Annals of Diagnostic Pathology 34 (2018) 72–76

Contents lists available at ScienceDirect

Annals of Diagnostic Pathology

journal homepage: www.elsevier.com/locate/anndiagpath

MCM-2 expression differentiates potentially malignant verrucous lesions from oral carcinomas

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ARTICLE INFO

Keywords:
Mcm-2
Hyperplasia
Dysplasia
Carcinoma
Proliferation

ABSTRACT

Background: Mcm-2 is a biomarker belonging to Mcm family of proteins which has rarely been used in oral potentially malignant and malignant lesions of the verrucous type. The objective of this study is to assess the expression of Mcm-2 in Normal Oral Mucosa (NM), Verrucous Hyperplasia (VH), Verrucous Carcinoma (VC) and Oral Squamous Cell Carcinoma (OSCC) and compare it with the clinicopathological characteristics.

Methodology: A total of 70 formalin fixed paraffin embedded tissue samples (10 cases of Normal Mucosa NM-Group A, 10 cases of Verrucous Hyperplasia- VH without Dysplasia- Group B, 10 cases of Verrucous Hyperplasia-VH with Dysplasia- Group C, 20 cases of Verrucous Carcinoma VC-Group D, 20 cases of Oral Squamous Cell Carcinoma OSCC- Group E) were subjected to immunohistochemistry with Mcm-2 antibody. Statistical analysis was carried out with various tests like ANOVA, Tukey HSD, Chi-Square and Shapiro-Wilk test by using the SPSS software.

Results: There was a significant difference in Mcm-2 expression with quantitative analysis among all the groups ($p < 0.05$). There was a significant progressive increase in nuclear Labelling Indices (nLI) from NM (49.08%), VC (60.45%), VH with Dysplasia (64.10%), and OSCC (89.22%).

Conclusion: The findings suggest that Mcm-2 may be a sensitive proliferation marker in oral potentially malignant and malignant lesions which may be useful for differentiating between VH with/ without dysplasia, VC and OSCC.

1. Introduction

Cell proliferation is a process which is vital to all living organisms. The control of this process is dysregulated in cancer, pre-cancer and hence these can be used as effective markers for early detection and prognosis of potentially malignant lesions and oral carcinomas. The commonly used cell proliferation markers include Ki-67, PCNA, Geminin, Cyclin D1, and Cyclin B1 for various oral lesions. But the literatures on these markers show that they have several limitations [1]. Hence there is an utmost need for a more sensitive proliferative marker to overcome these limitations. Mcm (Mini chromosome maintenance) group of proteins are such a group of cell proliferation markers which are a pre-requisite for DNA replication and cell cycle initiation and are expressed throughout the cell cycle. From this family of Mcm proteins, the preferred target for phosphorylation of Mcm hexamer is the Mcm-2 subunit which induces a conformational change in the Mcm complex and thus has an essential function in DNA replication [2]. Mcm-2 protein can be a favourable marker for early detection of altered abnormal cells in potentially malignant and malignant lesions having a tremendous potential for prognostication.

Oral verrucous lesions have been classified in the past and the most recent classification based on the nature of the lesion includes benign, potentially malignant and malignant verrucous lesions [3]. Verrucous hyperplasia, proliferative verrucous leukoplakia are classified as potentially malignant lesions, and verrucous carcinoma as a malignant lesion. Oral Verrucous lesions are clinically and histologically a diverse group of lesions as they present a difficulty in differentiating between these [4,5]. Although several conventional cell proliferation markers like Ki-67, PCNA, and Geminin have been used in the past to differentiate the potentially malignant lesions and malignant lesions, this is a first study reported using Mcm-2 as a marker in oral verrucous lesions (OVL).

Hence the present study aims to assess the expression of Mcm-2 in oral potentially malignant and malignant verrucous lesions and comparison with oral squamous cell carcinoma (OSCC).

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https://doi.org/10.1016/j.anndiagpath.2018.03.001

2. Methodology

A retrospective study was carried out on the routinely processed, paraffin-embedded tissue blocks obtained from the archives of the Department of Oral Pathology at SDM College of Dental Sciences, Dharwad. Clinical data were recorded retrospectively of the histologically diagnosed cases of Verrucous Hyperplasia (with and without dysplasia), Verrucous Carcinoma and Oral Squamous Cell Carcinoma. The Haematoxylin and Eosin sections were examined for histopathology and the cases were selected. The study was approved by the Institutional review board (No: 2015/P/OP/42) of SDM College of Dental Sciences and Hospital, Dharwad.

