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ORIGINAL ARTICLE

Minichromosome maintenance-2 (MCM2) expression differentiates oral squamous cell carcinoma from pre-cancerous lesions

Seyed Mohammad RAZAVI, Maryam JAFARI, Mitra HEIDARPOOR* and Saeedeh KHALESI**

*Department of Oral and Maxillofacial Pathology, School of Dentistry,*Department of Pathology, School of Medicine, Isfahan University of Medical Sciences, and **Department of Oral and Maxillofacial Pathology and Torabinejad Dental Research Center, School of Dentistry, Isfahan University of Medical Sciences, Isfahan, Iran*

Abstract

Background: Proteins necessary for DNA replication and normal regulation for the cell cycle include minichromosome maintenance-2 (Mcm-2). Overexpression of this protein in several premalignant and malignant lesions has been observed. In this study, the diagnostic value of Mcm-2 expression in distinguishing histologically-proven normal oral mucosa (NOM), oral benign keratosis (OBK), oral epithelial dysplasia (OED), and oral squamous cell carcinoma (OSCC) was investigated. *Materials and Methods*: In this descriptive analytical study, 73 archived specimens of oral tissues, including 20 OBK, 20 OED, 20 OSCC, and 13 NOM cases were selected. The means of labeling indices (LIs) of Mcm-2 expression by immunohistochemistry in each category of lesions were calculated. The data was analyzed by one–way ANOVA, discriminant analysis, and Fisher's exact tests. *Results:* The means of labeling indices (LIs) of Mcm-2 expression show statistically significant difference between the four studied groups (P<0.001). Mcm-2 had overexpression and higher positivity in OSCCs. A cut-off point of 67% was determined in order to distinguish OSCC from precancerous lesions. *Conclusion:* The findings indicated that Mcm-2 could be a useful marker for early detection of oral SCC and dysplasia. Also, due to the overexpression of this marker in OSCC, there exists the possibility of application of Mcm-2 for molecular target therapy in these patients.

Keywords: dysplasia, hyperkeratosis, squamous cell carcinoma, minichromosome maintenance proteins

INTRODUCTION

Oral squamous cell carcinoma (OSCC) is considered as the most common malignant lesion of the oral cavity.[1] The most common precancer lesions are white patches of the oral mucosa.[2] Despite various new treatment methods, 40% of patients with OSCC have a poor prognosis.[3] Early diagnosis of precancer lesions can help proper management and prognosis.[4] There are various unknown molecular mechanisms causing excessive proliferation, followed by the transformation process from normal mucosa to dysplasia and consequently to SCC. Recognition of cell proliferation regulating factors may be helpful in the early detection of dysplastic lesions and proper management, including treatment strategies with fewer side effects such as molecular targeted therapy.[5,6]

Minichromosome maintenance-2 (Mcm-2) cell-cycle regulatory proteins play on important role in regulating cell differentiation and cell proliferation.[7] Mcm-2 is expressed in four phases of the cell cycle.[8] Antibodies against Mcm-2 identify more cells in tissues in comparison with other proliferations markers such as Ki67.[9,10] According to molecular studies, Mcm-2 proteins identify both cycling cells and non-cycling cells with proliferative potential.[11,12] Many studies have showed Mcm-2 expression in neoplastic cells from different anatomical sites such as kidney, colon, and larynx.[2,8] Furthermore, recent studies conducted on precancerous and malignant lesions of oral cavity, and salivary gland tumours showed high expression of Mcm-2.[13,14]

Thus, considering the superiority and usefulness of this marker as a prognostic and

Address for correspondence: Saeedeh Khalesi, Assistant Professor, Department of Oral and Maxillofacial Pathology and Torabinejad Dental Research Center, School of Dentistry, Isfahan University of Medical Sciences, Isfahan, Iran. E mail: s_khalesi@dnt.mui.ac.ir. Tel: 00989131079487

diagnostic tool and the importance of diagnosing malignant lesions at early stages, this study was conducted to investigate the value of this marker in distinguishing normal oral mucosa (NOM), oral benign keratosis (OBK), oral epithelial dysplasia (OED) and oral squamous cell carcinoma (OSCC).

