Altered CD40 and E-cadherin expression – putative role in oral lichen planus

E. Neppelberg1,2,3, L. L. Loro1, G. Øijordsbakken1, A. C. Johannessen1,4

1Oral Pathology and Forensic Odontology, Institute of Oral Sciences, Faculty of Dentistry, University of Bergen, Bergen; 2Oral Surgery and Oral Medicine, Faculty of Dentistry, University of Bergen, Bergen; 3Department of Oral and Maxillofacial Surgery, Haukeland University Hospital, Bergen; 4Department of Pathology, Haukeland University Hospital, Bergen, Norway

BACKGROUND: Oral lichen planus (OLP) is characterized among other features by apoptosis of basal keratinocytes. To identify potential regulatory mechanisms associated with basal cell apoptosis in OLP, we investigated the expression of CD40, CD40 ligand (CD40L), CD44 and epithelial (E)-cadherin.

METHODS: Biopsies from 22 patients with OLP were investigated by immunohistochemistry for detection of CD40, CD40L, E-cadherin, CD44, Laminin-5 and Collagen IV, double-labelling for CD40 and CD3, and in situ mRNA hybridization for CD40 and CD40L.

RESULTS: In actively diseased areas of OLP lesions, basal keratinocytes did not express CD40 and were focally E-cadherin-negative, in contrast to non-diseased areas and normal oral mucosa. Demonstration of intraepithelial T cells expressing CD40 and CD40L, indicates a potential role in inflammatory cell responses involved in the disease process of OLP.

CONCLUSION: T cells may orchestrate inflammatory cell responses in OLP via CD40–CD40L interactions. As basal keratinocytes downregulate CD40, they may escape CD40–CD40L-induced apoptosis in OLP. On the other hand, loss of E-cadherin expression may contribute to epithelial basal cell destruction and T-cell migration into the epithelial compartment in OLP.

Keywords: apoptosis; CD40; CD40 ligand; CD44; epithelial cadherin; oral lichen planus

Abbreviations: Ab, antibodies; BSA, bovine serum albumin; DAB, diaminobenzidine; ISH, in situ hybridization; LP, lichen planus; mAb, monoclonal antibodies; OLP, oral lichen planus; OM, oral mucosa; pAb, polyclonal antibodies; PBS, phosphate-buffered saline; TBS, Tris-buffered saline.

Introduction

Oral lichen planus (OLP) is a chronic inflammatory mucocutaneous disorder of unknown aetiology. Characteristic histological features include a subepithelial band-like inflammatory cell infiltrate, and epithelial basal cell destruction. Changes in the basal cell compartment have been a matter of particular interest in recent research on OLP, and increased apoptosis, most often confined to the basal cell region has been demonstrated (1–3). Previous studies indicate that intraepithelial cytotoxic T cells are involved in direct targeting of keratinocytes for apoptosis (3–5). Apoptosis of basal keratinocytes is, however, a complex process involving several biological pathways regulating apoptosis and cell survival.

CD40, a transmembrane protein belonging to the tumour necrosis factor receptor (TNFR) superfamily, is constitutively expressed by cells with high proliferative potential including keratinocytes, T cells, dendritic cells and macrophages (6–8). CD40 and its ligand (CD40L/CD154/gp39) have been reported to play roles in the regulation of normal squamous epithelial cell growth and cell survival as well as inflammatory cell responses (8–10). CD40–CD40L interaction may either induce (via Fas and/or TNFR-1) or inhibit (via nuclear factor-κB) apoptosis in different cancer cell lines (8, 11, 12). Normal oral mucosa (OM) basal keratinocytes express CD40 (9, 13, 14), but its specific functional biological role has not yet been clarified, neither in normal nor in diseased OM.

Normal epithelial keratinocytes express several adhesion molecules that have important functions in co-ordinating cell-to-cell and cell-to-matrix contact (15, 16). One such adhesion molecule is epithelial (E)-cadherin, a transmembrane adhesion protein belonging to the cadherin family, involved in cell-to-cell adhesion in stratified squamous epithelium among other functions (16). E-cadherin is expressed in normal OM (17), and its maintenance of cell contacts has been shown to prevent apoptotic cell death in immortalized cell lines.
(18, 19). However, the specific biological role of E-cadherin in apoptosis of OM keratinocytes has not been elucidated. Another transmembrane adhesion protein is CD44, which belongs to a family with multiple isoforms that take part in various biological functions including cell-to-cell and cell-to-matrix interactions in stratified squamous epithelium (20). CD44 is expressed by keratinocytes in normal OM (21), but its specific biological roles in normal or diseased OM including the direction and mechanisms in which CD44 may regulate apoptosis have not been elucidated. On the one hand, CD44 has been shown to prevent apoptosis in human colonic cancer cell lines (22) and normal mouse colonic epithelium in vivo (23), and on the other hand, CD44 may promote apoptosis in human synovial cell lines from patients with rheumatoid arthritis (24). There are to our knowledge no reports regarding the expression of CD40, E-cadherin or CD44 in OLP.

