# Assessment of Proliferative Index Between the Tumor Margin, Center of Tumor, and the Invasive Tumor Front of Oral Squamous Cell Carcinoma With the Help of Mcm-2: An Immunohistochemical Study

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**Aim:** The knowledge of cellular proteins that involves cell cycle and its control system is essential for understanding tumor biology. Minichromosome maintenance protein (Mcm-2), a component of prereplicative complex, essential for initiating DNA replication, is deregulated in different malignant lesions, and is expressed throughout the whole cell cycle including the G0 and G1 phases. This characteristic cell cycle event is not found in other proliferative markers such as geminin, AgNOR, Ki-67, and proliferating cell nuclear antigen. The aim of the present study was to analyze and compare the expression of Mcm-2 in normal oral mucosa (NM) and oral squamous cell carcinomas at tumor margins (TM), the tumor center (TC), and the invasive tumor front (ITF), with correlation of clinicopathologic features.

**Materials and Methods:** Tissues from 50 oral squamous cell carcinomas and 10 NM were archived retrospectively and stained with an antibody directed against the Mcm-2 antigen. A quantitative method was used to score the Mcm-2 expression in NM, TM, TC, and ITF. Nuclei labeling index for each case was estimated as the percentage of immunoreactive nuclei among 500 cells separately for NM, TM, TC, and ITF.

**Results:** Nuclei labeling index increases progressively from NM (49.08%), TM (67.79%), and TC (76.87%) to ITF (87.77%).

**Conclusions:** Cell proliferation by Mcm-2 at the ITF had a strong positive relationship with TC, TM. Mcm-2, a pan-cell cycle marker, is more sensitive in comparison with other conventional proliferative markers, which can be a better prognostic indicator.

**Key Words:** Mcm-2, prereplicative complex, oral squamous cell carcinomas, invasive tumor front, cell proliferation

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Oral squamous cell carcinoma (OSCC) represents the majority of malignant lesions of the oral cavity, particularly in developing countries, where large populations are exposed to carcinogens such as tobacco, smoke, and betel nut extracts.[1] Cell proliferation is a vital biological process to all living organisms. Abnormal cell proliferation seems to be a precursor and a possible predictor of tumorigenesis. The assessment of cell proliferation in many types of tumors is a significant adjunct to histologically based tumor cataloging and has potential relevance as an indicator of treatment response and relapse. Therefore, acquaintance with cellular proteins that control cell proliferation is essential for understanding the tumor biology.[2]

The benefit of minichromosomal maintenance (MCM) proteins as cell proliferative proteins is that they are pan-cell cycle markers owing to their presence throughout the cell cycle and are lost after their exit from the cell cycle, with quick loss after differentiation and slower loss in the quiescent (G0) cells[3]; therefore, it is a superior marker of cells in the cell cycle.[4] Other advantages of MCMs are that they do not detect cells undergoing DNA repair,[4] and they are not downregulated in proliferating cells by nutritional deprivation[5]; they identify a greater number of cells in the cycle than do staining for proliferating cell nuclear antigen and Ki-67.[6]

MCM proteins belong to the family of 6 heterohexameric major isoforms (MCM-2-7), are a key component of the prereplication complex, which is vital for the initiation of DNA replication, and epitomize the point of convergence of numerous signaling pathways involved in cell growth.[4] In the early G1 phase, the Cdc6 protein functionally networks with origin recognition complex and heaps the MCM proteins. The association of origin recognition complex, Cdc6, and MCM proteins in pre-RCs makes chromatin competent or "licensed" for replication.[7] The MCM proteins progressively dissociate from chromatin as S phase proceeds, consistent with their anticipated function as a DNA helicase. Dissociation of Cdc6 and MCM proteins from chromatin warrants that DNA is replicated only once during a single cell division cycle, as replicated chromatin is bereft of functional pre-RCs and thus not "licensed" for replication.[8]

Mcm-2 is a member of the MCM protein family that plays an important role in 2 crucial steps of the cell cycle,

namely the onset of DNA replication and the cell division. The desired target of phosphorylation of the MCM hexamer is the Mcm-2 subunit, which convinces conformational change in the MCM complex and thus has dynamic function in DNA replication. Unlike other MCM subunits, Mcm-2 binds specifically to chromatin.[9] As a consequence, the present study intended to evaluate the immunohistochemical expression of Mcm-2 in normal oral mucosa (NM) and OSCC at tumor margins (TM), tumor center (TC), and invasive tumor front (ITF) with clinicopathologic corelation.

