Original Article

Immunohistochemical expression and evaluation of cyclin D1 and minichromosome maintenance 2 in oral squamous cell carcinoma and verrucous carcinoma

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Abstract

Background: The study of cell proliferation is important for assessing the tumor behavior, prognosis and patient survival of oral carcinomas. As literature search did not reveal sufficient studies of immunohistochemical expression of cyclin D1 and minichromosome maintenance 2 (MCM2) in oral squamous cell carcinoma (OSCC) and verrucous carcinoma (VC), the present study was undertaken.

Materials and Methods: The study group included 20 cases of histopathologically diagnosed OSCC, 10 cases of VC and 10 cases of normal mucosa (NM). All samples were evaluated for the expression of cyclin D1 and MCM2 using standard Immunohistochemistry (IHC) procedure.

The present study involved both qualitative and quantitative analyses. Qualitative analysis was done by evaluation of intensity and area of staining. Quantitative analysis was done by calculating the percentage of positively stained cells and assessing the labeling index (LI). Data obtained were subjected to statistical analysis using SPSS statistical package (version 23.0).

Results: On evaluating and comparing the intensity of staining and area of staining of cyclin D1 and MCM2 between the study groups, statistically significant values ($P < 0.05$) were obtained using Kruskal–Wallis ANOVA. Comparison of LI of cyclin D1 and MCM2 in NM, OSCC and VC statistically significant results ($P < 0.05$) was obtained using Mann–Whitney *U*-test. Mean LI of MCM2 was found to be significantly higher than mean LI of cyclin D1 in all the study groups.

Conclusion: From the present study, we conclude that MCM2 has the potential to serve as a novel cell proliferation biomarker in OSCC and VC when compared to cyclin D1.

Keywords: Cell proliferation, cyclin D1, minichromosome maintenance 2, oral squamous cell carcinoma, verrucous carcinoma

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INTRODUCTION

Oral squamous cell carcinomas (OSCCs) belonging to a larger subgroup of tumors termed head-and-neck squamous cell carcinomas represent over 90% of malignant oral neoplasms.[1] According to the International Agency for Research on Cancer, the incidence rate of oral cancer in India is 12.6/100,000 people.[2] The high incidence of OSCC in India has been attributed to a variety of etiological factors such as tobacco smoking, tobacco chewing, alcohol consumption and human papillomavirus infections.[1] These factors may act individually or synergistically in oral carcinogenesis.[3]

Verrucous carcinoma (VC), a rare tumor first described by Ackerman[4] in the year 1948, is a low-grade variant of OSCC and is being considered as a separate clinicopathologic entity distinct from OSCC because of its unique biologic behavior and slow-growing nature. VC has a limited propensity to metastasize, hence with a better prognosis than OSCC.[5] Few studies reveal that some foci of OSCC may be observed in 20% of VC cases, making it a hybrid tumor, thus conferring it a metastatic potential.[6]

The prognosis of the patients decreases with increasing tumor stage, hence it is of great importance to detect the tumor as early as possible. If OSCC is diagnosed at an early-stage (T1N0) survival rate of up to 80% is noted, but in the later stages (T3–T4), it falls to about 20%–30%.[7] Studies have supported that oral carcinogenesis emerges from the accumulation of genetic alterations and epigenetic abnormalities in the expression of genes involved in cell proliferation.[8] Hence, the study of cell proliferation is important for assessing the tumor behavior, prognosis and patient survival.[9]

Numerous proliferation markers have been developed to detect and quantify the proliferation of cells in oral carcinoma.[9] Indeed, the strongest connection between cyclins and oncogenesis has been reported in studies conducted in OSCC.[10] Among the cyclins, cyclin D1 appears to be important in the G1 phase which is the only phase where the extracellular stimuli like growth factors can have an effect on the cell cycle.[11] Amplification and overexpression of cyclin D1 have been reported in head-and-neck, oral, laryngeal and nasopharyngeal carcinoma.[12] Similarly, because of its expression in the early G1 phase, few studies have demonstrated that minichromosome maintenance (MCM) proteins can be used as proliferation markers for determining the tumor behavior.[13] MCM2 protein can be used to estimate the proliferative index and also as a prognostic factor to determine the survival rate of patients with OSCC.[9]

Although few studies have been carried out to detect the expression of cyclin D1 and MCM2 in different grades of OSCC, literature search reveals very few studies on the expression of these markers in VC. With this background, the present study has been undertaken to evaluate the immunohistochemical expression of MCM2 and cyclin D1 in OSCC and VC.

