Deparaffinized sections were washed in phosphate-buffered saline (PBS), and endogenous peroxidase activity was blocked using 0.3% solution of hydrogen peroxidase at room temperature for 5 minutes. After antigen retrieval, by microwave treatment, the primary antibody, MCM5 monoclonal mouse antihuman antibody (clone CRCT 5.1; Thermo Fisher, Massachusetts, USA) was applied for 60 minutes at room temperature, and the sections were washed in PBS. PathnSitu's PolyExcel HRP/DAB detection system was used for the detection of bound antibodies. Linking antibody and HR–peroxidase complex were added consecutively for 20 minutes at room temperature and again washed in PBS. The peroxidase activity was visualized with diaminobenzidine (DAB) and applied for 5 minutes.

Interpretation of staining

For the evaluation of MCM5 protein expression, the sections were initially scanned at low power. A prominent brown nuclear staining was considered as positive for MCM5 protein expression in our samples. For the evaluation of MCM5 protein expression, in the first three groups (group I, group II and group III), the superficial oral epithelium was divided into three compartments:
- Zone I – basal and parabasal layers
- Zone II – spinous layer
- Zone III – superficial layer.

A total of nine high-power fields (three fields from each zone) were evaluated in each tissue sample. In each high-power field, the number of positively stained cells

was counted in 100 epithelial cells. In cases of OSCC, nine high-power fields of malignant epithelial cells in the connective tissue stroma were selected randomly and positively stained cells among 100 cells were counted in each field. The counting of the immunopositive cells was independently conducted by two investigators. The interobserver variation noticed in 5% of sections was reassessed using a double-headed light microscope to achieve consensus. From the total number of cells counted, the MCM5 labelling index (LI) was calculated as a ratio of immunopositive cells to the total number of cells (900 cells).

Statistical analysis

All statistical analyses were performed by statistical software IBM SPSS version 20.0. To test the statistical significance of the differences in the mean nuclear MCM5 LI between the groups, the Kruskal–Wallis test was applied. The *post hoc* analysis was conducted using the Dunn–Bonferroni multiple comparison test for pairwise comparison between the four main groups and the subgroups of oral epithelial dysplasia and OSCC. The correlation between mean MCM5 labelling indices (LIs) with clinical parameters (TNM stage) was analysed using Spearman's rank correlation coefficient. A *P*-value <0.05 was considered statistically significant.

OBSERVATION AND RESULTS

In this study, of 30 cases of OSCC, 24 were males and six were females, while in dysplasias, of 27 cases, 21 were males and six were females. The mean age of patients in OSCC was 55 years, whereas in dysplasias, the mean age was 57 years. In OSCC, the tongue (63%) was the most frequent site of involvement followed by buccal mucosa (16%). About 40% of the dysplastic lesions included in the study were from the tongue followed by buccal mucosa (33%).

Expression of MCM5

The immunohistochemical analysis of various samples studied showed nuclear as well as cytoplasmic MCM5 expression. Nuclear positivity was seen as dark-brown stains in two patterns – darkly stained homogenous compact pattern and granular pattern. In normal mucosa among the 10 samples studied, six samples showed nuclear MCM5 expression in the basal and parabasal layers (zone I) and a complete absence of cytoplasmic staining was noticed in all these 10 cases. In epithelial hyperplasia, as with the normal mucosa, the nuclear expression of MCM5 was restricted to the basal and parabasal layers. An increase in both nuclear and cytoplasmic staining intensity was found with increased proliferative activity in the basal and parabasal layers. In oral epithelial dysplasias, a progressive

