Expression of High Mobility Group A Proteins in Oral Leukoplakia

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Abstract. *Background: Oral leukoplakia (LPL) is considered a potentially malignant disorder in the oral cavity and the gastric tract. High mobility group A (HMGA) proteins are important in the transformation of normal cells into cancer cells, but there is a lack of knowledge on their importance in oral cancer development. The aim of the current project was to investigate HMGA expression in LPLs with different levels of dysplasia. Materials and Methods: Biopsies were histologically processed to visualize the expression of HMGA1 and HMGA2 using immunohistochemistry. Results: An increase of HMGA1-positive cells correlating to the degree of dysplasia was registered in the epithelium and in the connective tissue. HMGA2 expression was seen in the epithelium and in the connective tissue but with no obvious correlation to the level of dysplasia. Conclusion: This is, to our knowledge, the first study showing the expression of HMGA proteins in healthy and non-healthy oral mucosa.*

Leukoplakias (LPLs) are pre-malignant mucosal lesions, out of which 5% develop into oral squamous cell carcinomas (OSCC) (1). LPLs in the oral mucosa are a clinical challenge since there is a lack of well-established clinical and histological criteria that can identify lesions with risk for malignant transformation (2).

One of the hallmarks of cancer is the ability of cancer cells to avoid growth suppressors, thereby avoiding apoptosis (3). A central role in this process is the dysregulation of the retinoblastoma-associated (RB) and p53 proteins, resulting in inhibition of apoptosis and chromatin instability (3). The gene expression of RB and p53 is, in turn, regulated by the high mobility group A (HMGA) proteins (4). Overexpression of HMGA1 and HMGA2 is a key feature of cancer and is correlated with poor prognosis (5). The expression of HMGA1 and HMGA2 has been reported to be related to tumor differentiation, invasion and cell proliferation (6).

The HMGA proteins are architectural transcription factors involved in chromatin remodeling and cancer transformation (4, 7). The HMGA family consists of HMGA1a and HMGA1b, produced by a gene on chromosome 6p21, and HMGA2, a product of a gene on chromosome 12q14-15 (7-8). The HMGA proteins are able to regulate gene expression either through DNA-binding motifs, so-called AT-hooks, recognizing A/T-rich sequences of DNA or by interacting with other proteins such as nuclear factor kappa-light-chain-enhancer of activated B-cells (NFκB) and p53 (7, 9, 10).

The role of HMGA proteins in tumorigenesis differs, with HMGA1 being suggested as an important factor in cell transformation and an indicator of potentially malignant cells (7). The expression of HMGA1 was found to increase with the level of atypical cells in pancreatic and breast cancer (11, 12). Based on these findings Chiappetta and co-workers suggested that the expression of HMGA1 precedes a malignant phenotype and could therefore be used as a marker for cancer diagnosis (12).

HMGA2 proteins are involved in transcription activation of certain cell proliferation genes contributing to tumor formation (13). The expression of HMGA2 is found in the invasive front of cancer tissue, thereby contributing to tumor invasion and epithelial-to-mesenchymal transition (EMT) (14, 15). HMGA2 was, therefore, suggested as a potential biomarker for EMT during malignant progression (16).

There is limited knowledge regarding the role of HMGA proteins in LPLs and their potential role in the development of LPLs into oral cancer. Since the expression of HMGA proteins is suggested to correlate with malignant transformation, it is important to investigate their role in dysplasia development. The aim of the current project was to investigate the expression of HMGA1 and HMGA2 in LPLs with different levels of dysplasia.

Materials and Methods

Paraffin-embedded biopsies from 29 patients with the clinical diagnosis LPL were retrieved from the archives of the Department of Oral Medicine and Pathology, Institute of Odontology at Gothenburg University. Previous histopathological diagnosis of