MATERIALS AND METHODS

Study design and patient selection

A retrospective cross-sectional immunohistochemical analysis was carried out on 40 archival retrieved formalin-fixed paraffin-embedded tissue blocks. The study comprised 20 histopathologically diagnosed cases of OSCC (10 cases of well-differentiated squamous cell carcinoma [WDSCC] and 10 cases of moderately differentiated squamous cell carcinoma [MDSCC]), 10 cases of VC and 10 cases of normal mucosa (NM), and the immunohistochemical expression of cyclin D1 and MCM2 was analyzed in all the three groups.

Immunohistochemistry

The expression of cyclin D1 and MCM2 was evaluated using standard IHC procedure with anti-cyclin D1 (rabbit monoclonal antibody – EP12 [PathnSitu Biotechnologies Private Limited]) and anti-MCM2 (rabbit monoclonal antibody – EP40 [PathnSitu Biotechnologies Private Limited]). Positive control sections included tonsil for cyclin D1 and MCM2 and were treated in the same manner as the test groups.

Immunohistochemical analysis

The presence of brown-colored end product at the site of target antigen indicated positive staining. All the cases showed variable intensities of nuclear staining. To know the extent of stain uptake, intensity of staining was analyzed. Ten random fields were selected at ×40 magnifications in each slide. Sections were scored for staining intensity and scaled as follows:[14-16] 0 – no stain, 1 – mild stain, 2 – moderate stain and 3 – intense stain.

To know the expression pattern and also to determine the levels of protein expression in the epithelial layers, area of staining was determined by scanning the entire section of the epithelium and area of stained epithelial cells was recorded as:[17] 0 – 0%, 1 – <25%, 2 – 25%–49%, 3 – 50%–74% and 4 – 75%–100%.

To determine the labeling index (LI), the slides were examined under a light microscope (Olympus CX21) at ×40 magnification and representative photomicrographs were taken in five hotspot areas for each slide. The

photomicrographs were then analyzed using image processing program (ImageJ, http://imagej.nih.gov/ij/). Percentage of IHC-positive tumor cells per hot spot (A) was calculated and total number of tumor cells in each slide was calculated till a minimum of 400 cells were reached, i.e., the sum of the denominators (x).[17] LI was calculated using the formula:[18]

$$LI\% = \frac{\text{LI\%} = \text{A} \times 100}{\text{Total no. of tum or cells}(\times)}$$

Data obtained were subjected to statistical analysis using SPSS Version 17.0 (SPSS, Inc, Chicago, IL, USA). Kruskal–Wallis ANOVA and Mann–Whitney *U*-test were performed, and $P < 0.05$ was considered statistically significant.

RESULTS

Cyclin D1 and MCM2 positivity was seen in all cases. On comparison of staining intensity of cyclin D1 and MCM2 among the study groups, a statistically significant value ($P < 0.05$) was obtained using Kruskal–Wallis ANOVA [Table 1]. Similarly, on comparing the area of staining of MCM2 among the study groups, a statistically significant value ($P < 0.05$) was obtained using Kruskal–Wallis ANOVA. However, comparison of area of staining of cyclin D1 among the study groups was statistically not significant ($P > 0.05$) [Table 1].

On comparing the intensities of staining and area of staining between cyclin D1 and MCM2 in OSCC, a statistically insignificant value ($P > 0.05$) was obtained by using Mann–Whitney U-test. This could be because both markers predominantly showed intense staining (score 3) with 74%–100% positive cells (score 4) in OSCC [Table 2].