increase in the nuclear MCM5 expression was noticed from mild to severe dysplasias [Figure 1]. In mild dysplasias, the expression of MCM5 nuclear staining was noticed in zones 1 and 2 in seven cases, whereas three cases showed expression only in zone 1. In moderate dysplasias, though the expression of MCM5 was restricted to zones 1 and 2 as with mild dysplasia, the number of positive cells was more and the mean nuclear MCM5 LI was higher. Cytoplasmic staining was noticed in all these cases in zones 1 and 2 but with a less staining intensity compared to mild dysplasia. In severe dysplasia, nuclear MCM5 positivity was restricted to zones 1 and 2 in all the cases but there was a definite increase in the mean LI compared to moderate dysplasia. The comparison of the mean nuclear LI of MCM5 expression showed a progressive increase from mild to severe epithelial dysplasias as shown in Figure 2. When statistically analysed using the Kruskal–Wallis test, the difference in the mean value was found to be statistically significant with a *P*-value <0.001 [Table 1] following which the Dunn–Bonferroni multiple comparison test was conducted for pairwise comparison between the grades of dysplasia [Table 1.1].

The nuclear MCM5 expression was noticed in the entire thickness of the superficial epithelium in all the three grades of squamous cell carcinoma. In well-differentiated squamous cell carcinoma, the malignant epithelial islands in the superficial connective tissue showed positive nuclear staining only in the peripheral cells [Figure 3]. Cytoplasmic staining was noticed in the central cells especially in the islands with keratin pearls. The keratin pearls also showed positive immune reaction, and cytoplasmic staining intensity of tumour cells was more in the cells closer to the keratin pearls but, in the deeper islands and islands in the invading front, nuclear staining was noticed in all the cells and there was minimal cytoplasmic staining. In moderately differentiated

Table 1: The mean nuclear MCM5 labelling index in various grades of oral epithelial dysplasia

Dysplasia	No:	MCM5		*P*
		Mean	**SD**	
Mild	10	15.54	2.63	<0.001
Moderate	10	19.12	1.82	
Severe	7	27.90	4.85	

Table 1.1: Pairwise comparison of mean nuclear MCM5 labelling index among the various grades of oral epithelial dysplasia

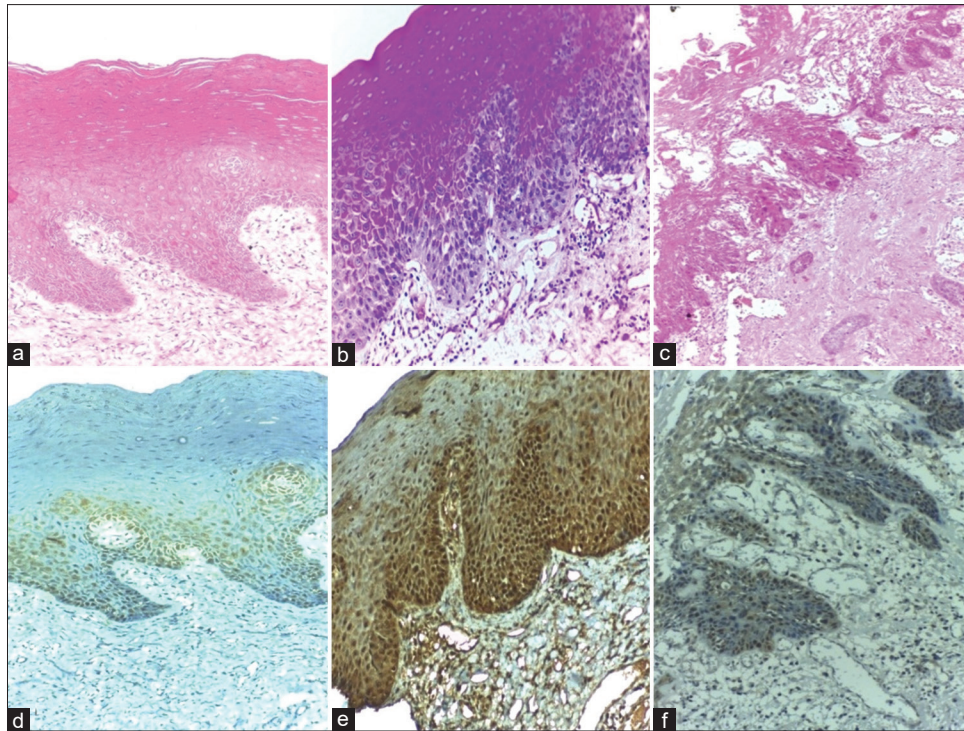| Dysplasia		*P*
Mild	Moderate	0.051
	Severe	<0.001
Moderate	Severe	0.012

