

## Expression of Mcm7 and Cdc6 in Oral Squamous Cell Carcinoma and Precancerous Lesions

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**Abstract.** *Background:* The present study aimed at evaluating the clinical importance of Mcm7 and Cdc6 expression in oral squamous cell carcinoma (OSCC) and precancerous lesions. *Materials and Methods:* RT-PCR and immunohistochemistry analysis were performed on 47 frozen samples and 98 paraffin-embedded samples to evaluate the mRNA and protein expressions of Mcm7 and Cdc6. *Results:* RT-PCR and immunohistochemistry indicated positive expressions of Mcm7 mRNA and protein in normal oral mucosa, precancerous lesions and OSCC. Significant differences were found between all the groups. Cdc6 mRNA and protein had low expressions in normal oral mucosa but were highly expressed in precancerous lesions and OSCC. Mcm7 and Cdc6 expressions in the lymph node metastasis cases were significantly higher than those of the non-metastatic carcinomas. *Conclusion:* High expressions of Mcm7 and Cdc6 are correlated with the development and metastasis of OSCC and may become a molecular marker for the early diagnosis and prognosis prediction for OSCC.

A major problem in the management of patients with oral squamous cell carcinoma (OSCC) and precancerous lesions is the need for early diagnosis and an exact prediction of tumour behaviour and prognosis. The consistent transformative process through dysplasia of the normal differentiated epithelium to malignant cancer is often presented as overzealous proliferation. It is possible to study the developmental mechanisms of oral cancer by studying the activity of cellular proliferation. A key step in the regulation of cell proliferation is the control of the initiation

of DNA synthesis. Minichromosome maintenance protein 7 (Mcm7) and cell division cycle 6 (Cdc6) are closely related proteins that are components of the prereplicative complex (Pre-RC) (1). They are essential for initiating eukaryotic DNA replication and serve as useful markers of proliferating cells (2, 3).

Mcm7 is a member of a family of six structurally related proteins, Mcm2 to Mcm7, that possess helicase activity (4, 5) and which are evolutionarily conserved in all eukaryotes. The Mcm2-7 proteins appear to form hetero-hexamers and play important roles in initiation and elongation during DNA replication. Mcms have been demonstrated in replicating cells, but not in quiescent, differentiated or senescent cells (4-7). Cdc6 is essential for the initiation of DNA replication and, in combination with other regulatory proteins, also limits cells to a single round of synthesis per cell cycle. Its best-characterized function is the assembly of Pre-RC at origins of replication during the G1-phase of the cell division cycle. Cdc6 also plays important roles in the activation and maintenance of the checkpoint mechanisms that coordinate S-phase and mitosis and recent studies have unveiled its proto-oncogenic activity. In addition, Cdc6 overexpression in primary cells may promote DNA hyperreplication and induce a senescence response similar to that caused by oncogene activation (8, 9). Cdc6 proteins are highly expressed in various tumour cells and tumorigenesis is suppressed through suppression of Cdc6 expression (10-12). Since down-regulation of Mcm7 and Cdc6 proteins is known to result in loss of cellular proliferation ability, both proteins have been applied as biomarkers for G1-staged cells in different fields for their ability to detect "growth potential" (13-21). Recent studies have shown that Mcm7 and Cdc6 may also be useful proliferation markers in dysplasia and carcinoma in various tissues (13-19).

Because Mcm7 and Cdc6 expressions have not previously been assessed in OSCC together (22, 23), the aim of this study was to assess the expression of both proteins in a range of oral lesions to determine their potential value as proliferation and prognosis markers of

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oral lesions, especially for OSCC and to determine whether they are useful in predicting tumour proliferation rates and prognosis.

## Materials and Methods

### *Experiments using frozen tissues.*

**Materials.** All the experimental samples were procured from the First Affiliated Hospital's Oral-Maxillofacial Surgery Department of Sun Yat-Sen University and included patients diagnosed with precancerous lesions (leukoplakia, 15 cases) and OSCC (22 cases, no radiotherapy or chemotherapy was performed before surgery). Normal oral mucosa samples were collected from peripheral mucosa tissues of benign oral tumours from ten consenting patients. All the samples were pathologically substantiated.