Thus, as the CD40–CD40L system, E-cadherin as well as CD44 may be involved in epithelial keratinocyte biology and apoptosis, we investigated their expression in OLP compared with normal OM. In order to further characterize the CD40-expressing cells in OLP, an immunohistochemical double labelling with the T-cell marker, CD3 was also performed.

Materials and methods

Tissue specimens

Biopsies from 22 admitted patients (seven men and 15 women, age range: 30–79, mean age 59 years), with clinically and histologically diagnosed OLP, were investigated in this study. Clinically, all OLP lesions were of the reticular type, 14 of which were also erythematous. Another two patients had ulcerous lesions. All patients presented with bilateral buccal lesions, seven had gingival lesions in addition, five had tongue lesions and four had lip lesions. Biopsies were taken from reticular lesions of the buccal mucosa, not associated with dental restorations. Ten patients reported a history of cutaneous LP. None of the patients had been treated with either topical or systemic steroids. Normal OM samples from healthy individuals (n = 5), human tonsils (n = 5) and oral squamous cell carcinomas (n = 3) served as control specimens. The biopsy specimens were divided into two halves: one was snap frozen in isopentane, pre-cooled in liquid nitrogen and stored at −70°C, and the other was fixed in 4% buffered formalin (pH 7.2) and embedded in paraffin.

The regional committee for medical ethics in research approved the project, and patients included in this study gave their informed consent.

Immunohistochemistry

CD40 and CD40 ligand

Immunohistochemical avidin-biotin-peroxidase complex technique (ABC-technique) was performed on 5-µm-thick cryostat sections fixed in 50% acetone at 4°C for 30 s then 100% acetone for 5 min. Endogenous peroxidase activity was blocked with 2% H2O2 in phosphate-buffered saline (PBS) for 15 min. Non-specific binding was blocked for 30 min with either 5% normal horse serum or 5% normal goat serum, diluted in 4% bovine serum albumin (BSA/PBS, pH 7.2). The sections were incubated for 60 min with the following antibodies (Ab): mouse monoclonal Ab (mAb) to human CD40, Clone EA-5, isotype IgG1, 1:80 (Calbiochem, La Jolla, CA, USA), Clone HB-14, isotype IgG1, 1:100 (gift from Tedder TF, Department of Immunology, University Medical Center, Durham, NC, USA), rabbit polyclonal Ab (pAb) CD40, 1:100 (Santa Cruz Biotecnology Inc., Santa Cruz, CA, USA), mouse mAb CD40L. Clone 24–31, isotype IgG1, 1:100 (Calbiochem) or rabbit pAb CD40L 1:100 (Santa Cruz). Ab were diluted in 4% BSA/PBS. Biotinylated secondary Ab (horse antimouse IgG or goat antirabbit IgG; Vector Laboratories, Burlingame, CA, USA) were applied for 30 min. Incubation with avidin-biotin complex (DAKO A/S, Glostrup, Denmark) for 60 min was followed by development in 3-amin-9-etylcarbazole (AEC kit, Vector Lab. Inc., Burlingame, CA, USA) for 15 min. Sections were counterstained with haematoxylin, rinsed in tap water and mounted with aqueous mounting medium. Sections were washed in PBS after each step, except after incubation with blocking serum. Cryostat sections of tonsils were included as positive controls.

E-cadherin, CD44, Laminin-5 and Collagen IV

Five µm formalin-fixed, paraffin-embedded sections were incubated using the DAKO autostainer – Universal Staining System (DAKO-USA, Carpinteria, CA, USA) as previously described (25). The following mAb were used: antihuman E-cadherin, clone HEC-1, isotype IgG1, 1:15000 (R&D Systems Inc., Minneapolis, MN, USA), antihuman CD44 (pan Ab), Clone DF1485, isotype IgG1, 1:1500 (DAKO), anti-Laminin-5, Clone D4B5, isotype IgG1, 1:100 (CHEMICON International, Inc., 28820 Single Oak Drive, Temecula, CA, USA), antihuman Collagen IV, Clone CIV 22, isotype IgG1, 1:5 (DAKO). Normal OM (n = 5) and oral squamous cell carcinomas (n = 3) were included as positive controls. Salivary glands deeper in the tissue biopsies of OLP served as internal positive controls for E-cadherin.