## MATERIALS AND METHODS

### Ethical Approval

The research project was approved by the institution's IRB with the number 2014/*P*/OP/24.

### Patient Consent

Patients consent was not required.

### Tissue Samples

The scientific and ethics committee of the institution (IRB) has approved the present retrospective study. A total of 50 cases, which included histopathologically diagnosed cases of OSCC having ITF and TC; among 50 cases, 29 cases having TM with a site specification of buccal mucosa, were retrieved from the Department of Oral and Maxillofacial Pathology and Microbiology, SDM College of Dental Sciences and Hospital, Dharwad, between the year 1998 and June 2016. In total, 10 cases of NM were also included in the study.

### Immunohistochemistry

For immunohistochemistry, 3-μm-thick formalin-fixed paraffin-embedded sections were deparaffinized in xylene and then rehydrated in graded ethanol solutions. Antigen retrieval was accomplished by microwaving at 100°C in Tris-EDTA buffer (pH 9) for 15 minutes. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide for 10 minutes, followed by rinsing with phosphate-buffered saline (pH 7.4, 3 times), before immersing in blocking solution (Biogenex life sciences limited, CA) for 5 minutes at room temperature. Sections were incubated with the primary protein rabbit monoclonal antibody Mcm-2 protein (clone EPR4120; Biogenex Life Sciences Limited, CA). The antigenic sites were visualized using diaminobenzidine substrate chromogen and counterstained with Mayer's hematoxylin. Known positive sections of cervical carcinoma were used as positive controls. For negative control, sections were treated as above but without the primary antibody.

### Scoring of Mcm-2 Expression

A quantitative method was used to score the Mcm-2 expression in NM, TM, TC, and ITF. Cell counts were made at ×400 magnification using a 10×10 squared eye piece graticule (Leica Ltd) with a conventional light microscope to count the cells proficiently and without bias. Two individual observers carried out all the observations to eliminate the interobserver bias. Nuclei labeling index (nLI) for each case was estimated as the percentage of immunoreactive nuclei among 500 cells separately for NM, TM, TC, and ITF. Counting over 500 cells according to Brown and Gatter[10] is generally accepted as a minimal requirement.

### Statistical Analysis

Data collection was conducted using the Microsoft Office Excel package and processed with the SPSS 20.0 software package for statistical analyses. The student *t* test was used to evaluate the differences in the means of Mcm-2 proliferative index at NM, TM, TC, and ITF. ANOVA test and student *t* test was conducted to correlate the clinicopathologic features with expression of Mcm-2. $P < 0.05$ was considered statistically significant.

## RESULTS

Mcm-2-positive cells were clearly and easily identified by their brown nuclear staining.

The ages of the patients with OSCC ranged from 26 to 70 years (mean of 48 y); the patients were predominantly male individuals (45; 90%). The expression at the ITF (nLI = 87.77%) was greater than that at NM (nLI = 49.08%), TM (nLI = 67.79%), and TC (nLI = 76.87%), and this difference was statistically significant ($P < 0.05$) (Table 1). Mcm-2 nuclear expression did not show significant association with any of the clinicopathologic parameters ($P > 0.05$); this could be due to the small sample size, but a striking difference was observed with overexpression of Mcm-2, particularly at ITF, and in the clinicopathologic features. The mean Mcm-2 nLI for the selected ITF is higher in older age groups (above 45 y = 87.96%; below or equal to 45 y = 87.51%) with different levels of lymph nodes involved (lymph node involvement = 88.27%; no lymph node involvement = 87.58%), with endophytic tumor growth (endophytic = 89.13%; exophytic = 87.24%) in late TNM stage [early stage (I and II) = 86.88%; late stage (III and IV) = 88.19%], and with greater tumor size (< 4 cm = 86.76%; > 4 cm = 88.39%). Lastly, an increase in Mcm-2 nLI is observed at advanced grade of OSCC [Broder's grading (moderately differentiated = 88.94%; well differentiated = 87.05%), Bryne's grading (well