**RNA extraction and RT-PCR.** The primer sequences were as follows:  $\beta$ -actin (115 bp), F: 5'-CATTGGCAATGAGCGGTTC-3' and R: 5'-CCACGTACACATTCATGATG G-3'; Mcm7 (268 bp), F: 5'-GAGCATCGGCTAATGATGGAG-3' and R: 5'-GTGACGA TTCCACGCACAGTT-3' and Cdc6 (363 bp), F: 5'-TGCTCTTGA TCAGGCAGTTG A-3' and R: 5'-GGCCCGAATGTGTAAGCA CT-3'. RNA extraction and cDNA synthesis were performed with frozen samples. The mRNA levels of the target genes were assessed with the use of a semiquantitative multiplex RT-PCR protocol.

**Statistical analysis.** The genetic expression level was represented by the relative expression. The relative expression levels of Mcm7 and Cdc6 equalled the Mcm7 or Cdc6 grey value of each band/ $\beta$ -actin grey value of each band. SPSS Software 16.0 was used (SPSS Inc. Chicago, IL, USA) for statistical analysis; *t*-tests were performed on the quantitative data and variance analysis and rank-sum tests were performed for heterogeneous variance.

### *Experiments using paraffin-embedded tissues.*

**Materials.** Eighty-eight paraffin-embedded samples of oral precancerous lesions (leukoplakia) and OSCC collected between 2004 and May 2007 by the Pathology Department of the First Affiliate Hospital of Sun Yat-Sen University were included for analysis. The specimens were divided into two groups based on the histological type: 34 specimens of precancerous lesions (mild, moderate and severe dysplasia 11, 13, and 10 cases, respectively, representing 15 males and 19 females; ages 17-82, with an average age of 51.4 years) and 54 patients with OSCC (29 males, 25 females; ages 21-78, with an average age of 53.1 years).

The specimens were separated based on TNM staging criteria (Union Internationale Contre Cancer, UICC, 2002): stage I: 7 cases, stage II: 18 cases, stage III: 18 cases and stage IV: 11 cases; lymph node metastasis: 29 cases and non-lymph node metastases: 25 cases; pathological criteria: well-differentiated: 30 cases, moderately-differentiated: 12 cases and poorly-differentiated: 12 cases. All the samples were collected before laser therapy, radiotherapy or chemotherapy. Ten specimens of normal oral mucosa were collected as above and embedded in paraffin.

**Immunohistochemistry.** Immunohistochemistry was performed using a standard streptavidin-biotin-peroxidase-diaminobenzidine immunohistochemical technique. The primary reagents included mouse anti-human Mcm7 monoclonal antibody and mouse anti-human Cdc6 monoclonal antibody (Santa Cruz Biology, Santa Cruz, CA USA;

1:100 dilutions), the positive controls were cervical tumour tissue specimens and PBS was used in lieu of the first antibody as a negative control. The Mcm7 immunohistochemistry staining results were interpreted as positive in the presence of brown-yellow granules in the nucleus. The Cdc6 results were positive in the presence of brown-yellow granules in the nucleus or yellow stained cytoplasm. At  $\times 400$  magnification, three non-overlapping microscopic fields were examined and the number of positive cells and the total cells in the field of view were calculated. A labelling index (LI) was calculated for Mcm7 and Cdc6 expression. The LI equalled the total number of positive cells divided by the total number of cells times 100.

**Statistical analysis.** SPSS software 16.0 (SPSS Inc.) was used for variance analysis and *t*-tests to evaluate experimental results and their relationships with clinical and pathological markers. Statistical significance was established at a  $p < 0.01$  threshold.