2.1. Immunohistochemical analysis

The paraffin embedded sections of 3 μm thickness from the blocks on silanized slides were collected and kept for overnight incubation. Sections were deparaffinised in xylene, rehydrated in alcohol. Antigen retrieval was carried out by heating in a pressure cooker using Tris-Edta buffer for 3 whistles. The sections were allowed to cool, after which incubation with 3% hydrogen peroxide was done to block the endogenous peroxidase activity following which incubation with primary antibody Mcm-2 (Biogenex, San Ramon, USA) was carried out for 1 h at room temperature. Further incubation with super-enhancer and secondary antibody (30 mins) was done followed by visualization by freshly prepared DAB (Diamino benzidine) chromogen for 10 min. The sections were not allowed to be dry until this stage and phosphate buffer saline (PBS) was used as a wash buffer after each step. The slides were then counter-stained with Harris Haematoxylin. The stained slides were examined by optical microscopy and the positive cell distribution in the different levels of epithelium was observed. Nuclear staining was considered as positive for Mcm-2.

2.2. Evaluation of Mcm-2 staining

The presence of brown coloured end product at the site of target antigen indicated positive-staining. Representative areas were selected in each case based on the highest number of positively stained nuclei. The manual cell count was performed using an eyepiece graticule × 10 oculars × 40 objective and a counting grid (Lawrence & Mayo) containing 100 blocks. The cases were scored by counting the positive cells per maximum of 500 tumor cells per case. The percentage nuclear labelling index (nLI) (No. of positive cells/total no. of tumor cells expressed as a percentage) was calculated per case. The intra and inter-observer reliability was assessed.

The stained slides were examined by an optical microscope, and the positive cell distribution in the different levels of the epithelium was also analysed. As shown in Fig. 6 the epithelium was divided into 3 levels: Level I (lower one-third of the epithelium i.e. Stratum Basale and Stratum Parabasal), Level II (lower two-third of the epithelium i.e. Stratum Basale, Stratum Parabasal and Stratum Spinosum) and Level III (extending to the upper 1/3rd of the epithelium i.e. Stratum Basale, Stratum Parabasal, Stratum Spinosum and Stratum Superficiale or Stratum Corneum). The positively stained nuclei were counted as described earlier in all the 3 levels and nuclear labelling indices were calculated in Groups B, C and D [14]. (See Figs. 1–4.)

2.3. Statistical analysis

Chi-square test was performed to compare the association of patient's clinicopathological parameters and nuclear labelling indices (nLI) of the Mcm-2 proteins whereas, Kolmogorov–Smirnov, Shapiro–Wilk test were done to evaluate the distribution of the tests. Tukey post-hoc test was also done to compare the nuclear labelling indices between the above mentioned groups A to E. The nLI of Mcm-2 was compared with the patient's age, gender, site, tumor size and betel-quid, tobacco habits with chi-square test. SPSS software version 20.0 was used for statistical analysis. A value of $p < 0.05$ was considered to indicate a statistically significant result.

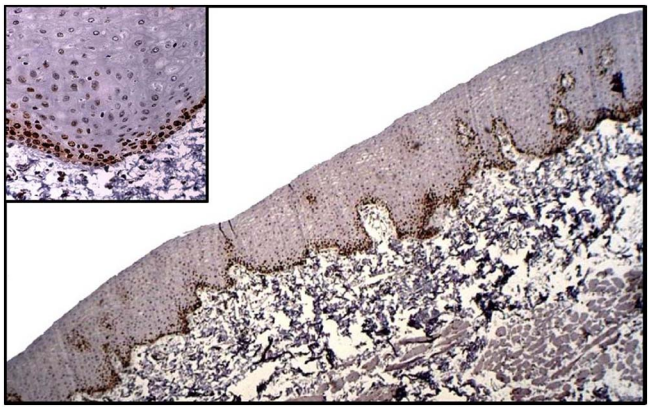

Fig. 1. (NM) Stratified squamous epithelium showing nuclear staining of Mcm-2 in basal and parabasal cell layer. (IHC-Monoclonal Anti-body, Mcm-2; Original Magnification 10×, inset 40×).