MATERIALS AND METHODS

Patients and tissue selection

In this descriptive analytical study, paraffin-embedded, formalin-fixed biopsy tissues of 73 patients in the archives of the Department of Oral and Maxillofacial Pathology, Dental School at Isfahan University of Medical sciences were retrieved. The 73 cases selected for analysis comprised 20 oral benign keratosis (OBK), 20 oral epithelial dysplasia (OED), 20 oral squamous cell carcinoma (OSCC), and 13 samples of normal oral mucosa (NOM).

The microscopical haematoxylin-eosin stained slides of all cases were reviewed by two oral pathologists to confirm the diagnosis. Furthermore, because inflammation may destroy the architecture of tissues, samples with more than 50 inflammatory cells in a field at ×100 magnification were excluded from this study.

Immunohistochemical staining

For the detection of Mcm-2 expression by immunohistochemical (IHC) staining, 3-4μm sections were cut from the paraffin-embedded tissues. The tissue sections were deparaffinized with xylene, and rehydrated with graded ethanol. For antigen retrieval, the sections were heated in a microwave oven at 96°C for 15 min in citrate buffer (pH 6.0), and then cooled at room temperature for 20 min. Endogenous peroxidase activity was blocked by incubation with 3% H2O2 in methanol for 20 min and the sections were washed with phosphate-buffered saline (PBS). The sections were then incubated with anti-Mcm-2 (BM28) mouse anti-human monoclonal antibody (clone 46; BD transduction laboratories, Lexington, KY, USA) at 1:50 dilution for 60 min. After that, the immunocomplexes were treated with post primary block and then detected by Novolink polymer (Novacastra, Germany) for 30 min, followed by washing in PBS. The immunoreactivity was visualized by diaminobenzidine (DAB) (DAKO, Denmark). In the final stage, the sections were counterstained with hematoxylin.

The Mcm-2 expression was confirmed by the presence of brown-stained nuclei in the epithelial cells in the tissue sections. The negative control for all tissues was based on staining by omitting the primary antibody. Sections of cervix with different grades of intraepithelial neoplasia were used as positive control.[9]

Assessment of immunohistochemical staining

IHC-stained slides were evaluated by two pathologists in a blinded manner with light microscopy (Olympus BX41TF, Tokyo, Japan). Ten representative fields per slide were examined at ×400 magnification. The most dysplastic areas of OED and the invasive front of the tumour in OSCC were selected for examination. For NOM and OBK, the most involved areas were selected as their representative fields.

An eyepiece graticule was used and the epithelial connective tissue interface was taken as the base of the square. All epithelial cells inside the area of the graticule were counted. Positive and negative immunoreactive cells and the total number of epithelial cells were counted. A mean of 1000 cells was counted for each case.

Expression of Mcm-2 protein was recorded into labeling indices (LI). The labeling index (LI) was calculated by dividing the number of immunopositive cells by the total number of cells per case and multiplying by 100.[13] Furthermore, the Mcm-2 expression level was evaluated using the semi-quantitative scale: 0 (negative: no immunostained cells), +1 (weak: <25% immunostained cells), +2 (moderate: 25 to 50%) and +3 (strong: >50%).[15,16] When there were considerable discrepancies regarding the results of IHC expression, slides were reviewed jointly by two pathologists and the final result was recorded according to the diagnosis agreed by both parties.

Statistical analysis

The obtained data from clinical and immunohistochemical studies were analyzed by the Statistical Package for the Social Sciences, version 18.0 (SPSS Inc., Chicago, IL, USA) using one-way ANOVA, discriminant analysis and Fisher's tests. The Mcm-2 protein expression, indicated by the mean of LI, in the four studied oral pathologies was compared using ANOVA test. Scheffe post-hoc test was used to compare pairs of tissues. A P-value <0.001 was considered statistically significant.