## Results

**RT-PCR.** Positive expression of Mcm7 mRNA was observed in the normal oral mucosa, precancerous lesions and OSCC with expression levels of  $1.932 \pm 0.104$ ,  $2.448 \pm 0.103$  and  $3.123 \pm 0.070$ , respectively. Significant differences were found between all the groups ( $p < 0.01$ ). Positive expressions of Cdc6 mRNA were observed in the normal oral mucosa, precancerous lesions and OSCC with expression levels of  $1.798 \pm 0.096$ ,  $2.448 \pm 0.117$ , and  $2.903 \pm 0.118$ , respectively. Significant differences were found between all the groups ( $p < 0.01$ ) (Figure 1).

**Immunohistochemical staining characteristics of Mcm7 and Cdc6.** Figure 2 depicts positive Mcm7 protein expression in the nucleus. In the normal oral mucosa epithelium, positive staining was distributed in the basal layer. Staining distribution was generally the same with the mild dysplasia cases. A progressive increase of positive cells along the epithelium and towards the middle was observed in cases of moderate dysplasia. In OSCC, strong positive staining was primarily distributed around cancer nests as differentiated basal cells and in poorly differentiated cancer cell bands.

Figure 3 depicts positive Cdc6 protein expression. No expression was noted in the normal oral mucosa. Some positive cells were observed only in the basal layer and 1-2 layers above the sub-basal layer in mild dysplasia tissues. Other layers were negative for expression. Positive cells were heterogeneously expressed at different levels in the various stages of dysplasia. Several well-differentiated OSCC showed staining around cancer nests or cornified pearls in monolayer or multilayer formation. Positive moderate to poorly differentiated OSCC exhibited loss of regularity, with diffuse distribution.

**Immunohistochemical expressions of Mcm7 and Cdc6.** Mcm7 protein-positive expression was observed in the normal oral mucosa (8/10), precancerous lesions (30/34) and OSCC