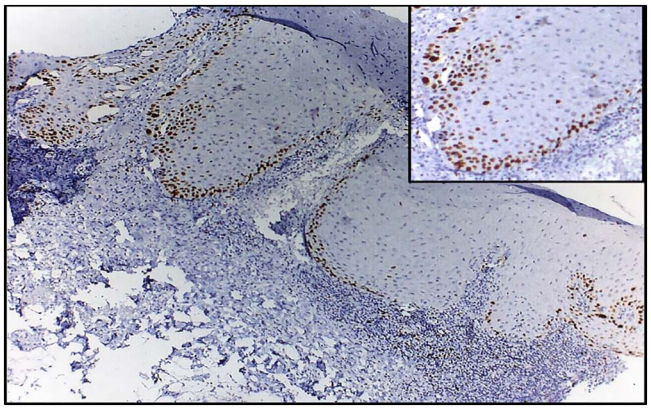

Fig. 2. (VH without Dysplasia) Hyperkeratinized stratified squamous epithelium showing prominent uniform nuclear staining of Mcm-2 in the basal, parabasal cell layers with randomly stained cells in the suprabasal cell layers. (IHC-Monoclonal Anti-body, Mcm-2; Original Magnification 10×, inset 40×).

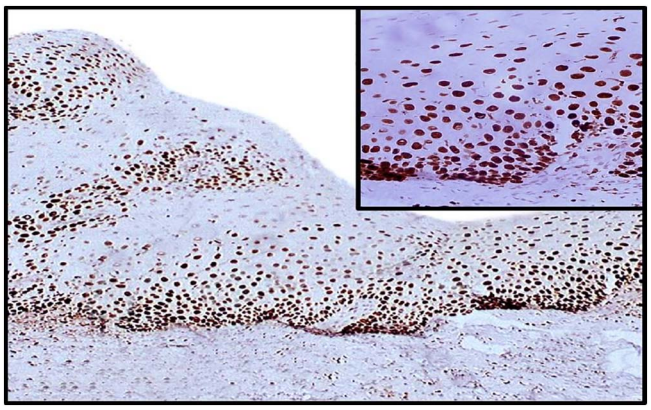

Fig. 3. (VH with Dysplasia) Hyperkeratinized dysplastic stratified squamous epithelium showing prominent nuclear staining of Mcm-2 in the basal to superficial cell layers involving > 2/3rd of the epithelium. (IHC-Monoclonal Anti-body, Mcm-2; Original Magnification 10×, inset 40×).

3. Results

This study comprised of 70 patients whose clinicopathological

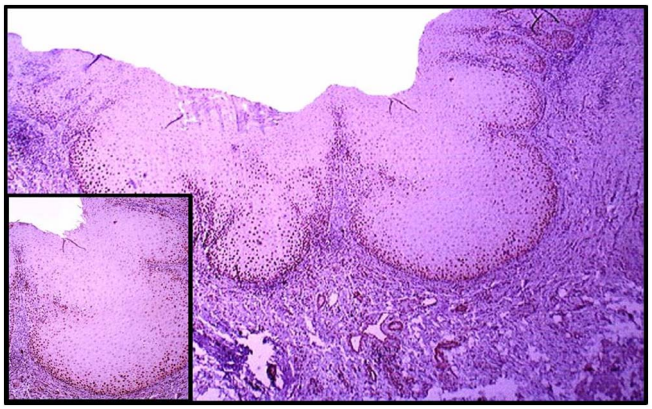

Fig. 4. (VC) Hyperplastic stratified squamous epithelium with pushing broadened rete ridge showing prominent nuclear staining of Mcm-2 in the basal, parabasal and several other cells of the suprabasal cell layers. (IHC-Monoclonal Anti-body, Mcm-2; Original Magnification 10×, inset 40×).

Table 1
Distribution of tissue samples in the study groups.

Groups (n = 70)	Tissue samples for Mcm-2 expression
A (10)	Normal Mucosa (NM)
B (10)	Verrucous Hyperplasia without dysplasia (VH without Dysplasia)
C (10)	Verrucous Hyperplasia with dysplasia (VH with Dysplasia)
D (20)	Verrucous Carcinoma (VC)
E (20)	Oral Squamous Cell Carcinoma (OSCC)

n = total number of tissue samples.

Table 2
Statistical analysis between the clinicopathological parameters and Mcm-2 expression in the study groups.