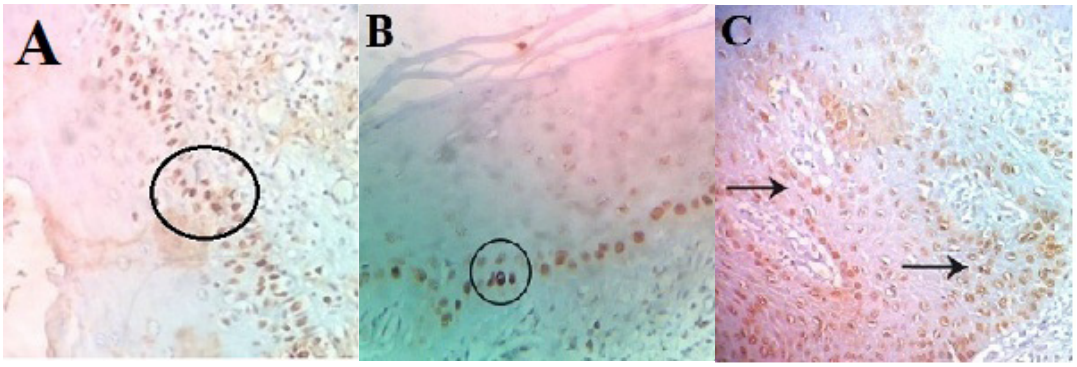

FIG. 1. Immunoexpression pattern of protein Mcm-2 staining in NOM (A), OBK (B) and OED (C) (original magnification ×100). Circles and arrows showing high immunoreactive cells.

RESULTS

The 73 cases in this study included 40 males (54.8%) and 33 females (45.2%). The mean age of the studied patients was 49.5. The cases of oral epithelial dysplasia (OED) comprised of 18 cases of mild dysplasia while the remaining cases had moderate dysplasia. The cases of oral SCC included 14 well-differentiated SCC and the others were moderately-differentiated according to the WHO classification.[17]

In NOM and OBK samples, the Mcm-2 protein was generally restricted to the basal and parabasal compartments (Fig. 1A and B). In OED, Mcm-2 protein was expressed at a higher frequency in the basal and parabasal compartments and extended to the mid-prickle cell region (Fig 1C). It should be noted that the Mcm-2 expression in the one of the mild dysplasia cases was of high frequency in all layers of the epithelium.

The Mcm-2expression in the OSCC samples was seen in a high number of epithelial cells with stronger staining intensities at the invasive front (Fig 2A and B). In tissue samples with histological keratin pearls, the higher Mcm-2 expression appeared around the periphery of the islands (Fig 2C). The moderately-differentiated (MD) OSCCs had a diffuse positive immunoreactive cell distribution (Fig 3). Evaluation of Mcm-2 expression level of the four studied groups was showed in Fig 4.

The means of labeling indices (LIs) of Mcm-2 was progressively higher from ONM and OBK through OED to OSCC (Table 1). The means of labeling indices (LIs) of Mcm-2 expression in OSCC was higher than NOM, OBK and OED. These differences were statistically significant ($P< 0.001$). Furthermore, the means of labeling indices (LIs) of Mcm-2 between OED and OBK was significantly different.

A cut-off point of 67% with 85% sensitivity and 92.5% specificity was determined for differentiating OSCC from pre-cancerous lesions by using the Statistical Package for the Social Sciences, version 18.0 (SPSS Inc., Chicago, IL, USA). The coefficients of discriminant functions

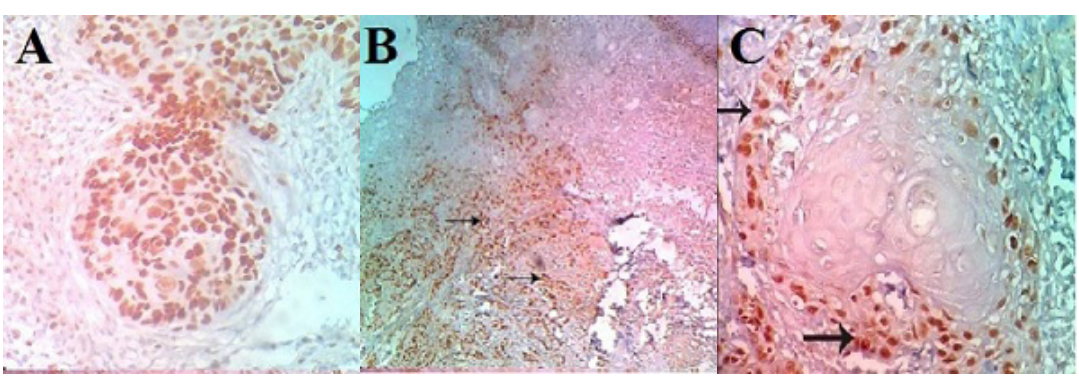

FIG. 2. (A) Immunohistochemical staining showing Mcm-2-positive cells in well-differentiated SCC (original magnification ×100), (B) Distribution of Mcm-2 at the invasive front of OSCC (×40), (C) Arrow showing the distribution of Mcm-2 around a keratin pearl (×400).