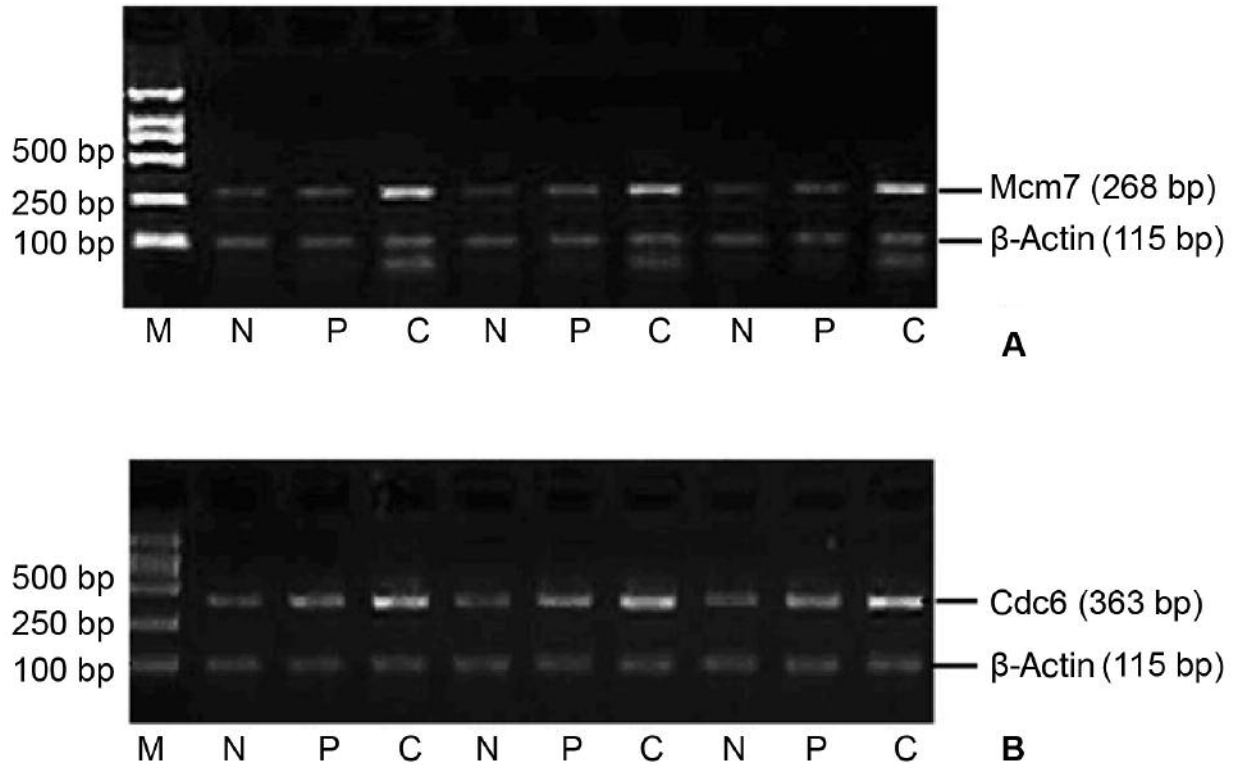


Figure 1. Expression analysis of *Mcm7* (A) and *Cdc6* (B) mRNA in normal oral mucosa (N), precancerous lesions (P) and oral squamous cell carcinoma (C), 3 samples of each. The  $\beta$ -actin lanes demonstrate equal loading.

(51/54), with LI values of 3.6%, 22.3% and 45.9%, respectively. The average LI values differed significantly between groups ( $p < 0.01$ ). The LI of the moderate dysplasia group (13/13) and the severe dysplasia group (10/10) was 22.5% and 37.4%, respectively ( $p < 0.01$ ) and both were statistically significantly different from the mild dysplasia group (LI=4.4%,  $p < 0.01$ ). In the OSCC group, the LI values of *Mcm7* were correlated with the N-staging of metastasis of the lymph nodes ( $r = 0.634$ ,  $p < 0.01$ ) and the clinical staging of combined TNM criteria ( $r = 0.648$ ,  $p < 0.01$ ), but were not correlated with patient gender, age, T-staging of the primary lesion, or histological differentiation (Table I).

*Cdc6* protein was rarely expressed in the normal mucosa group (0/10) or the mild dysplasia group (3/11), but was positively expressed in the moderate-severe dysplasia group (13/23) and the OSCC group (33/54). The LI values for the mild dysplasia group, the moderate and severe dysplasia group and for the OSCC group were 1.5%, 24.7% and 31.2%, respectively ( $p < 0.01$ ). In the OSCC group, LI values for *Cdc6* were also correlated with N-staging ( $r = 0.697$ ,  $p < 0.01$ ) and clinical staging ( $r = 0.707$ ,  $p < 0.01$ ), but not correlated with patient gender, age, T-staging of the primary lesion or histological differentiation (Table I).

Table I. Correlations of *Mcm7* and *Cdc6* expression to clinicopathological features of OSCC.

Parameter	N	<i>Mcm7</i> LI ( $\bar{x} \pm s$ )	<i>P</i> -value	<i>Cdc6</i> LI ( $\bar{x} \pm s$ )	<i>P</i> -value
Gender					
Male	29	47.0 $\pm$ 23.1		31.6 $\pm$ 12.8	
Female	25	44.6 $\pm$ 24.5	0.713	30.7 $\pm$ 16.9	0.894
Tumor status					
$\leq 2$ cm	13	38.0 $\pm$ 23.0		19.3 $\pm$ 9.0	
$> 2$ cm	41	48.4 $\pm$ 23.5	0.166	34.9 $\pm$ 14.8	0.044
Lymph node status					
N0	25	30.6 $\pm$ 16.5		19.7 $\pm$ 8.5	
Positive	29	59.1 $\pm$ 20.6	$< 0.001$	41.0 $\pm$ 15.1	$< 0.001$
Pathological status					
I	7	22.4 $\pm$ 14.0		7.0 $\pm$ 4.2	
II	18	34.1 $\pm$ 16.6		24.7 $\pm$ 11.5	
III	18	55.2 $\pm$ 16.7		37.1 $\pm$ 16.1	
IV	11	65.6 $\pm$ 25.4	$< 0.001$	47.4 $\pm$ 18.3	$< 0.001$
Histological grade					
G1	30	41.7 $\pm$ 24.1		28.5 $\pm$ 12.9	
G2	12	48.8 $\pm$ 19.8		36.4 $\pm$ 19.0	
G3	12	53.7 $\pm$ 25.1	0.298	32.5 $\pm$ 14.0	0.673