Category	Group B nLI	p value	Group C nLI	p value	Group D nLI	p value	Group E nLI	p value
Age								
< 50 yrs	38.92		65.54		67.16		80.66	
> 50 yrs	28.26	0.200	61.95	0.200	56	0.154	89.86	0.487
Gender								
Male	31.47		59.23		63.77		94.4	
Female	45.65	0.200	75.46	0.012*	41.66	0.068	80	0.072
Habit								
Tobacco	32		62.59		61.45		89.26	
BQ	44.05		74.8		53.8	0.80	–	
Combi	39.21	0.153	64.62	0.436	–		90.2	0.856
Size								
< 2 cm	32.36		61.3		44.6		90.1	
> 2 cm	37.12	0.200	64.8	0.542	62.21	0.020*	89.01	0.200
Site								
BM	35.73	0.200	60.75		56.11		89.22	
Others	–		71.92	0.339	70.58	0.839	–	0.60

* $p < 0.05$ (BQ- Betel Quid, Combi- Combination, BM- Buccal Mucosa).

parameters were collected from the case records and are listed in Table 1 and Table 2. The age of the patients at the time of diagnosis ranged from 18 to 71 years. Males were predominantly affected than females and buccal mucosa was the most commonly affected site in the oral cavity in all the study groups. The habit prevalence was also taken into consideration and tobacco chewing habit was the most common adverse habit seen in all the study groups. The nLI of Mcm-2 was compared with the clinicopathological parameters as shown in Table 2.

The nuclear labelling indices were compared between the groups and within the groups. There was a substantial progressive increase in the nuclear expression of Mcm-2 from VH without dysplasia to normal mucosa to VC and VH with Dysplasia and highest in OSCC. Highly significant results were obtained when the nuclear labelling index of NM was compared with that of OSCC ($p < 0.01$), similarly VH without Dysplasia had significant result when compared to VH with dysplasia, VC and OSCC. The nuclear labelling index of VC when compared to that of OSCC also yielded significant results ($p < 0.01$).

The nuclear labelling index was compared between the groups and significant differences were noted. The nLI of VH with dysplasia (64.10%) was found to be more than VH without dysplasia (35.73%), VC (60.45%) showed lesser nLI as compared to VH with dysplasia, whereas the highest nLI was seen in OSCC (89.22%) as compared to all other groups. The same has been shown in the Table 3. (See Table 4.)

Table 3
Statistical analysis using ANOVA welch test for comparison of Mcm-2 expression in the study groups.

	Groups (n = 70)	Mean	Std. Dev	Df2	p value
nLI (%)	A (10)	49.08	3.95	26.303	< 0.001*
	B (10)	35.73	11.45		
	C (10)	64.10	13.27		
	D (20)	60.45	15.93		
	E (20)	89.22	5.51		
	Total (70)	64.39	21.43		

* $p < 0.001$ (Highly Significant). n = Total number of tissue samples; Std. Dev = Standard deviation; Df = Degree of Freedom.

4. Discussion

Oral verrucous lesions are a diverse group of lesions with a wide range of differential diagnosis which creates a diagnostic dilemma for both the clinician and pathologist [4,5]. It becomes necessary to differentiate the potentially malignant verrucous lesions from oral carcinomas, for the better treatment and prognosis of these lesions [4]. As researchers believe that a great help could be offered by molecular approaches and immunohistochemistry, conventional cell proliferation markers including Ki-67, PCNA, Cyclin D1 have been the area of research in this diverse group of lesions.

Mcm-2, a member of Mcm group of proteins is recognized to be a novel proliferation marker in prediction of prognosis of potentially malignant verrucous lesions and oral carcinomas. Mcm-2 as compared to conventional proliferation markers such as Ki-67, PCNA [11], and Geminin is more sensitive as it identifies all the cells in the cycle, hence a pan-cell cycle marker [2].

Mcm-2 expression was seen in all the study groups- from Group A to Group E (Table 1). In the literature, Mcm-2 positive immunoreaction is reported in the different cell compartments including the nucleus, cytoplasm and the cell membrane [2], but in the present study positive Mcm-2 expression was restricted to the nucleus of cells in all the groups. Similar to the present study Mcm-2 nuclear expression was noted by Kodani [6], Chatrath [7], Torres Rendon [12]. This can be explained by the fact that when cells exit mitosis Mcm-2 accumulates in the nucleus (early G1 phase) and form the pre-replicative complexes with Cdc6, Cdt1, Cdc45 allowing CDK activated initiation of DNA synthesis during the subsequent phases thus licensing the cells to proliferate [2]. Mcm-2

Table 4
Quantitative analysis by the level wise distribution of Mcm-2 expression in the study groups.