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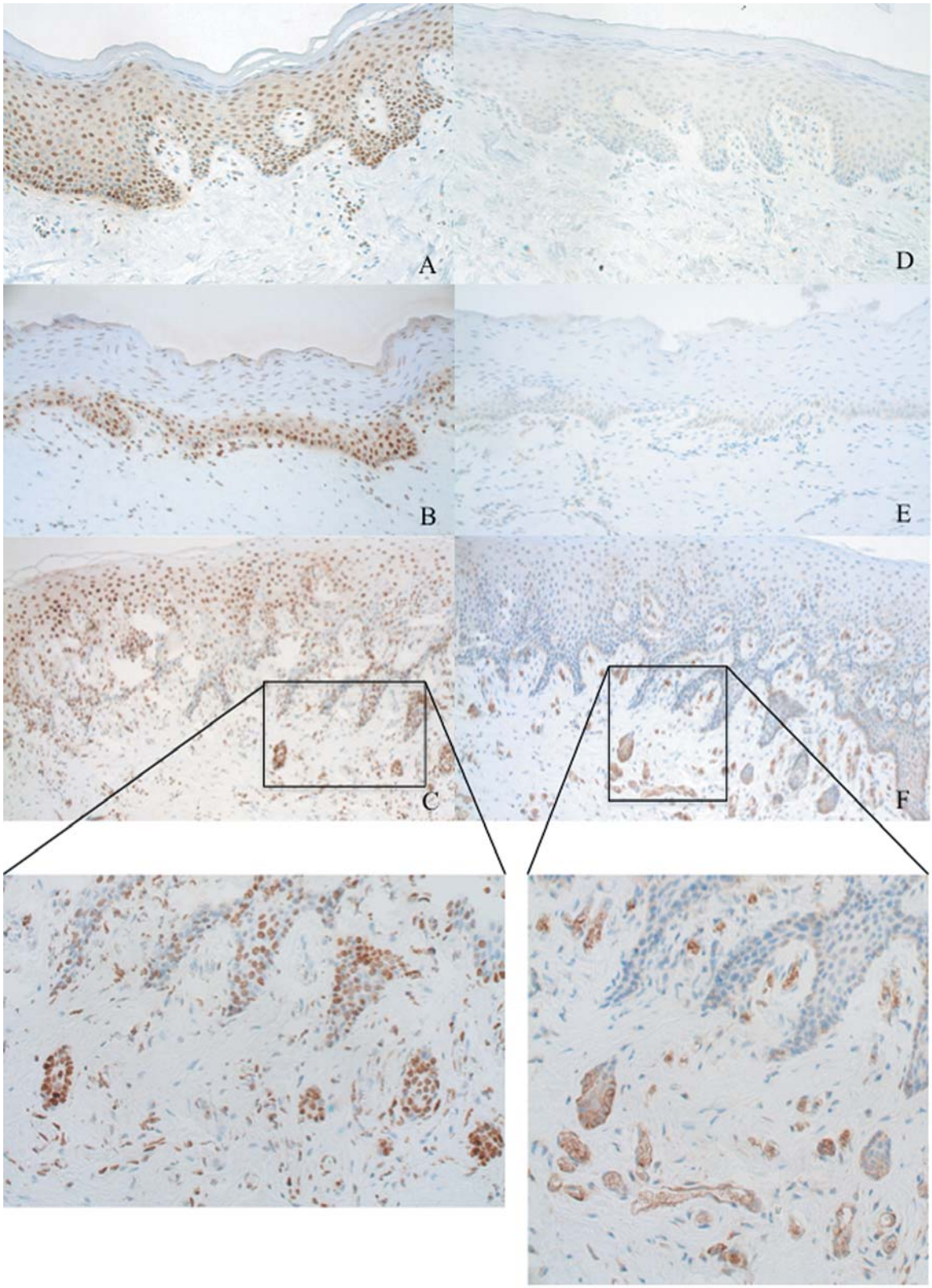

Figure 1. *Illustration of immunohistochemical staining of high mobility group-A1 (HMGA1) (A-C) and HMGA2 (D-F) in healthy oral mucosa (biopsies from three different individuals). Magnification, ×100. Insets C and F, connective tissue illustrating positive staining of inflammatory cells and endothelial cells. Magnification ×200.*