**TABLE 1.** Comparison of Mcm-2 nLI in NM With TM, TC, and ITF Using *T* Test

| Group | N | nLI (%) | SD (%) | *T* | *df* | *P* |
|---|---|---|---|---|---|---|
| NM | 10 | 49.08 | 3.95 | −4.871 | 37 | < 0.001* |
| TM | 29 | 67.79 | 11.83 | — | — | — |
| NM | 10 | 49.08 | 3.95 | −16.57 | 26.188 | < 0.001* |
| TC | 50 | 76.87 | 7.92 | — | — | — |
| NM | 10 | 49.08 | 3.95 | −24.89 | 20.484 | < 0.001* |
| ITF | 50 | 87.77 | 6.55 | — | — | — |

df indicates degrees of freedom; ITF, invasive tumor front; Mcm-2, minichromosome maintenance protein; nLI, nuclei labeling index; NM, normal oral mucosa; TC, tumor center; TM, tumor margins.

*$P = 0.001 < 0.05$.

**TABLE 2.** Correlation of Clinicopathologic Features With Expression of Mcm-2 (nLI) at ITF, TC, and TM in Oral Squamous Cell Carcinomas

| Clinicopathologic Features | Mean Value of Mcm-2 at TM (%) | Mean Value of Mcm-2 at TC (%) | Mean Value of Mcm-2 at ITF (%) | *P* |
|---|---|---|---|---|
| Age (y) | | | | |
| ≤45 | 66.35 | 76.35 | 87.51 | NS |
| >45 | 68.67 | 77.24 | 87.96 | NS |
| Sex | | | | |
| Male | 67.43 | 76.92 | 87.98 | NS |
| Female | 78.00 | 76.40 | 85.90 | NS |
| Lymph node involvement | | | | |
| Involved | 65.71 | 75.44 | 88.27 | NS |
| Not involved | 68.58 | 77.43 | 87.58 | NS |
| Type of the tumor | | | | |
| Exophytic | 68.76 | 76.76 | 87.24 | NS |
| Endophytic | 65.23 | 77.14 | 89.13 | NS |
| Stage of the tumor | | | | |
| Early | 67.60 | 75.52 | 86.88 | NS |
| Late | 67.86 | 77.50 | 88.19 | NS |
| Size of the tumor (cm) | | | | |
| <4 | 67.60 | 74.95 | 86.76 | NS |
| >4 | 67.86 | 78.04 | 88.39 | NS |
| Broder's grading | | | | |
| Well differentiated | 69.26 | 76.81 | 87.05 | NS |
| Moderately differentiated | 65.98 | 76.97 | 88.94 | NS |
| Bryne's grading | | | | |
| Well differentiated | 65.66 | 75.58 | 85.29 | NS |
| Moderately differentiated | 70.07 | 77.20 | 89.24 | NS |
| Poorly differentiated | 62.76 | 81.63 | 91.61 | NS |

Early stage = TNM stage I and II; late stage = TNM stage III and IV.

ITF indicates invasive tumor front; Mcm-2, minichromosome maintenance protein; nLI, nuclei labeling index; NS, nothing significant; TC, tumor center; TM, tumor margins.

differentiated = 85.29%; moderately differentiated = 89.24%; poorly differentiated = 91.61%)] (Table 2).

## DISCUSSION

Cell cycle aberration seems to be a precursor and a possible prognosticator of tumorigenesis. OSCC represents the majority of malignant lesions of the oral cavity, particularly in developing countries, where large populations are exposed to carcinogens such as tobacco, smoke, and betel nut extracts. In the current study, the immunohistochemical reactivity of Mcm-2 in all the cases of NM was intensely expressed to the basal and suprabasal cells (Fig. 1). These results are in accordance with Chatrath et al[3] and Feng et al.[11] These findings indicate that the cell division is confined to the basal and suprabasal cells, which constitute the population of least differentiated stem cells, which continue to be in cell cycle, whereas the superficial cells have lost their proliferative ability due to transition from progenitor to terminally differentiated cells, and thus are functionally unlicensed. Takeda et al[12] have suggested that stem cells are present in the basal layer of NM, on the basis of expression of stem cell markers, cytokeratin 19 and p63.