\*G1, well-differentiated; G2, moderately differentiated; G3, poorly differentiated. Status: UICC, 2002.

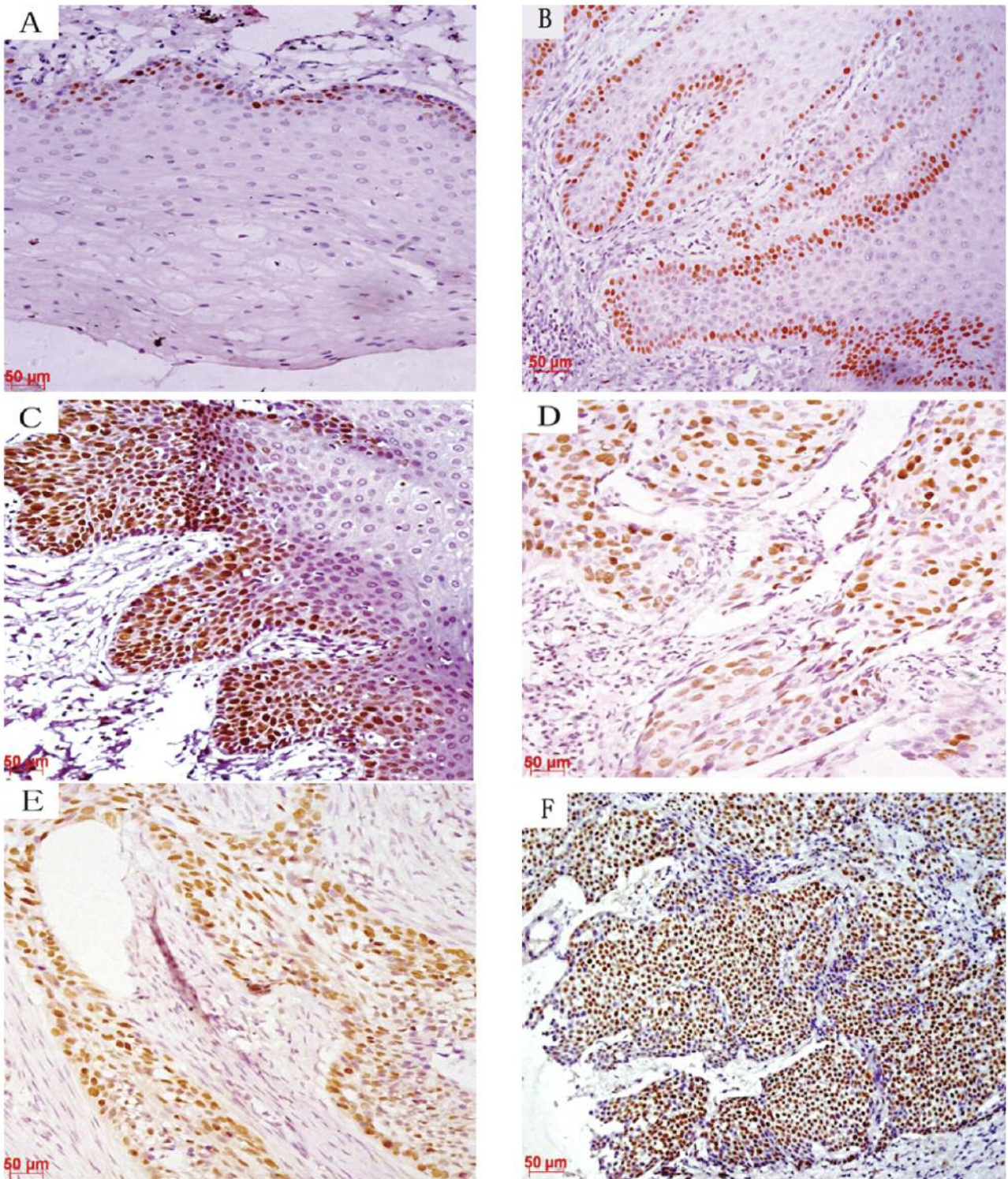


Figure 2. *In situ* immunohistochemical staining of Mcm7 protein. A, Normal oral mucosa epithelium; B, mild dysplasia; C, moderate dysplasia; D, E, squamous cell carcinoma; F, cervical cancer, positive control. Magnification:  $\times 200$ .