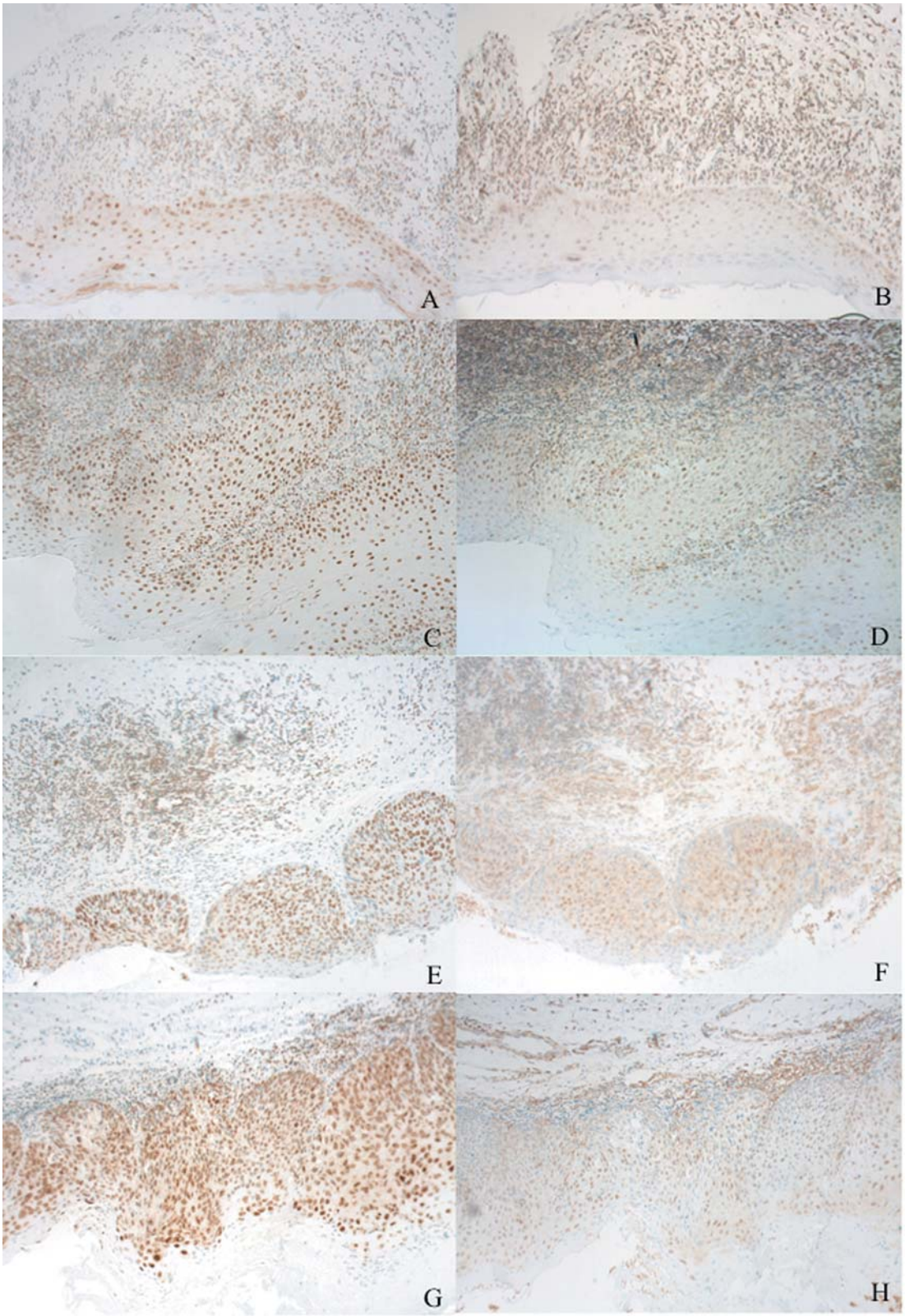

Figure 2. *Illustration of immunohistochemical staining of high mobility group-A1 (HMGA1); A, C, E, G and HMGA2 (B, D, F, H). A and B: leukoplakia without dysplasia; C and D: leukoplakia with moderate dysplasia; E and F: leukoplakia with severe dysplasia; and G and H: cancer in situ. Magnification ×100.*

biopsies revealed 12 samples with hyperkeratosis without dysplasia and 17 samples with hyperkeratosis with dysplasia. The degree of dysplasia in all samples was re-evaluated by an experienced pathologist and found to be in concordance with the primary histopathological diagnoses. Tissue samples from clinically-healthy oral mucosa of eight other individuals were collected in conjunction with minor oral surgical procedures after signed approval from the respective patient.

Immunohistochemistry. Sections, 4-μm thick, were de-waxed and incubated in DIVA antigen retrieval solution (Biocare medical, Concord, CA, USA) at 60˚C overnight. The sections were incubated with primary antibody for 30 min followed by incubation with Envision horseradish peroxidase (HRP)-labeled polymer (DakoCytomation A/S, Glostrup, Denmark) for 30 min. Positive cells were detected using 3,3'-Diaminobenzidine (DAB) substrate (DakoCytomation). The sections were counterstained using hematoxylin. The antibodies used were HMGA1 (1:2000; ab129153; Abcam, Cambridge, UK) and HMGA2 (1:50; ab52039; Abcam). Omission of primary antibodies served as a negative control.