Groups (n = 70)	No. of tissue samples	Level I	Level II	Level III	p Value
Group B	10	5	5	0	
Group C	10	1	6	3	< 0.001*
Group D	20	0	15	5	

* $p < 0.001$ (highly significant).

expression was also noted in cytoplasm and cell membrane alongwith a nuclear expression in studies conducted by Vargas [8]. This could be due to the fact that during S phase of the cell-cycle nearly the entire amount of Mcm proteins dissociate from the chromatin, leaving only a small amount bound to the region of unreplicated DNA [2]. Later during the G2/M phase, these Mcm proteins are not present on chromatin and are only detectable from the cytoplasm where they later undergo enzymatic degradation [2]. The membranous expression of Mcm-2 proteins could be due to presence of surface receptors on the cell membrane. In the present study no cytoplasmic or membranous staining was noted and purely nuclear expression was seen.

In the present study, the nuclear Mcm-2 immunoexpression in normal buccal mucosa was seen in all differentiating cells except the completely differentiated cells although an intense nuclear staining was seen in the basal and parabasal layers of the epithelium. This indicates that the normal process of maturation is maintained in the cells and the Mcm-2 protein expression is confined to the proliferative compartment with down regulation as epithelial cells undergo terminal differentiation. The Mcm-2 nLI was 49.08% in normal mucosa in the present study. Similar nLI in normal tissues have also been observed by other authors for Mcm-2 in oral mucosa in the range of 25%–45% [2,4,8,and]. This suggests that the epithelial basal and suprabasal compartments in NM have a low and controlled proliferation rate but with a continuous proliferative capacity.

Since the nLI of NM was 49%, this value was used as a cut-off value to estimate the overexpression in other study groups. The nLI of Mcm-2 expression was observed to be more than the cut off in Groups C, D and E. The nuclear labelling index showed a substantial progressive increase from NM to VC to VH with Dysplasia and the highest in OSCC.

In Group B, the Mcm-2 expression was seen in basal and parabasal layers (Level 1) with few cells in the middle third of the epithelium. This indicates that there is a controlled rate of proliferation in verrucous hyperplasia without dysplasia in the basal and parabasal cell layers with mostly differentiated cells in the superficial layers. The nLI of Mcm-2 in Group B (36%) was less than the nLI (49%) in normal mucosa in the present study. This could be due to the fact that most of the cells in verrucous hyperplasia without dysplasia are completely differentiated with a higher degree of hyperkeratosis. Since there are no studies reported in the literature till date with respect to Mcm-2 in Oral verrucous lesions [9,10], it is beyond the scope of our study and further studies with more number of samples should be evaluated to comment on expression of Mcm-2 in these lesions.

Mcm-2 expression in Group C was observed in basal, parabasal and middle third layers of the epithelium (Level 2) except the most differentiated cells in the superficial most layers. The nLI was found to be 64.10% which was more than the nLI of Group A and Group B. This signifies a constant cell-cycle re-entry of dysplastic cells that causes more cells to be licensed to proliferate. Although there are no reports of studies done by using Mcm-2 as a marker in Verrucous Hyperplasia with dysplastic changes, Torres-Rendon [12], has compared the expression of Mcm-2 in Normal Mucosa and Oral epithelial dysplasia and has found the nuclear expression of Mcm-2 to be increased in oral epithelial dysplasia (73.62%) due to the constant cell-cycle re-entry with some cells licensed, which is in agreement with the current study. The expression pattern was studied level-wise and it was seen that it extended up to level 2 and in few cases up to level 3 of the epithelium. This might be related to the presence of more number of less differentiated cells and more cells in a constant state of re-entry in the cell-cycle in VH with dysplasia. In a similar study done by Habiba [14], he found the expression of Hur and Ki-67 restricted to level 2 in oral verrucous hyperplasia although in oral borderline verrucous lesions had an expression pattern extending up to the level 3 similar to that found in our study.