Although cyclin D1 predominantly showed moderate-to-intense staining (score 2 and 3) and MCM2 predominantly showed intense staining (score 3), on comparing the intensities of expression of staining between cyclin D1 and MCM2 in VC, a statistically insignificant value ($P > 0.05$) was obtained by using Mann–Whitney *U*–test [Table 2].

Similarly, on comparing the area of staining between cyclin D1 and MCM2 in VC, a statistically insignificant value ($P > 0.05$) was obtained by using Mann–Whitney U-test. This can be because, in VC, cyclin D1 showed mostly <24% positive cells (score 1), whereas MCM2 showed both <24% and 74%–100% positive cells (score 1 and 4) [Table 2].

On comparison of labeling index (LI) of cyclin D1 and MCM2 between the study groups, a statistically significant value ($P < 0.05$) was obtained [Table 3] using Kruskal–Wallis ANOVA.

On comparing the LI between cyclin D1 and MCM2 in NM, OSCC and VC, a statistically significant value was obtained ($P < 0.05$) using Mann–Whitney *U*-test [Table 4].

Table 1: Comparison of cyclin D1 and minichromosome maintenance 2 intensity and area of staining between the study groups

	n	Mean	SD	Mean rank	χ^2	*P*
Cyclin D1-intensity of staining						
NM	10	1.500	0.707	12.60	8.249	0.016*
OSCC	20	2.450	0.887	24.73		
VC	10	2.100	0.876	19.95		
Cyclin D1-area of staining						
NM	10	1.500	0.707	16.50	4.281	0.118
OSCC	20	2.350	1.309	24.10		
VC	10	1.600	0.843	17.30		
MCM2-intensity of staining						
NM	10	2.100	0.738	13.05	9.249	0.01*
OSCC	20	2.800	0.616	24.15		
VC	10	2.600	0.699	20.65		
MCM2-area of staining						
NM	10	1.700	0.675	11.55	12.012	0.002*
OSCC	20	3.250	1.070	26.23		
VC	10	2.400	1.174	18.00		

*Statistically significant using Kruskal-Wallis ANOVA.
MCM2: Minichromosome maintenance 2, SD: Standard deviation, NM: Normal mucosa, OSCC: Oral squamous cell carcinoma, VC: Verrucous carcinoma

DISCUSSION

Cell cycle progression is regulated by factors such as cyclins, cyclin-dependent kinases (CDKs), inhibitory enzymes, the retinoblastoma protein, p21, p27 and p53.[19] Among the cyclins, cyclin D1, a 45 kDa, 295 amino acid protein, is encoded by CCND1 gene located at chromosome 11q13. Overexpression of cyclin D1 is thought to provide the tumor cells with a selective growth advantage.[15]

MCM proteins were first reported by Maine in 1984 in an attempt to identify factors that originate DNA replication.[20] MCM2–7 are imported into the nucleus when CDK activity is low in early G1 and exported from the nucleus during S phase when CDK activity is high.[21]

According to molecular studies, MCM2 proteins identify both cycling cells and noncycling cells with proliferative potential.[22] Therefore, detection of cyclin D1 and MCM2 can be used to distinguish cells that exhibit aberrant cell proliferation activity.[23]

In the present study, in NM, 60% of the cases showed mild staining intensity of cyclin D1 in the nucleus of basal cells and few cells in the parabasal layer which is similar to

Table 2: Comparison between cyclin D1 and minichromosome maintenance 2 staining intensity and area of staining in normal mucosa, oral squamous cell carcinoma and verrucous carcinoma

Comparison	*n*	NM			OSCC			VC		
		Mean	SD	*P*	Mean	SD	*P*	Mean	SD	*P*
Cyclin D1 - I	10	1.5	0.707	0.079	2.45	0.887	0.155	2.1	0.876	0.173
MCM2 - I	10	2.1	0.738		2.8	0.616		2.6	0.699	
Cyclin D1 - A	10	1.5	0.707	0.525	2.350	1.309	0.022*	1.6	0.843	0.097
MCM2 - A	10	1.7	0.675		3.25	1.070		2.4	1.174	

*Statistically significant using Mann-Whitney U-test. MCM2: Minichromosome maintenance 2, SD: Standard deviation, NM: Normal mucosa, OSCC: Oral squamous cell carcinoma, VC: Verrucous carcinoma