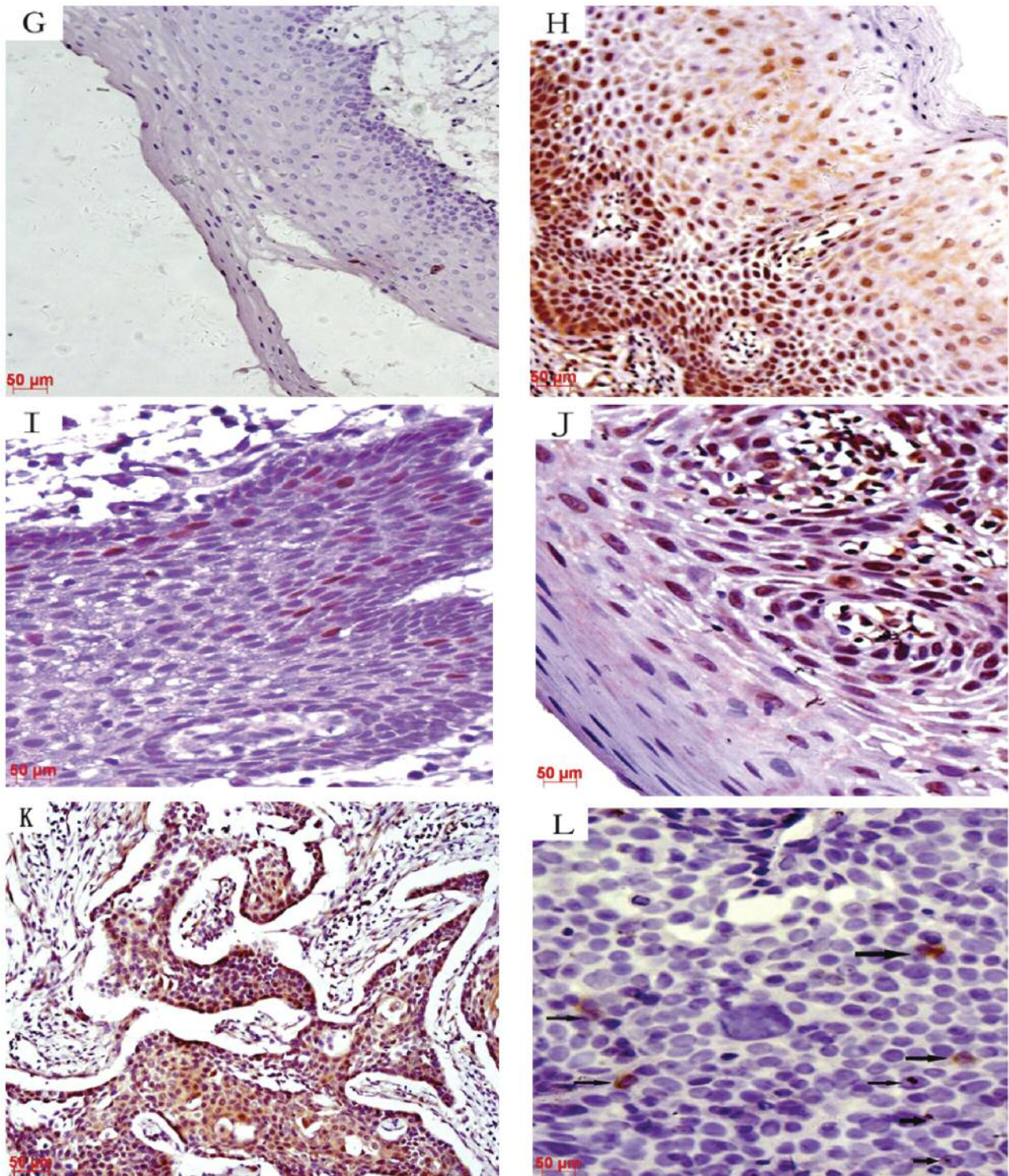


Figure 3. *In situ* immunohistochemical staining of Cdc6 protein. G, Normal oral mucosa; H, severe dysplasia; I, well-differentiated squamous cell carcinoma cancer nests; J, K, moderate- to poorly differentiated squamous cell carcinomas; L, cervical cancer, positive control. Magnification:  $\times 200$ .

## Discussion

In this study RT-PCR indicated that mRNA of *Mcm7* and *Cdc6* were not or only little expressed in normal mucosa, and their expression was significantly up-regulated in precancerous lesions and OSCC, suggesting that both proteins may be involved in the occurrence and development process of OSCC.

Mcm proteins have been shown to be expressed locally in normal cellular proliferation zones, while in dysplasias and malignant tumours, they are expressed in most cells and throughout the entire layer of the epithelium (24). In the present immunohistochemical examination, Mcm7 expression in the normal oral mucosa epithelium was mainly centralized in the basal layer and 1-2 layers above the sub-basal layer, while absent from other layers, indicating the presence of cell division and proliferation ability in and above the basal layer. Mcm7 expression was progressively elevated during the transformation of normal cells to moderate-severe dysplasia and invasive carcinoma, suggesting an increasing number of cells entering the proliferation cycle during tumorigenesis. The Mcm7 protein level directly reflected cellular proliferative activity and thus the detection of Mcm7 expression may diagnostically assist the determination of OSCC, particular with regard to precancerous lesions. An earlier immunohistochemical study of 101 cases of oral cell smears similarly revealed differences in Mcm2 and Mcm5 expression in oral normal mucosa, different levels of dysplasia and carcinogenesis (22).