An intense Mcm-2 nuclear staining pattern was seen up to the superficial layers of epithelium in Group D. The nLI in group D was found to be (60.45%) which is marginally less as compared to VH with dysplasia. This marginal decrease in the nLI of VC than VH with dysplasia could be due to the minimal atypia or dysplastic features in VC as compared to VH with dysplasia. Also a fact mentioned by Gimenez Conti [13], VC is characterized by a differentiation of a high order and epithelium shows little mitotic activity, hence the cells taking up Mcm-2 are lesser as only the least differentiated cells or the cells in the cell cycle will show Mcm-2 expression. The level-wise expression showed a similar pattern as in case of VH with dysplasia, with Mcm-2 positive expression extending up to level 3 of the epithelium in most of the cases. In the study by Habiba [14], only Hur expression was seen to be extending till the level 3 of the epithelium whereas, p53 and Ki-67 expression was seen only till level 2.

Gouvea [15] studied the expression of Mcm-2 in Proliferative Verrucous Leukoplakia, a potentially malignant verrucous lesion. Till date there have been no other studies in the literature with the help of Mcm-2 on Oral Verrucous Lesions involving NM, Hyperplasia, Dysplasia and Carcinoma.

Tumor cells located in the ITF have been suggested to be more aggressive in terms of metastatic potential and influence prognosis in OSCC [16]. In the present study, in Group E, the nuclear expression of Mcm-2 was assessed at the Invasive Tumor Front (ITF) of well-differentiated OSCC. At the ITF, Mcm-2 nuclear expression was observed to be more distributed among all the malignant epithelial cells with greater intensity. The nLI of Group E was found to be the highest among all the groups- 89.22%. This increase in Mcm-2 expression in the peripheral tumor cells and at the invasive fronts (Fig. 5) suggests a high rate of cellular proliferation and assists the subsequent invasion of the surrounding structures. This was in accordance with the study by Shalash [17]. Moreover statistically significant results were obtained when the nLI of OSCC was compared with the other groups including the NM, VH and VC.

A Clinico-pathological correlation was done with the groups C, D, E in which an over-expression of Mcm-2 was seen when compared to the cut-off value (nLI = 49%) of normal mucosa (Group A). The parameters used when comparing the Mcm-2 expression included age, gender, habit association, size of the lesion and site involved. In the present study, the nLI of Mcm-2 in Groups C and D when compared to the age was found to be higher in cases below 50 years whereas it was higher in cases above 50 years of age in Group E. Similarly in Group C, the nLI of Mcm-2 was higher in females, whereas the Mcm-2 expression was seen to be higher in Males in Groups D and E. Also, in the current study, the nLI of Mcm-2 when compared to the habit association showed an increase in Mcm-2 expression in cases with betel quid consumption in Group C. In Group D, the nLI of Mcm-2 was higher in cases with tobacco consumption whereas nLI was higher in a combination of both tobacco and betel-quid chewing in Group E. The size of the lesion whether < 2 cm or > 2 cm was compared with nLI of Mcm-2 expression. The nLI was found to be more in case of lesions > 2 cm in size in Groups C and D with a marginally higher Mcm-2 expression in cases < 2 cm in Group E. The buccal mucosa was the most commonly involved site and there was an increase in the Mcm-2 expression in the other sites involved in the oral cavity in Groups C and D. Although the Mcm-2 expression did not show statistically significant results in the Clinico-pathological parameters except in case of Mcm-2 expression in females of Group C and size of the lesion and Mcm-2 expression in Group D, but further studies with a larger sample size should be done to come to a definitive clinico-pathological correlation.

These findings indicate that the degree of Mcm-2 expression in oral verrucous lesions may be an effective diagnostic factor that determines the potential of a lesion for malignant transformation. It is important to emphasize that appropriate diagnosis of oral potentially malignant verrucous lesions and oral carcinomas can prevent wide surgical resection of the lesions. Early detection would offer better treatment, increased survival and improved quality of life.

The present study was the first initiative with Mcm-2 to differentiate between oral potentially malignant verrucous lesions and oral