Table 3: Comparison of labeling index of cyclin D1 and minichromosome maintenance 2 between the groups

	n	Mean	SD	Mean rank	χ^2	*P*
Cyclin D1 LI						
NM	10	8.41	5.10	8.30	14.663	0.002*
OSCC	20	21.05	5.44	25.70		
VC	10	21.68	10.90	24.10		
MCM2 LI						
NM	10	18.33	3.99	8.40	14.869	0.002*
OSCC	20	43.53	11.69	25.50		
VC	10	40.67	19.84	23.30		

*Statistically significant using Kruskal-Wallis ANOVA.
MCM2: Minichromosome maintenance 2, SD: Standard deviation, NM: Normal mucosa, OSCC: Oral squamous cell carcinoma, VC: Verrucous carcinoma, LI: Labeling index

the study results of Swaminathan *et al*.[24] and Angadi and Krishnapillai.[15]

However, in the present study, 80% of cases showed intense staining for cyclin D1 in WDSCC [Figure 1] which is similar to the results of Ohnishi *et al*.[25] This is in contrast to the study of Patel *et al*.,[26] Angadi and Krishnapillai[15] and Goto *et al*.[27] where mild-to-moderate intensity of staining was observed in WDSCC.

Similarly, 50% of cases showed intense staining and 50% of cases showed moderate staining for cyclin D1 in MDSCC [Figure 2] which is nearly similar to the observations of Swaminathan *et al*.[24]

Forty percent of cases showed intense staining for cyclin D1 in cases of VC and 30% of cases showed moderate and mild staining [Figure 3] in contrast to Angadi and Krishnapillai[15] where predominantly mild staining was observed.

The immunoreactivity for area of staining of cyclin D1 in NM showed <25% of positivity in the nucleus of basal and parabasal cells. Whereas, in WDSCC, 80% of samples showed 50%–100% of positivity [Figure 4]. On analyzing the immunoreactivity for area of staining of cyclin D1 in MDSCC [Figure 5] and VC [Figure 6], 60% of cases showed <25% of positivity.

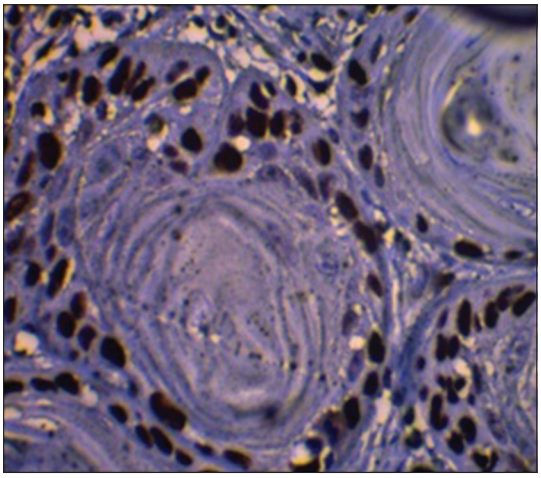

Figure 1: Cyclin D1 expression in well-differentiated squamous cell carcinoma with intense staining (score 3) at ×40 magnification

In our study, the intensity of staining expression for MCM2 in NM was found to be moderate, whereas 100% of WDSCC [Figure 7], 80% of MDSCC [Figure 8] and 70% of VC [Figure 9] showed intense staining. These findings are in accordance with Kodani *et al*.,[28] Chatrath *et al*.,[29] Shalash[21] and Torres-Rendon *et al*.[30] with regard to OSCC. The difference between the mean scores of intensity of staining of MCM2 between the study groups was found to be statistically significant.

MCM2 expression in NM shows that controlled cell division and proliferation ability occur only in basal and parabasal compartments while the superficial cells do not possess proliferative ability. This result was similar to that of Chatrath *et al*.[29] and Feng *et al*.[31] In contrast, Torres-Rendon *et al*.[30] investigated MCM2 expression in NM and found that MCM2 was mainly expressed at the suprabasal compartment only.