In contradiction, Torres-Rendon et al[13] found that the Mcm-2 protein was located mainly in the suprabasal compartment only. The absence of Mcm-2 expression in a significant proportion of basal cells was explained by assuming that these cells are in a temporary G0 state, as a part of a self-defense mechanism to maintain a controlled cell proliferation of the oral mucosa.

In all of the stratified squamous sections examined, there was an overall 49.08% mean Mcm-2 nLI, suggesting that there is close regulation of the proliferative components of these NM. Similar nLIs in normal tissues have also been observed by other authors for Mcm-2 and Ki-67 in oral mucosa by Kodani et al,[14] in the larynx by Chatrath et al,[3] in the prostate tissues by Meng et al,[15] and for geminin in breast tissue by Shetty et al.[16] This suggests that the epithelial basal and suprabasal compartments in NM have a low and controlled proliferation rate but with a continuous proliferative capacity.

In the present study, Mcm-2 nuclear expression was detected in all tissue sections, 50/50 (100%) of OSCC. Almost similar percentages of Mcm-2 immunoreactivity were previously detected in laryngeal squamous epithelial lesions,[17] in epithelial ovarian tumors,[18] breast cancers,[7] and colorectal cancer.[19] The present finding suggests that Mcm-2 may play a significant role in oral carcinogenesis.

In the current study, the positive Mcm-2 expression was detected only in the nuclei of the tumor cells in all the cases of OSCC and NM, and this is in accordance with the findings obtained by Kodani et al,[14] Chatrath et al,[3] and Torres-Rendon et al.[13] This could be explained by the notion that, when cells exit mitosis, these newly synthesized MCM proteins accumulate in the nucleus (early G1 phase) and assemble into prereplicative complexes.[20] The literature reports Mcm-2 as a nuclear marker; few studies showed Mcm-2 expression in the cytoplasm[12] and at the cell surface membrane of the tumor cells,[21] along with the nuclear one.[22]

The mean Mcm-2 nLI in NM in the present study was 49.08%, which is considered as a cut off for differentiating it from overexpression in OSCC. In contrast to NM, the increase in nuclear expression of Mcm-2 was observed in OSCC, and this difference was statistically significant ($P < 0.05$) (Table 1), indicating a constant ectopic cell cycle re-entry, with a high proportion of licensed and dividing cells in OSCC, which is a feature of malignancy. Increase in Mcm-2 nLI in OSCC compared with NM is in accordance with the result of previous studies conducted.[13,14]

According to Bryne et al[23] and Piffko et al,[24] the most useful prognostic information can be deduced from the invasive front of the tumors, where the deepest and presumably most aggressive cells reside. This is due to molecular events at the invasive tumor, which include increased angiogenesis, aberrant expression of adhesion-related molecules, overproduction of extracellular matrix–degrading enzymes, and increased expression of proliferation-related molecules.

Although the studies have been reported using Mcm-2 as a proliferative marker in OSCC, yet there is a lack of