Figure 1: Varying grades of dysplasia (a-c), (a) mild dysplasia, (b) moderate dysplasia and (c) severe dysplasia. H&E,10X. Progressive increase in nuclear MCM5 expression in varying grades of dysplasia (d-f). IHC, MCM5, 10X
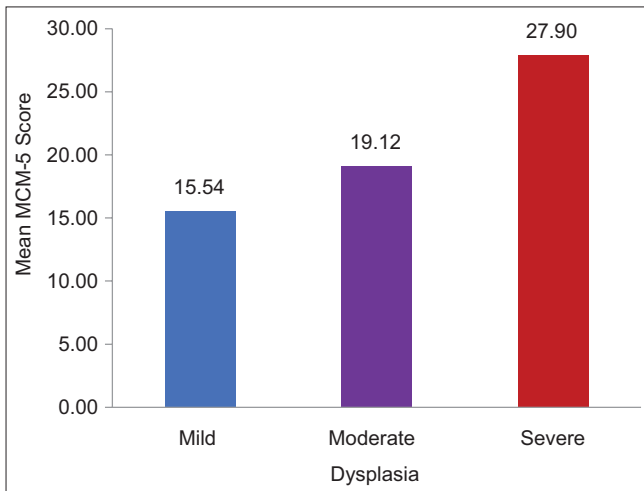

Figure 2: Bar diagram showing progressive increase in nuclear MCM5 expression with the increase in the grades of oral epithelial dysplasia
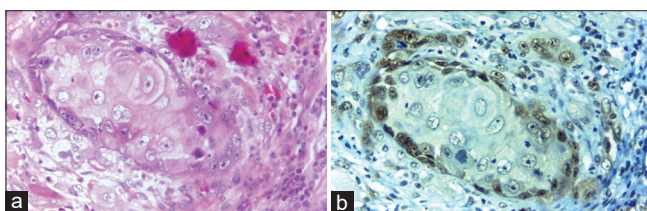

Figure 3: Well-differentiated squamous cell carcinoma. Positive MCM5 expression restricted to the peripheral cells of the island. (a) H&E, 20X and (b) IHC, 20X

squamous cell carcinoma, the tumour islands showed nuclear positivity in both peripheral and central cells with minimal cytoplasmic staining [Figure 4]. Intense nuclear staining with minimal cytoplasmic staining was noticed in the areas of muscular invasion and in islands closer to the nerve. In poorly differentiated squamous cell carcinoma, the MCM5-positive malignant cells were distributed throughout the connective tissue stroma [Figure 5]. The majority of the cells with positive MCM5 expression showed granular pattern of staining. A comparison of mean nuclear MCM5 expression of various grades of squamous cell carcinoma showed a progressive increase in expression from grade I to grade III [Figure 6], and the difference in the mean LI between various grades was found to be statistically significant with a *P*-value <0.001 [Tables 2 and 2.1].