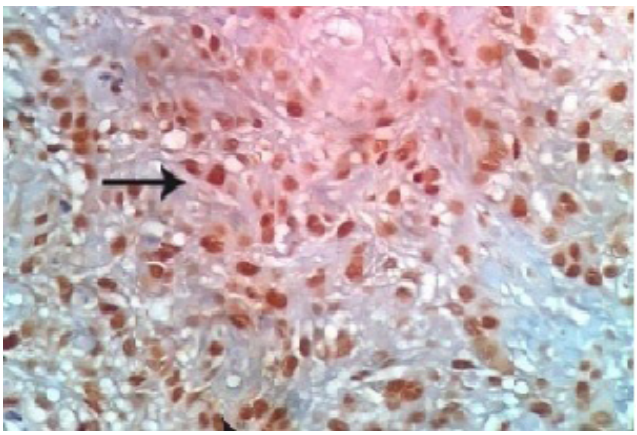

FIG. 3. Immunohistochemical staining showing Mcm-2-positive cells in moderately-differentiated OSCC (Original magnification ×400)

for different tissue types were calculated using discriminant analysis and Fisher test. The details of equations and calculated coefficients for the studied oral samples are presented in Table 2. The cases with greater value of equation were determined with using the obtained coefficient (Constant value; b0 and LI coefficient (b1) and equation y= b0+b1x, x=LI).

DISCUSSION

Mcms are sensitive and specific markers of cells in cycle.[2] The importance of pre-replication proteins such as Mcm-2 as prognostic markers of dysplasia and neoplasia various anatomical sites of the human body have been described. A few studies have been performed on the oral mucosa.[13] In this study, Mcm-2 expression was investigated three different oral pathologic lesions and compared with normal oral mucosa. The findings indicated that the Mcm-2 expression was significantly higher in OSCC than other oral categories. A cut-off point of Mcm-2 (67%) with acceptable sensitivity of 85% and specificity of 92.5% and an equation for differentiating OSCC from other benign lesions were introduced.

Mcm-2 expression in NOM and OBK epithelium was mainly in the basal and parabasal layers, while it was absent from other layers.