In the current study, 70% of the WDSCC [Figure 10] and 50% of MDSCC [Figure 11] showed more than 75% positivity of area of staining of MCM2, with expression along the periphery of the invaded epithelial islands, and at the invasive fronts. On the other hand, the central cores of the cell nests mostly showed negative MCM2 reaction.

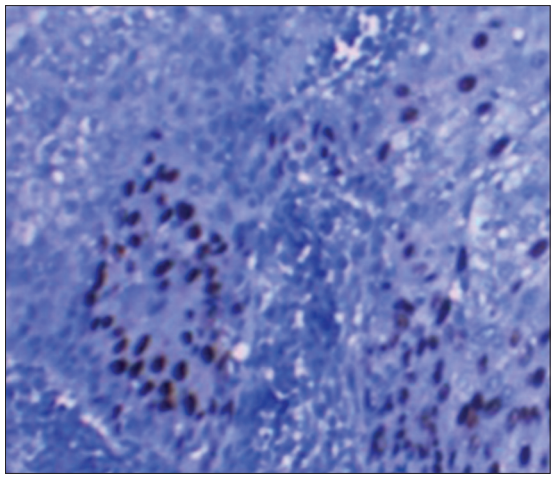

Figure 2: Cyclin D1 expression in moderately differentiated squamous cell carcinoma with moderate staining (score 2) at ×40 magnification

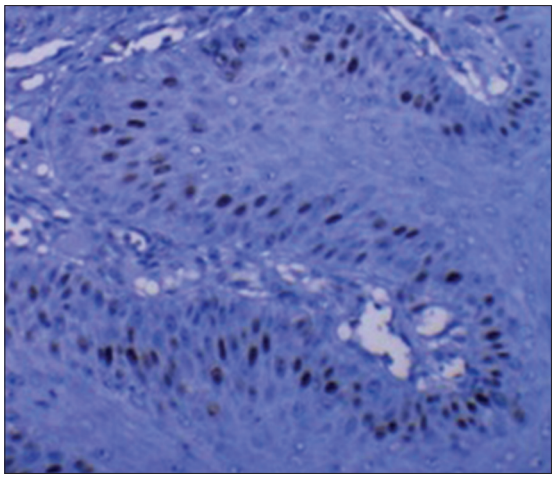

Figure 3: Cyclin D1 expression in verrucous carcinoma with mild staining (score 1) at ×40 magnification

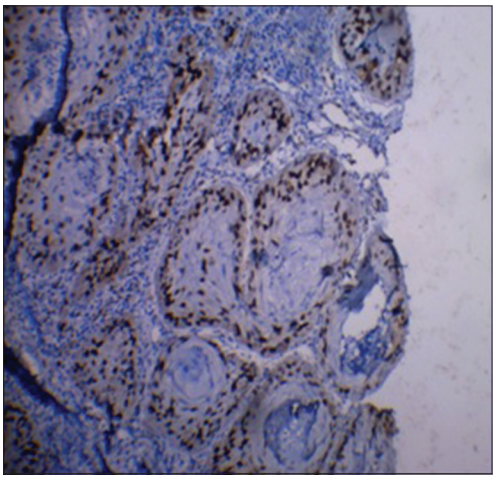

Figure 4: Cyclin D1 expression in well-differentiated squamous cell carcinoma with 50%–74% of area of staining (score 3) at ×10 magnification

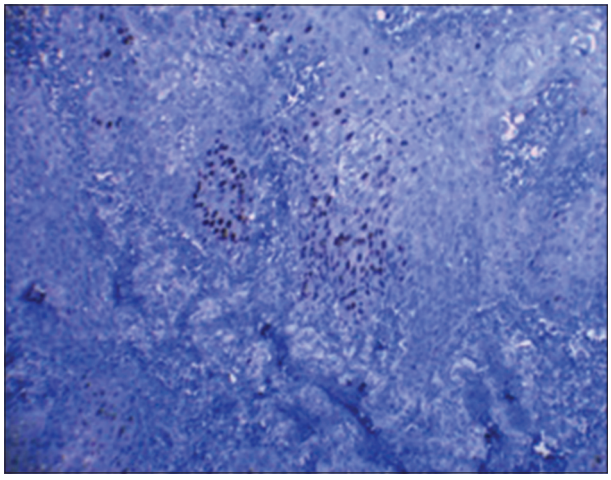

Figure 5: Cyclin D1 expression in moderately differentiated squamous cell carcinoma with <25% of area of staining (score 1) at ×10 magnification