The comparison of the mean nuclear MCM5 LI between the four main groups also showed a progressive increase in expression from group I to group IV [Figure 7]. On statistical analysis using the Kruskal–Wallis test, the difference in the mean nuclear MCM5 LI between the groups was found to be statistically significant with a *P*-value <0.001 [Table 3]. The pairwise comparison using the Dunn–Bonferroni multiple comparison test showed that the difference in the mean LI between normal mucosa and epithelial hyperplasia was not significant but the difference in the mean MCM5 LI of normal control group with oral epithelial dysplasia and OSCC was statistically significant [Table 3.1]. A statistically significant difference
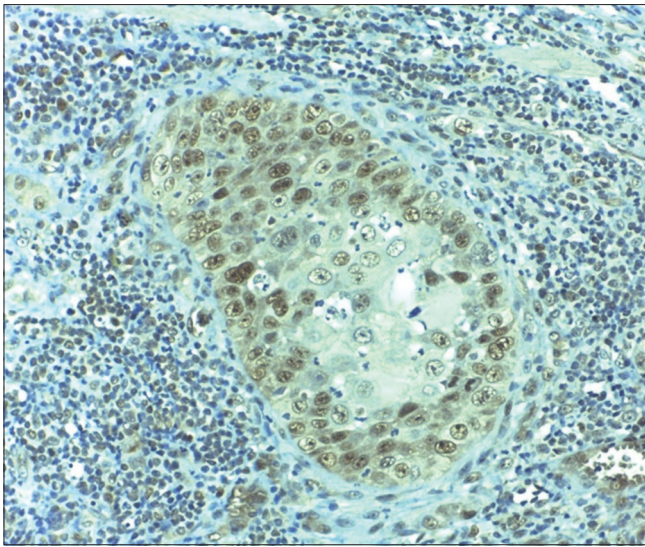
Figure 4: Moderately differentiated squamous cell carcinoma. Tumour islands showing positive MCM5 expression. Both compact and granular patterns of nuclear staining noted. IHC, 20X
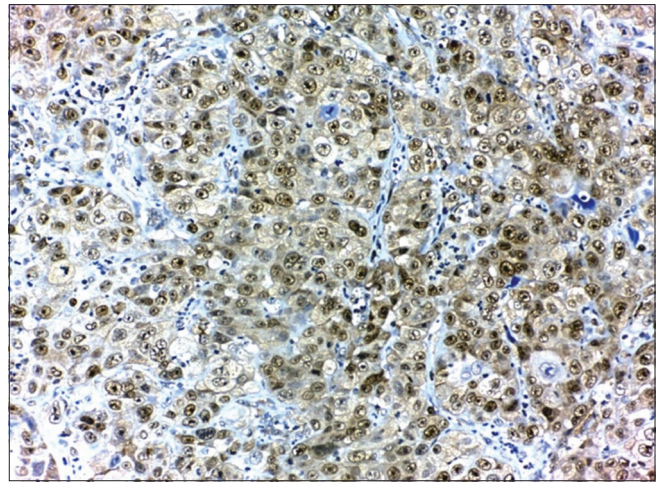

Figure 5: Poorly differentiated squamous cell carcinoma. Sheets of MCM5-positive tumour cells distributed within the connective tissue stroma. IHC, 10X
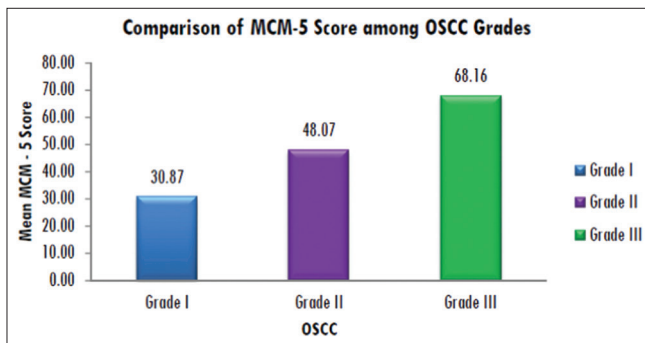

Figure 6: Bar diagram showing a progressive increase in nuclear MCM5 expression with increasing grades of oral squamous cell carcinoma
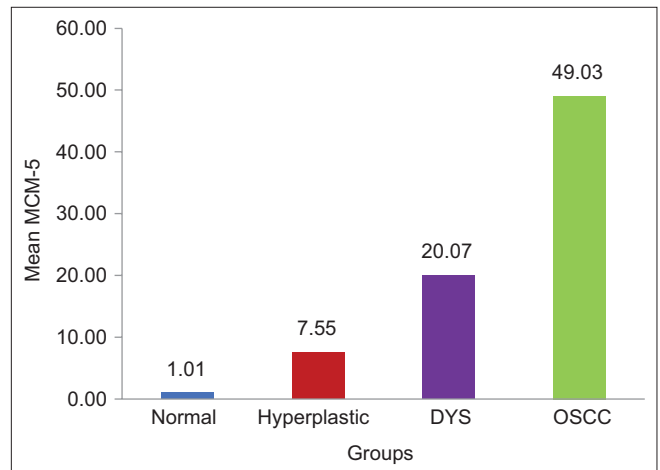