Histological analysis. Quantitative analysis was performed on digitalized images obtained using a light microscope (Leitz Wetzler; Leica Microsystems, Wetzlar, Germany) with a Leica DC100 camera (Leica Microsystems). Quantification of positive cells in two to three images (magnification, ×100) from the epithelium and the connective tissue respectively was performed using CellSense computer software (Olympus, Hamburg, Germany). Results are expressed as the number of positive cells/mm^2.

Statistical analysis. Analyses of differences between groups were carried out by Mann Whitney U-test using the statistical software SPSS v17 (SPSS Inc., Chicago, IL, USA). p-Values <0.05 were considered significant.

The study was approved by the regional Ethical Review Board at University of Gothenburg, with approval number Dnr: T247-13.

Results

Positive staining for HMGA1 and HMGA2 were found in both the epithelium and the connective tissues in both healthy individuals and patients with LPLs. In the connective tissue, positive staining of the HMGA proteins was seen in inflammatory cells and endothelial cells. An increased number of inflammatory cells in the connective tissue corresponded to a higher level of positive staining in the epithelium (Figures 1 and 2).

An increase of HMGA1-positive cells correlating to the degree of dysplasia was registered in the epithelium. Differences in HMGA1 expression were statistically significant between the groups (Figure 3A). A similar pattern was also found for the expression of HMGA1 in the connective tissue. However, this pattern was not seen for HMGA2 expression, for which no obvious correlation to the level of dysplasia was seen in the epithelium and in the connective tissue (Figure 3B). In general, more HMGA2-positive cells were found in the connective tissue, regardless of the level of dysplasia.

The highest number of HMGA1-positive stained cells was found in samples from healthy individuals, whereas the lowest number of such cells was observed in samples from patients with LPLs without dysplasia (Figure 3A). In sections from LPLs with dysplasia, an intermediate number of HMGA1-positive cells was seen (Figure 3A).

For HMGA2, the highest number of positively stained cells was also found in samples from healthy individuals (Figure 3B). The variations in non-healthy epithelium were smaller in comparison to these for HMGA1.

Discussion

HMGA proteins have been shown to play important roles in transcription regulation, DNA repair and chromatin remodeling, as well as RNA processing (4). The presence of HMGA proteins has been investigated in several types of cancers (8, 11, 15, 17), and they are suggested to be key factors in tumor progression, including EMT. Since the level of HMGA proteins has been suggested to correlate to the development of cancer, we investigated the presence of HMGA1 and HMGA2 in the pre-cancerous lesions of leukoplakia and healthy oral mucosa in order to investigate their potential role in cell transformation.

In the current study positive staining for both HMGA1 and HMGA2 was found in tissue samples from patients with LPL with and without dysplasia. An increase in HMGA1 staining was found to correlate with an increasing level of dysplasia. This is in accordance with the results presented by Sarhadi *et al.*, who found lower levels of HMGA1-positive cells in metaplasia and higher levels in dysplasia and carcinoma *in situ* (18). Low HMGA1 expression was also found in early-stage adenomas but expression was high in adenomas with dysplasia (8). In relation to the level of dysplasia, a less distinct pattern was found for HMGA2 compared to that of HMGA1. It has been suggested that HMGA1 rather than HMGA2 may be responsible for cell transformation. HMGA1 expression was, therefore, suggested as a biomarker for cancer diagnostics (7).

The expression of HMGA proteins in OSCC was investigated by Miyazawa and co-workers (14). They found that HMGA2 was expressed in 73.8% of the carcinoma tissue samples, while normal gingival tissues did not express HMGA2. The results also showed that in oral carcinoma tissues, HMGA2 staining was observed in the invasive front towards the connective tissue, indicating that HMGA2 expression may contribute to the aggressiveness of carcinoma (14). Overexpression of HMGA2 and a correlation with poor prognosis have further been shown in various types of cancer, including OSCC (15). Not only may the presence of HMGA proteins contribute to tumor development, but their location in cells has also been suggested to influence cancer prognosis. Esposito and co-workers found that cancer cell lines express