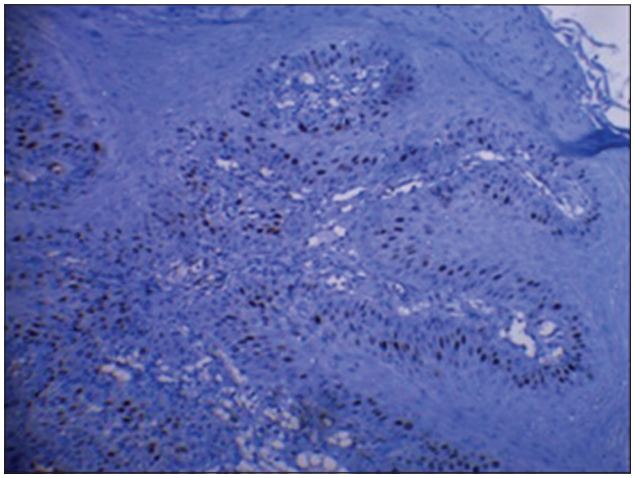

Figure 6: Cyclin D1 expression in verrucous carcinoma with 50%–74% of area of staining (score 3) at ×10 magnification

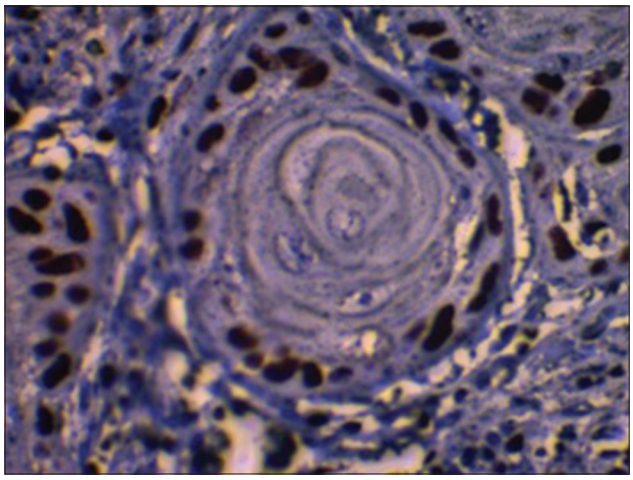

Figure 7: Minichromosome maintenance 2 expression in well-differentiated squamous cell carcinoma with intense staining (score 3) at ×40 magnification

The observations in WDSCC are in accordance with Shalash,[21] Szelachowska *et al*.,[32] Scott *et al*.[33] and Gouvêa *et al*.[34] Whereas, the findings in MDSCC are in accordance with Kodani *et al*.,[28] Chatrath *et al*.,[29] Shalash[21] and

Table 4: Comparison of labeling index between cyclin D1 and minichromosome maintenance 2 in normal mucosa, oral squamous cell carcinoma and verrucous carcinoma

Comparison	*n*	NM			OSCC			VC		
		Mean	SD	*P*	Mean	SD	*P*	Mean	SD	*P*
Cyclin D1 LI	10	8.41	5.098	0.007*	21.048	5.436	0.005*	21.681	10.902	0.017*
MCM2 LI	10	18.331	3.993		43.532	11.689		40.667	19.836	

*Statistically significant using Mann-Whitney U-test. MCM2: Minichromosome maintenance 2, SD: Standard deviation, NM: Normal mucosa, OSCC: Oral squamous cell carcinoma, VC: Verrucous carcinoma, LI: Labeling index