Figure 7: Bar diagram showing a progressive increase in MCM5 expression between normal oral mucosa, hyperplastic oral epithelium, oral epithelial dysplasias and OSCCs

Table 2: The mean nuclear MCM5 labelling index in various grades of oral squamous cell carcinoma

OSCC	No:	MCM5 score		P
		Mean	SD	
Grade I	10	30.87	9.27	<0.001
Grade II	10	48.07	8.86	
Grade III	10	68.16	13.19	

Table 2.1: Pairwise comparison of mean nuclear MCM5 labelling index in the various grades of OSCC

OSCC		P
Grade I	Grade II	0.017
	Grade III	<0.001
Grade II	Grade III	0.031

was also noticed between the mean nuclear MCM5 LI of oral epithelial dysplasias and OSCCs (*P*-value <0.001).

Spearman's rank correlation analysis showed a significant positive correlation ($R^2 = 0.268$, $P = 0.029$) between the expression of MCM5 protein and higher TNM status in OSCC [Figure 8]. A positive correlation was also noticed between MCM5 expression and tumour size ($R^2 = 0.191$, $P = 0.005$) [Figure 9] but no clinically significant correlation could be observed between MCM5 expression and nodal and distant metastasis when analysed separately. Similarly, no significant association was observed between MCM5 expression and other clinical parameters such as age, sex and site of the tumour.

DISCUSSION

MCM proteins are DNA-dependent ATPases required for the initiation of eukaryotic DNA replication, specifically the formation and elongation of the replication fork.[10] They function as a replication licencing factor and control the 'once per cell cycle' DNA replication in eukaryotic cells.
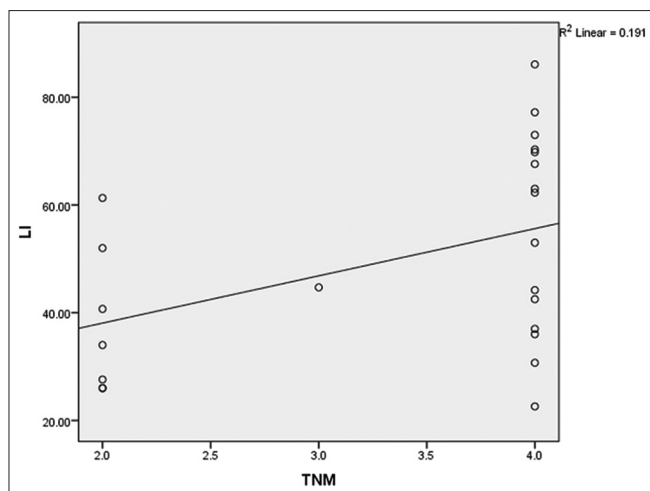
Figure 8: Scatter plot showing the correlation between mean nuclear MCM5 LI and TNM staging
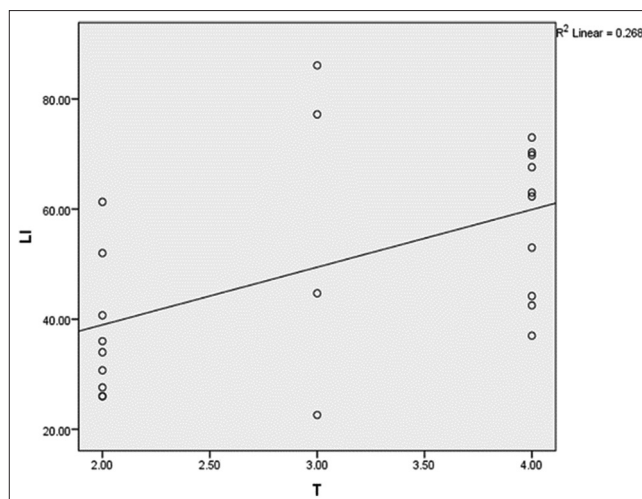