The present results also indicated that Mcm7 expression was correlated with metastasis of lymph nodes and clinical TNM staging in the patients with oral cancer, suggesting that highly expressed Mcm7 may indicate a poor prognosis. Szelachowska *et al.* (23) in their retrospective study also indicated the possibility of Mcm2 as a prognostic factor in OSCC patients.

Cdc6 protein level, to a certain degree, reflected cellular proliferation activity, but compared to Mcm7, the Cdc6 protein-positive expression was lower in all the samples. Even though Cdc6 expression was progressively elevated during normal-moderate-severe dysplasia and invasive carcinogenesis, a lack of staining did not necessarily indicate low proliferative activity due to the high negative rates of the methodology. The results suggest that it would be difficult to employ Cdc6 as a marker of OSCC and precancerous lesions in clinical pathology. However, high expression of Mcm7 was usually present in most of the samples with positive Cdc6 expression. Therefore, Cdc6 may be valuable with Mcm7 in the early diagnosis and prognosis of OSCC. This study also suggested a correlation between Cdc6 expression and metastasis of lymph nodes and clinical staging of TNM in the oral cancer patients.

In conclusion, high expressions of Mcm7 and Cdc6 are correlated with OSCC development and metastasis, suggesting that both may act as important factors in OSCC and metastasis to the lymph nodes. Expression and LI values of both proteins may help distinguish normal tissues from dysplasia and tumour lesions and, to a certain extent, predict metastasis to the lymph nodes. Therefore, the Mcm7 and Cdc6 expression may potentially become molecular markers for the early diagnosis and prognosis prediction of OSCC.

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