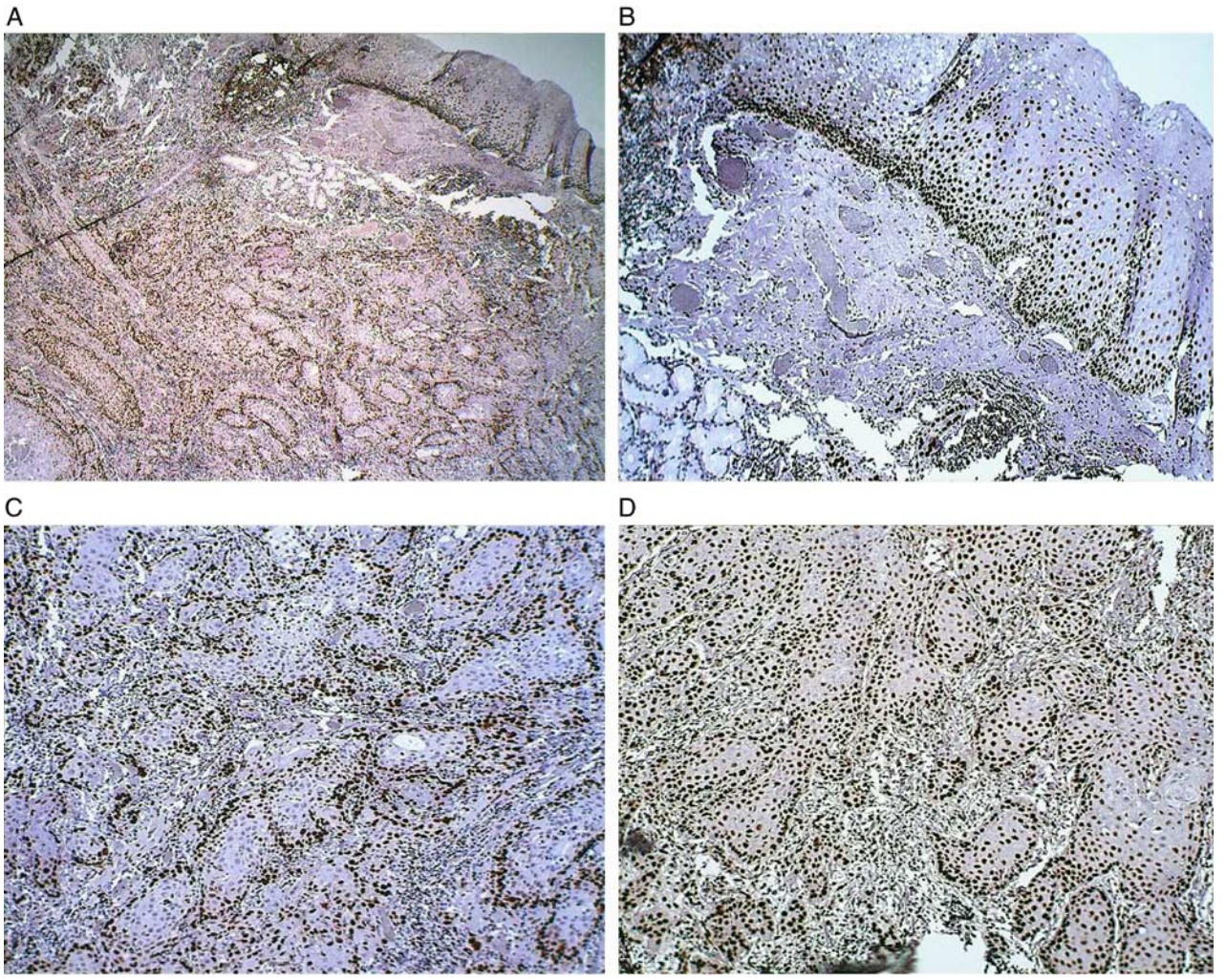


**FIGURE 1.** A, Expression of Mcm-2 protein in oral squamous cell carcinoma with TM, TC, and ITF (×40). B, TM shows nuclear expression of Mcm-2 in more than two third of the epithelium (×100). C, TC shows nuclear expression of Mcm-2 at peripheral cells with negative expression at the center of tumor islands (×100). D, ITF shows nuclear expression of Mcm-2 at peripheral cells and center of tumor islands (×100) (IHC-Mo Ab Mcm-2). ITF indicates invasive tumor front; TC, tumor center; TM, tumor margins.

literature with regard to its expression at ITF; hence, in the present study, Mcm-2 expression has been evaluated in NM, TM, TC, and ITF and compared with prognostic and risk factors in OSCC.

The nuclear expression at the ITF was greater than that at the TC and TM, and this difference was statistically significant ($P < 0.05$). TM shows increase in expression, as compared with NM, because it consists of a population of dysplastic cells, which continue to proliferate and to be in the cell cycle, while, at TC, peripheral cells are actively proliferating, but the center of islands with keratin pearl formation is differentiated, and hence shows loss of expression. The negative reaction at the superficial zone at TM and at the keratin pearls in TC suggests withdrawal of cells into the reversible state (G0) or terminally differentiated state where cells exist in the proliferative cycle and hence result in downregulation of origin licensing factor Mcm-2. ITF shows maximum positivity, as it consists of a population of undifferentiated cells that remain in cycle, as a result of deregulation of normal controls over cell proliferation. Similarly, Torres-Rendon et al[13] observed Mcm-2 expression in OSCC at ITF to be 81.16%, similar to the present study's 87.77% nLI.