Figure 9: Scatter plot showing the correlation between mean nuclear MCM5 LI and tumour size

Table 3: The mean nuclear MCM5 labelling index in the four main groups

Groups	No:	MCM5		P
		Mean	SD	
Normal epithelium	10	1.01	1.34	<0.001
Hyperplastic epithelium	10	7.55	1.62	
Dysplasia	27	20.07	5.81	
OSCC	30	49.03	18.58	

Table 3.1: Pairwise comparison of mean nuclear MCM5 labelling index among the four main groups

Groups		P
Normal	Hyperplasia	0.317
	Dysplasia	<0.001
	OSCC	<0.001
Hyperplastic	Dysplasia	0.017
	OSCC	<0.001
Dysplasia	OSCC	<0.001

During the late M to early G1 phase of the cell cycle, they prime chromatin for DNA replication by binding origins of DNA replication.[11] Activated by S phase-promoting protein kinases, the origin-bound MCM complexes unwind the double-stranded DNA at the origins, recruit DNA polymerases and initiate DNA synthesis. Coupled with the initiation of DNA replication in the S phase, the MCM complexes move away from replication origins as a component of the DNA replication fork, likely serving as DNA helicases. Their departure deprives replication origins the ability to re-initiate DNA replication for the remainder of the cell cycle.[12] The requirement for MCM proteins in cycling cells and their absence in quiescent cells support strong evidence for their potential clinical application as cell proliferation markers. The MCMs represent the point of convergence of multiple cellular signalling pathways in promoting cellular proliferation and hence might serve as a sensitive and specific marker of the cells in the cycle. As

they detect more cells in cycle, they are considered to be a more superior proliferative marker than PCNA and Ki-67 to detect the cells undergoing ectopic cell cycle entry in epithelial dysplasia and squamous cell carcinoma.

In this study, normal oral mucosa showed a low nuclear MCM5 LI and the expression of MCM5 was seen only in the basal layer (zone I). Restriction of MCM5 expression in the basal layers of normal oral mucosa had been documented earlier.[9] Similar basal cell MCM2 expression in various normal mucosae including colorectal mucosa[13] and normal laryngeal mucosa had also been reported.[14] The presence of MCM expression in the basal layer alone suggests that the epithelial basal compartment has a low and controlled proliferation rate but with a continuous proliferative capacity. The complete absence of expression or absence of expression in significant number of cells in the basal layer found in a few cases in our study, however, might be due to the occurrence of most cells in the temporary G0 phase (not in cycle) with a lesser number in a licenced G0–G1 transition phase. In epithelial hyperplasia, as with the normal mucosa, the nuclear expression of MCM-5 was noticed in zone 1, but there was a slight increase in the LI. The intensity and distribution of MCM5 staining was found to be more with increased proliferative activity in the basal and parabasal layers. MCM5 expression in oral hyperplasia was not studied previously but similar expression of MCM2 restricted to basal proliferative compartment in benign keratosis had been observed earlier.[15] All the three grades of oral epithelial dysplasia showed nuclear MCM5 expression in zones 1 and 2. Though there was no considerable difference in the distribution of positive cells between the various grades, there was a statistically