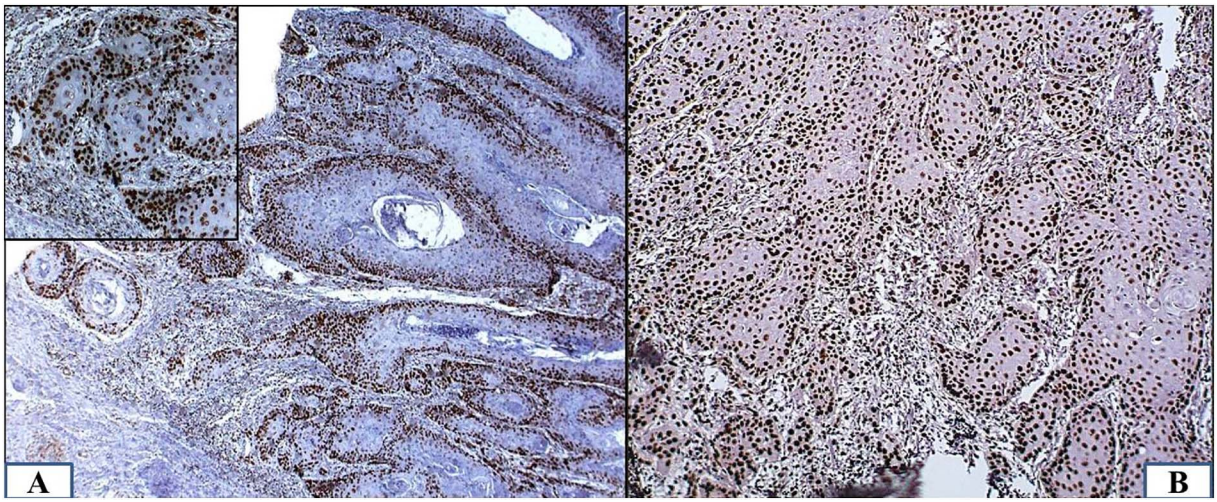

Fig. 5. (OSCC) A — ITF showing infiltrating solid cords, strands and islands with intense Mcm-2 nuclear expression in all the tumor cells except the cells (mature) in the centre of tumor islands. (IHC-Monoclonal Anti-body, Mcm-2; Original Magnification 10×, inset 40×). B — ITF showing infiltrating solid cords, strands and islands with intense nuclear expression of Mcm-2 in all cells of the tumor. (IHC-Monoclonal Anti-body, Mcm-2; Original Magnification 40×).

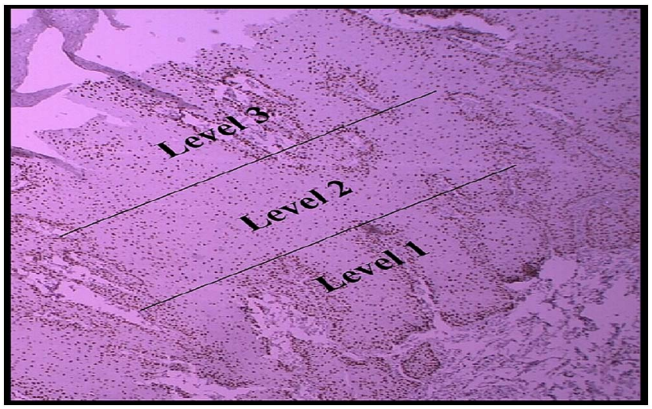

Fig. 6. Imaginary division of stratified squamous epithelium of Verrucous Hyperplasia with Dysplasia. The epithelium was divided into 3 levels to assess the distribution of positively stained cells with Mcm-2 antibody. The lower one-third (Stratum Basale and Stratum Parabasal), lower two-third of the epithelium i.e. Stratum Basale, Stratum Parabasal and Stratum Spinosum and extending to the upper 1/3rd of the epithelium i.e. Stratum Basale, Stratum Parabasal, Stratum Spinosum and Stratum Superficiale or Stratum Corneum were designated as Levels I, II and III respectively. (IHC-Monoclonal Anti-body, Mcm-2; Original Magnification 10×).

carcinomas. There was a substantial progressive increase in the nLI of Mcm-2 from Normal mucosa to VC to VH with Dysplasia to OSCC. In view of the present results, Mcm-2 can be used to differentiate among the diverse group of oral potentially malignant and malignant verrucous lesions. Mcm-2 is a pan-cell cycle marker which overcomes the shortcomings of conventional proliferation markers. Further studies need to be performed with larger sample sizes using conventional cell proliferation markers like Ki-67, Geminin and PCNA. In conclusion, these cell proliferation markers may serve as indicators for early detection which would offer better treatment, increased survival rate and improved quality of life.

Acknowledgements

The authors thank Prof. Srinath L Thakur, Principal, Dr. Kaveri Hallikeri, Professor and Head, Department of Oral Pathology, and Dr. MV Muddapur, Bio-statistician, SDM College of Dental Sciences and Hospital for their support and assistance throughout the study. Authors would also like to acknowledge Mrs. Hemalata Gudagudi for her technical assistance and Biogenex Lifesciences limited (California, USA) for providing Mcm-2 antibody with secondary Kit.

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