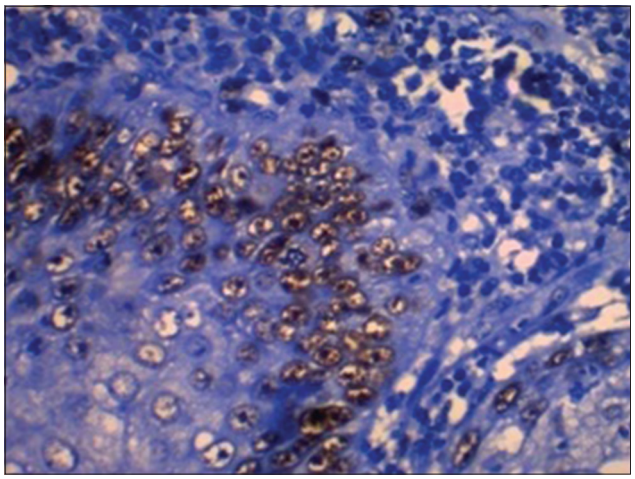

Figure 8: Minichromosome maintenance 2 expression in moderately differentiated squamous cell carcinoma with moderate staining (score 2) at ×40 magnification

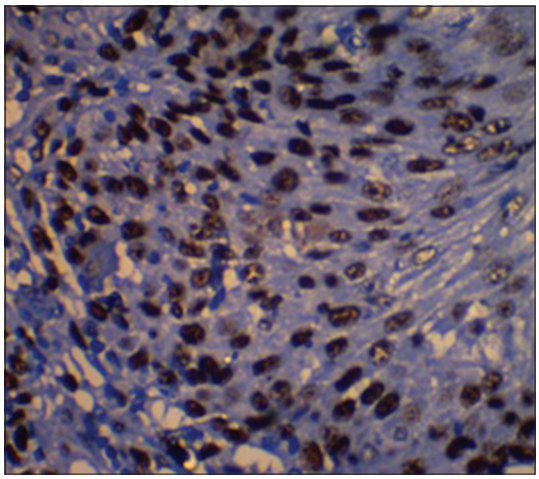

Figure 9: Minichromosome maintenance 2 expression in verrucous carcinoma with intense staining (score 3) at ×40 magnification

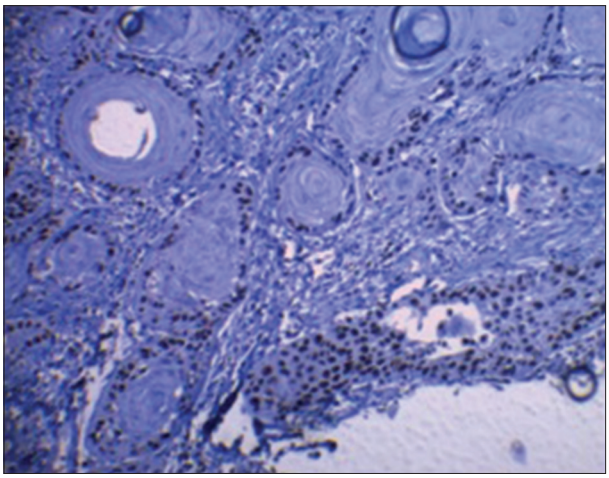

Figure 10: Minichromosome maintenance 2 expression in well-differentiated squamous cell carcinoma with more than 75% of area of staining (score 4) at ×10 magnification

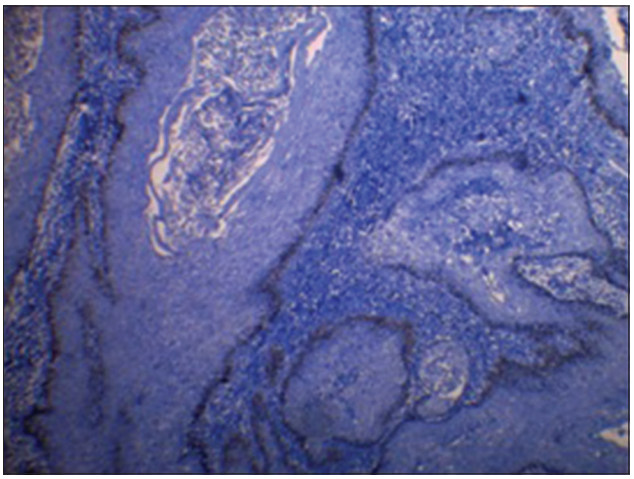

Figure 11: Minichromosome maintenance 2 expression in moderately differentiated squamous cell carcinoma with 50%–74% of area of staining (score 3) at ×10 magnification