A similar result has been previously reported for esophageal and OSCC using different a proliferative marker, Ki-67, wherein the deep margin showed high proliferative index than the central area by Kuwano et al.[25] According to Gerdes et al,[26] cells that are in early G1 phase could have been excluded from the estimate due to insufficient threshold of Ki-67 protein. This could be overcome by the new proliferative marker Mcm-2, which is expressed through the whole cell cycle, including the cell leaving the G0 phase to enter into the early G1 phase.

Increased expression from TNM stage I to IV of well to moderately differentiated carcinoma and that of

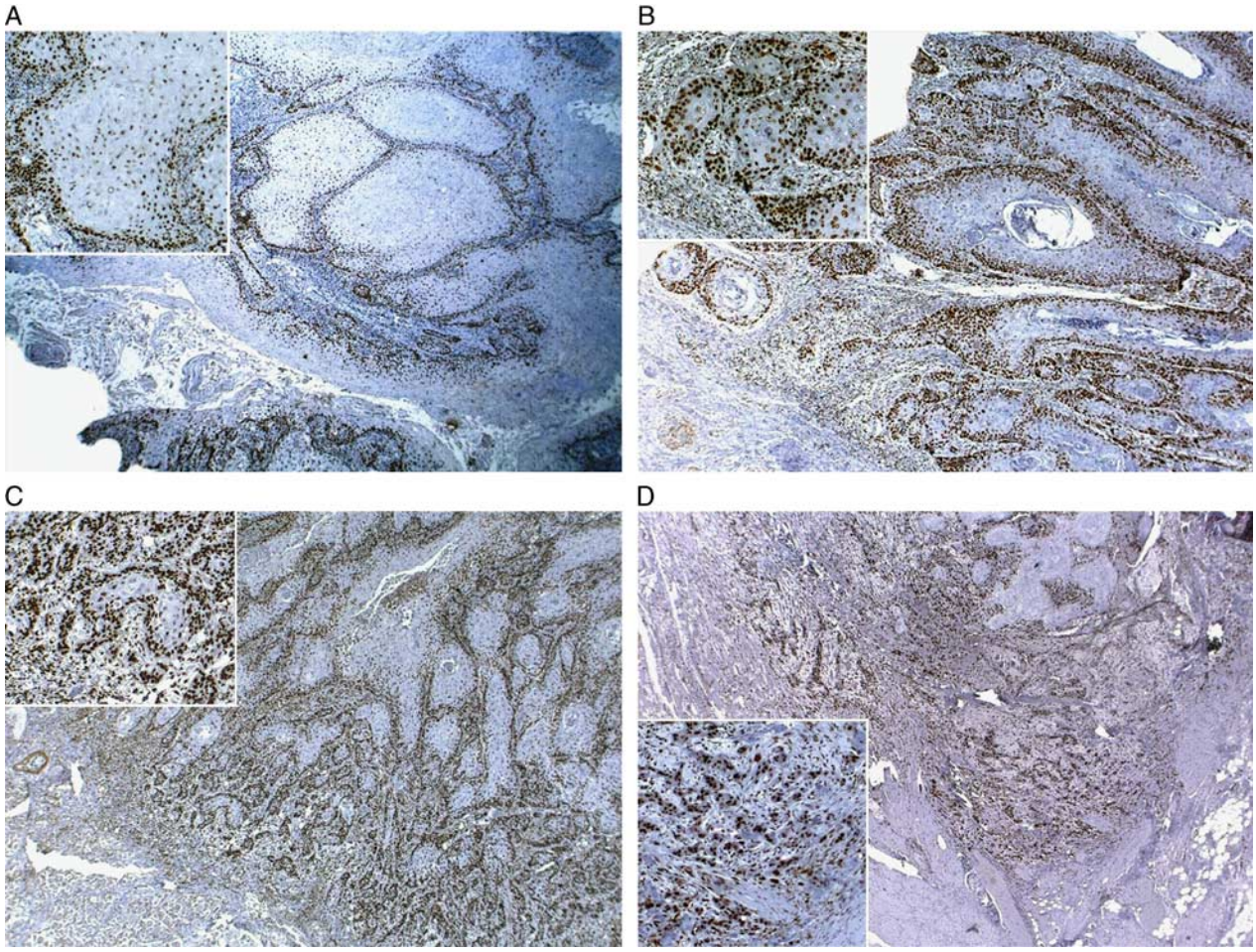


**FIGURE 2.** A, POI I (Bryne's criteria): pushing well-delineated infiltrating borders of tumor islands with Mcm-2 nuclear expressing only at peripheral basal and parabasal cells. B, POI II (Bryne's criteria): ITF shows infiltrating, solid cords, and strands of tumor islands with Mcm-2 nuclear expression diminishing toward the center. C, POI III (Bryne's criteria): ITF shows small groups or cords of infiltrating cells ($n > 15$) of tumor islands with Mcm-2 nuclear expression at the periphery and most of the cells in the center. D, POI IV (Bryne's criteria): ITF shows small groups and discohesive single cells ($n < 15$) of tumor islands with Mcm-2 nuclear expression of all the cells (×10 and ×40) (IHC-Mo Ab Mcm-2). ITF indicates invasive tumor front; POI, pattern of invasion. full color online

recurrence cases shows that Mcm-2 expression increases with the severity of tumor and thus can also predict about tumor aggressiveness and prognosis. Mcm-2 upregulation in ITF as well as in higher grade tumors suggests an early event that either is accentuated during dedifferentiation or, from the beginning, provides high-grade cells with a proliferative benefit, enabling them to evolve into high-grade carcinomas.