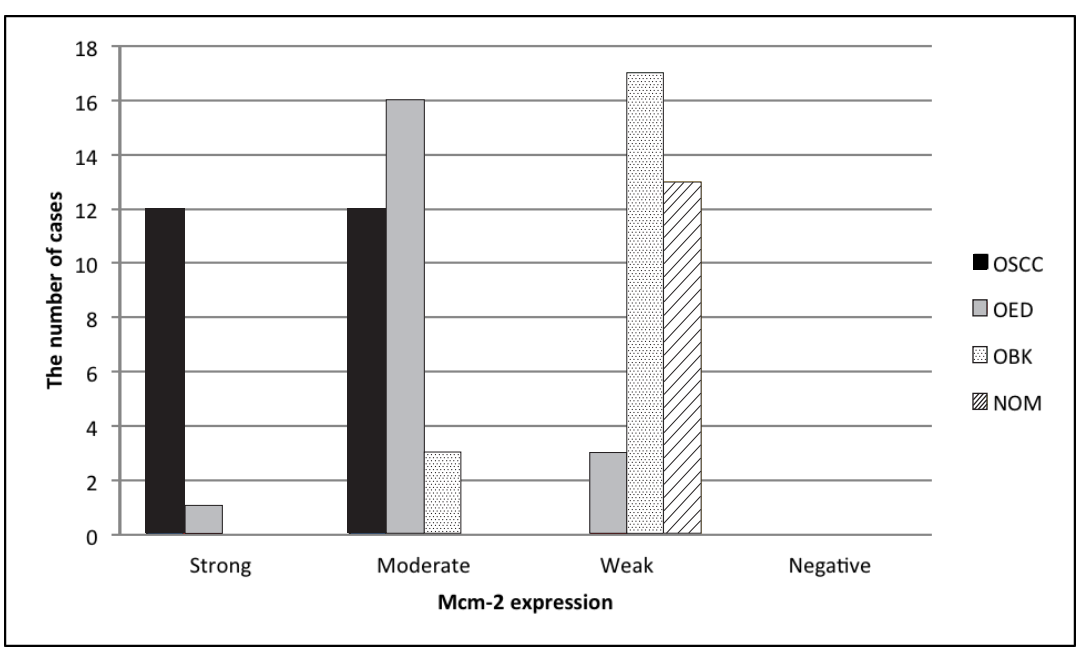

FIG. 4. Level of Mcm-2 expression in the various oral pathologies

TABLE 1: Mean and S.D. of Mcm-2 LI in the oral pathologies studied

Histological diagnosis	No. of biopsies	Labelling index mean (S.D.)
NOM	13	25.19 (7.25)
OBK	20	44.30 (10.3)
OED	20	59.15 (9.1)
OSCC	20	73.65 (11.2)

This result suggested that most cells of NOM and OBK are in the G0 phase. Also, controlled cell division and proliferation ability occur mainly in basal and parabasal compartment.

Torres-Rendon investigated Mcm-2 expression in NOM, OED and OSCC in order to determine the abnormal expression of Mcm-2 in the malignant progression of OED. According to this report, Mcm-2 could be a useful marker in this respect. Also, the location of Mcm-2 in NOM cases was mainly at the suprabasal compartment.[13] These results are in agreement with the findings of our study. But, Scott indicated a higher Mcm-2 expression in the superficial layers of moderate/severe dysplasia and OSCC compared to benign keratosis/mild dysplasia.[2] Unlike previous studies, there was a mild dysplasia with high frequency of Mcm-2 expression at all layers of the epithelium in this study. The result of the present study is in line with the findings of Gouveaet *et al.*[18]

Our findings regarding the higher Mcm-2 LI in OSCC compared to NOM are in agreement with the results of previous studies conducted by Torres–Rendon, Tamura and Kodani.[13,19,20] Most other studies indicated that an increasing number of cells enters the proliferation cycle during tumorgenesis. The invasive front is composed of tumour subpopulations with higher proliferative activity. Therefore, it was selected for protein evaluation.[13,17,19] Furthermore, this method may be better as it is easier to standardize the evaluation of the inferior limit (invasive front) of the lesion between the observers. The Mcm-2 LI was significantly higher in OSCC than in OED, OBK and NOM confirming that the application of this marker in differentiating malignant lesions from benign lesions is advantageous.[13,18,20,21] Similarly, the higher values of LI in OSCCs than OBK in this study confirm the importance of Mcm-2 in differentiating malignant epithelial lesions from benign reactive lesions such as benign keratosis.

The high value of Mcm-2 LI in some of the well-differentiated OSCCs as well as the similarity of LI in some of the mild dysplasia cases with some oral SCCs was also observed in Gouvea *et al*'s study. These observations indicate that lesions with strong positivity may have a higher potential for malignant transformation.[18]

In the current study, a cut-off point of 67% was determined for Mcm-2 with appropriate sensitivity and specificity for differentiating OSCC from other studied oral lesions. Another achievement of this study was determining the coefficients of discriminating functions for different types of the studied oral lesions. The coefficients and equations are useful for the threshold for differentiating OBK from OED.

Conclusion

This study indicated that Mcm-2 has the potential to be applied as a marker in differentiating the studied oral pathologies. Considering its overexpression in OSCC, there exists the possibility of applicability of Mcm-2 in molecular target therapy in patients with OSCC. More

TABLE 2: The coefficients of discriminant functions for different tissue types

Tissue type (y)	coefficients	LI	
	Constant value (b0)	LI coefficient (b1)	(x)
NOM	-4.690	0.262	X
OBK	-11.602	0.461	
OED	-19.599	0.616	
OSCC	-29.623	0.767	

studies with greater sample size and different grades of pathologies are recommended in order to achieve more precise results in this field.

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