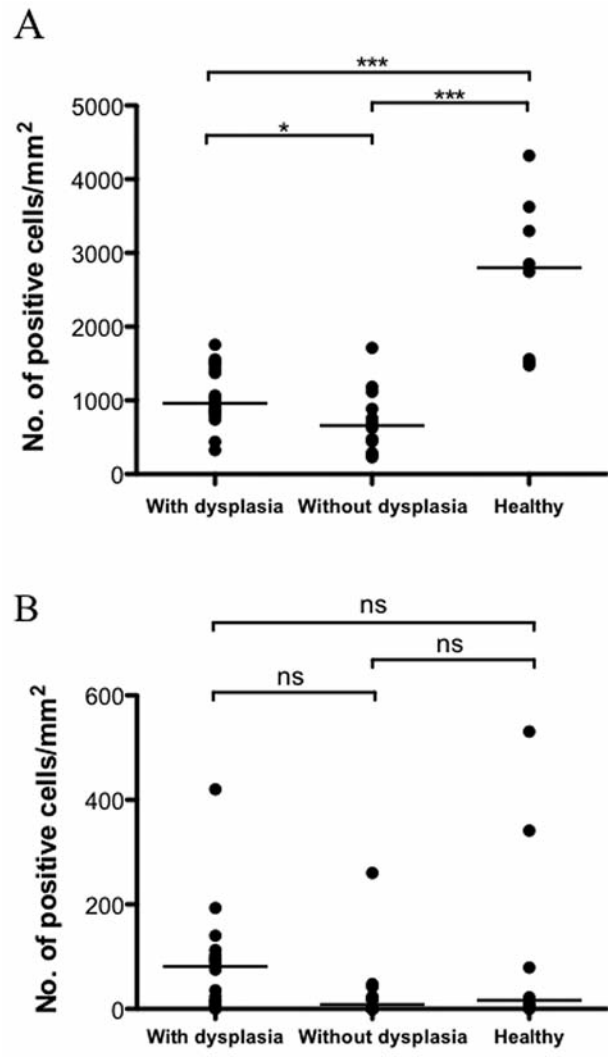

Figure 3. *The expression of high mobility group-A1 (HMGA1) (A) and HMGA2-positive (B) cells in the epithelium. Results are expressed as the number of positive cells/mm2. The graph shows the distribution and median values. *p<0.05; ***p<0.001; ns p>0.05.*

HMGA1 not only in the nucleus but also in the cytoplasm (19). This is in contrast to non-cancerous cell lines where HMGA1 staining was only present in the nucleus. Cells with HMGA1 protein expression in the cytoplasm also had a higher aggressiveness (19). In the current study, only nuclear staining of epithelial cells was seen in normal mucosa and LPLs for HMGA1. Positive staining of inflammatory cells was also seen in the nucleus, and for some cells, also in the cytoplasm for HMGA1. In contrast, positive staining for HMGA2 in inflammatory cells and endothelial cells was seen in the cytoplasm for most cells.

In head and neck squamous cell carcinoma (HNSCC), 87.5% of carcinomas were positive for HMGA1, while no staining was found in normal tissue (20). However, there are reports of weak staining in adjacent mucosa of tumor tissue as well as in non-tumor tissue (6, 20). In the review by Fusco and Fedele it was reported that HMGA1 is expressed in normal tissues but at low levels, while no staining for HMGA2 in normal tissue was reported (7). In the present study, high levels of HMGA1 were found in the epithelium of healthy oral mucosa, while HMGA2 was found at high levels in the connective tissue of healthy oral mucosa.

Absence of HMGA expression in healthy colon, breast, pancreatic and retina has been shown (5, 6, 11-12). Epigenetic mechanisms have been suggested as being important in the regulation of HMGA expression (15, 21-22). Since epigenetic mechanisms have been shown to be cell type-specific (23), this could provide an explanation the differences in HMGA expression in benign tissues. Additionally, epigenetic changes caused by bacteria have also been shown to influence development of head and neck carcinomas (24). In connective tissue, positive staining of HMGA proteins was seen in inflammatory cells and endothelial cells, which is in line with previous findings (25). HMGA proteins are important factors in the regulation of B-cell and plasma cell development and gene transcription of inflammatory cytokines in activated T-cells (26-27). Pathogens in the oral mucosa, such as bacteria, have also been shown to cause epigenetic changes influencing gene expression in chronic inflammation (28). The HMGA proteins were found not only to be involved in inflammation but also to induce expression of genes in inflammatory pathways in cellular development, lymphoid tumorigenesis and in hematopoiesis in both early and late stages of tumorigenesis (29).

In conclusion, this is, to our knowledge the, first study showing the expression of HMGA proteins in both healthy and non-healthy oral mucosa. Variation in the expression of HMGA subtypes was observed in epithelium and connective tissue. However, further studies are needed to elucidate the importance of HMGA in pre-malignant oral conditions.

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