Torres-Rendon *et al*.[30] The increase in MCM2 expression in the peripheral tumor cells and at the invasive fronts suggests a high rate of cellular proliferation and subsequent invasion into the surrounding structures.[35]

In our study, VC cases showed an average of 50% positivity for area of staining of MCM2 [Figure 12]. However, the results could not be compared due to the lack of published studies. VC is a tumor characterized by a differentiation of a high order in which the epithelium shows little mitotic activity.[36] This could be the reason for the cells taking up lesser MCM2 in our study.

In our study, the mean LI of cyclin D1 in NM was nearly similar to that of Moharil *et al*.,[37] while the mean LI of cyclin D1 for OSCC was nearly similar to the findings of Swaminathan *et al*.[24] The mean LI of cyclin D1 in VC in our study could not be compared directly due to lack of published reports.

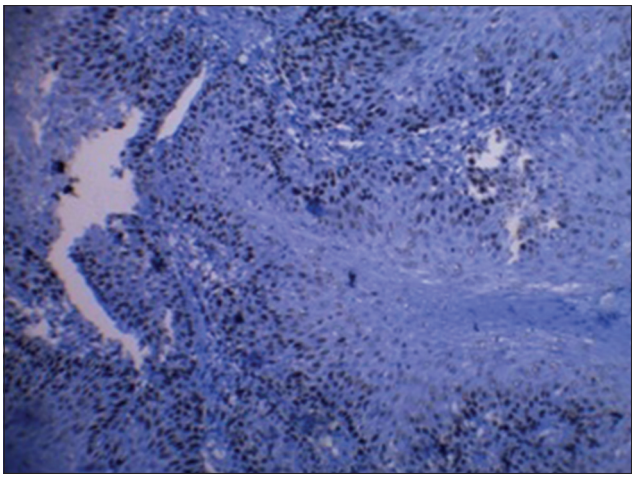

Figure 12: Minichromosome maintenance 2 expression in verrucous carcinoma with 50%–74% of area of staining (score 3) at ×10 magnification

In the current study, the mean value of LI of MCM2 in NM and OSCC was similar to observations by Kodani *et al*.[28] but lesser than the values published by Niranjan *et al*.,[35] Torres-Rendon *et al*.[30] and Razavi *et al*.[22] The mean LI of MCM2 for VC in the present study was lower than the value reported by Niranjan *et al*.[35] On comparing the mean LI of cyclin D1 and MCM2 between the NM, OSCC and VC, a statistically significant value was obtained ($P = 0.001$* and $P = 0.002$*, respectively).

In the present study, the mean LI of MCM2 in the study groups was found to be higher than the mean LI of cyclin D1. This could be because MCM2 proteins identify both cycling cells and noncycling cells with proliferative potential throughout the cell cycle and expressed in the cell nucleus from early G1 phase.[22] Hence, MCM2 can serve as a more potential biomarker for cell proliferation in OSCC and VC when compared to cyclin D1. Further studies need to be performed with larger sample size to validate the present findings.

CONCLUSION

The present study is probably an early initiative to evaluate the immunohistochemical expression of cyclin D1 and MCM2 in OSCC and VC. There was a substantial increase in the immunoexpression and mean LI of MCM2 and cyclin D1 from NM to OSCC. A similar progressive increase in the immunoexpression and mean LI of MCM2 and cyclin D1 was observed from NM to VC. A thorough literature search was done to find out the expression of cyclin D1 and MCM2 in VC-like lesions. However, only a very few articles were obtained for reference. Hence, the present study can have a place as one of the early studies attempted in expression of MCM2 and cyclin D1 in VC.

The interpretation of the present study also showed the highest expression of MCM2 in OSCC followed by VC, again showing the ability of the biomarker to be correlated with higher grade and establishing MCM2 as a better prognostic marker.

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Nil.

Conflicts of interest
There are no conflicts of interest.

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