significant difference in the mean nuclear MCM LI with mild dysplasia showing the lowest and severe dysplasia showing the highest value and a progressive increase in the expression of nuclear MCM was noted with progression of dysplasia. An increase in MCM5 expression with increasing grades of dysplasia was documented earlier.[9] Similar to their observation, we also noticed nuclear MCM5-positive expression in the lower one-third and the cytoplasmic MCM5 expression in the upper two-thirds of the epithelium. The superficial dysplastic epithelium in all the cases of squamous cell carcinoma showed positive nuclear MCM5 expression, and the expression was noticed in the entire thickness of the epithelium. This is in contrast with our findings in epithelial dysplasias where the expression was restricted to zone II, even in cases of severe dysplasias. The presence of nuclear MCM5 positivity in full thickness of epithelium at the areas of invasion suggests that full-thickness expression of MCM5 may be considered as a 'warning sign' for an impending malignant invasion and hence may be considered as a predictor of malignancy. Scott IS *et al*. reported high MCM2 expression in the surface layer in all the 10 cases of OSCC studied and suggested that the detection sensitivity in smears is likely to be very high as high frequency of expression of MCM2 was seen in surface layers in histological section of OSCC.[15] In well-differentiated OSCC, positive nuclear immunoreaction was evident at the periphery of the epithelial cell nests, while the core of the nests mainly the central keratinized cells showed cytoplasmic staining. Similar MCM protein expressions in well-differentiated OSCC had been described in many previous studies.[9,16-18] In moderately differentiated OSCC, all cells in the invading islands showed nuclear MCM5 expression irrespective of whether they are peripheral or central cells but cytoplasmic staining was minimal. Strict nuclear MCM2 expression with minimal or complete absence of cytoplasmic staining in moderately differentiated OSCC had been observed previously.[16,19] Total absence of both nuclear and cytoplasmic staining in some of the islands and few cells within some islands in the absence of differentiation probably suggests that these cells may be in the temporary G0 phase. In poorly differentiated squamous cell carcinoma, MCM5 expression was seen in most of the malignant cells distributed throughout the connective tissue stroma, in accordance with some of the previous studies,[16,18-20] indicating the considerable proliferative behaviour of OSCC of higher grades. The majority of the cells in all the cases of poorly differentiated SCC showed granular pattern of nuclear MCM5 with minimal cytoplasmic staining but, contrary to our findings, observed both nuclear and cytoplasmic

MCM2 immunoreactions in most cases of poorly differentiated OSCC cases included in their study.[18]

In our study, a stepwise and significant increased expression of nuclear MCM5 protein was noticed from normal oral mucosa to oral epithelial hyperplasia through oral epithelial dysplasia to OSCCs. Cytoplasmic staining, however, was seen more in well-differentiated squamous cell carcinoma compared to poorly differentiated squamous cell carcinoma suggesting that cytoplasmic staining can be taken as a marker of differentiation occurring in a cell which has increased proliferative potential. The cytoplasmic localization of MCM5 had been explained by the fact that in the S phase of the cell cycle, nearly the whole amount of MCM proteins dissociate from the chromatin, leaving only a fraction bound to unreplicated DNA. Subsequently, during the G2/M phase, MCM proteins are absent on chromatin and are detectable predominantly in the cytoplasm where they later undergo enzymatic degradation.[21] In this study, a positive correlation was noticed between higher TNM status and MCM5 protein overexpression suggesting their role as a prognostic marker to assess the biological behaviour of OSCC. The role of MCM5 in predicting the progression of OSCC had been suggested earlier by Yu SY *et al*. who noticed a significant correlation between the higher mean nuclear MCM5 LI and larger tumour size, positive lymph node metastasis and more advanced clinical staging.

Our findings suggest that MCM5 may be of great value in assessing the malignant potential of dysplastic lesions and may serve as progression marker in the potentially malignant oral lesions and OSCC. It may also serve as an important proliferative marker to evaluate the biological behaviour of OSCCs.

CONCLUSION

MCM5 may serve as an important marker to evaluate the biological behaviours of OSCC. The presence of this protein in all the phases of cell cycle helps in detecting more cells undergoing ectopic cell cycle entry in epithelial dysplasias and squamous cell carcinomas. Hence, MCM5 protein may turn out to be a promising marker superior to the conventional proliferative marker in use today as an effective diagnostic and prognostic tool in the assessment of oral potentially malignant disorders and OSCC.

Conflicts of interest
There are no conflicts of interest.

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