On the basis of the invasive front grading criteria proposed by Bryne, the pattern of invasion (POI) can be classified into 4 patterns: pushing well-delineated infiltrating borders; infiltrating solid cords, bands and/or strands; small groups or cords of infiltrating cells; and marked and widespread cellular dissociation in small groups and/or in single cells.[27] Although the result was not significant, an increasing value of mean Mcm-2 nLI was observed at different POI (POI I = 81.05; POI II = 88.49%; POI III = 89.18%; POI IV = 90%) (Fig. 2). This could be explained by the fact that noncohesive neoplasms infiltrate as small, irregular neoplastic cell aggregates or single cells consisting of a population of less differentiated cells and hence show diffuse Mcm-2 positivity.[28] A significant association between the POI and metastasis of the tumor to regional lymph nodes has been observed by several authors. Tumors that invaded in small groups or widespread cellular dissociation showed a higher tendency to metastasize to regional lymph nodes compared with tumors that invaded in pushing fronts or in solid cords and/or strands.[29]

Increased nuclear expression of Mcm-2 in cases of endophytic growth pattern, large size tumor, lymph node metastasis, TNM late stage (III and IV), moderately differentiated OSCC according to Broder's grading, and poorly differentiated OSCC according to Bryne's grading shows that Mcm-2 expression increases with the severity of tumor and thus can also predict about tumor aggressiveness and prognosis.

Similar findings in tumors of different locations reported that Mcm-2 protein represented a better proliferation

marker, and it carried higher predictive value than evaluations of lymph node involvement or the histologic grade of malignancy, and could serve as an independent prognostic factor.[7,15,30]

In conclusion, the presence of Mcm-2 protein as the prereplicative complex in the nuclei of neoplastic cells is consistent with the notion that these cells remain in cell cycle. Cell proliferation by Mcm-2 at the ITF had a strong positive relationship with TC and TM ($P < 0.001$), confirming that more cells from the ITF compared with other areas have been shown to be in the proliferative state, and thus informative in studies involving cell cycle control and other prognostic indicators. Further studies with Mcm-2 using larger sample size to determine clinical staging, histopathology grading, and recurrence of OSCC should be carried out to explore biological behavior and its prognosis.

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