Immunohistochemical CD40 and CD3 double-labelling

Cryostat sections were fixed in 50% acetone at 4°C for 30 s, followed by 100% acetone for 5 min and processed for double immunohistochemical labelling. The sections were incubated with mouse mAb CD40 1:1000 (Calbiochem) for 60 min, followed by 0.03% H2O2 (DAKO) for 5 min. Thereafter, the sections were incubated with Envision horseradish peroxidase-labelled polymer (DAKO) for 30 min, followed by development with diaminobenzidine (DAB) + chromogen (DAKO) for 7 min. After 5 min rinse in distilled water, sections were fixed in 4% formalin at room temperature for 2 min. Further, sections were placed in boiling TBS/EDTA (pH 9.0), and left for 1 min to cool at room temperature. Incubation with the second Ab, CD3 rabbit pAb 1:50 (Novocastra Lab. Ltd, Newcastle, UK), for 60 min was followed by incubation with Envision Alkaline-phosphatase (DAKO) for 30 min. After development
with New Fuchsin (DAKO) for 10 min, sections were counterstained with haematoxylin, rinsed in tap water and mounted with aqueous mounting medium. Sections were washed in TBS (pH 7.6) for 10 min after each step. The Ab were diluted in antibody diluent (DAKO). Serial sections were incubated with the two Ab separately for comparison.

Negative controls for immunohistochemistry included replacement of primary Ab with normal horse serum, Tris-buffered saline (TBS) or antibody diluent (DAKO). The various mAb of the same isotype served as negative control for each other.

In situ mRNA hybridization for CD40 and CD40L

CD40 or CD40L mRNA were detected using a set of probes (Table 1), each antisense probe detected a different region of CD40 or CD40L. One of the CD40 antisense probes has been previously described (26). The other probes were laboratory designed using NATIONAL BIOSCIENCES OLIGO PRIMER ANALYSIS software programme, version 5.0 for Windows (Molecular Biology Insights, Cascade, CO, USA). Total mRNA was detected using an 18-mer 'poly-T' probe. All probes were biotinylated at the 5'-end. The catalysed signal amplification system for in situ hybridization (ISH; Genpoint kit, DAKO) was used for detection of CD40 and CD40L mRNA as previously described (27). Sections were fixed in 4% buffered paraformaldehyde for 10 min, followed by washing in 0.1% diethylpyrocarbonate-treated H₂O. Probes at a concentration of 3 ng/μl in hybridization solution (DAKO) were applied on sections, covered with coverslips and incubated overnight under humid conditions at the appropriate hybridization temperature (Table 1). Probes were tested individually and as a cocktail. After hybridization, coverslips were removed by washing in TBS, 0.1% Triton-X-100, pH 7.6 for 10 min. The sections were subjected to a stringent wash for 20 min at 55°C (room temperature for 'poly-T'). The hybridization reaction was visualized with peroxidase substrate at room temperature for 3 min. Sections were counterstained with haematoxylin, rinsed in tap water and mounted with aqueous mounting medium. Incubation with biotinylated sense probes complementary to the antisense probes, were performed to test the specificity of the hybridization reaction. A biotinylated 18-mer 'poly-T' probe was used for detection of total mRNA in sections. To test the specificity of our detection system, particularly for non-specific staining due to endogenous biotin activity, sections for control were incubated with hybridization solution only. Cryostat sections of human tonsils were used for positive control.

Evaluation of tissue sections

Tissue sections were evaluated morphologically under light microscope (Leica DMLM, GmbH, Munster, Germany). Special attention was given to evaluate the epithelium, basal keratinocytes and subepithelial inflammation in diseased areas compared with non-diseased areas of OLP. Cell counts were performed for immunohistochemical CD40 and CD40L expression, as well as for mRNA CD40 and CD40L expression. The cell counts were performed manually in: (i) epithelial basal cells in OLP and OM and (ii) subepithelial cell infiltrate in OLP. A fitted ocular grid was placed along the basement membrane zone and at subsequent regions in the subepithelial cell infiltrate of OLP lesions. Up to 1000 cells were counted for each slide at x400 magnification. Before cell counting, sections was blinded and interobserver calibration was performed (EN, LLL, ACJ). Results are presented in percentage positive cells of total (number of positive cells:total cells).

Results

Loss of CD40 expression in basal keratinocytes in diseased areas of OLP

In OLP, most basal keratinocytes (93–95%) of non-diseased epithelium expressed CD40, while in areas exhibiting subepithelial inflammation and/or basal cell destruction only few keratinocytes (10–15%) expressed CD40 (Fig. 1a,b). Intraepithelial dendritic CD40-expressing cells, comparable with Langerhans cells were occasionally observed (Fig. 1c,d). In the subepithelial cell infiltrate CD40 was detected in more than half

Table 1 Primer sequence for the antisense probes used in the in situ mRNA hybridization

<table>
<thead>
<tr>
<th>Target</th>
<th>Probe sequence</th>
<th>Incubation temperature (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD40</td>
<td>1. 5'-CCT-CCT-GGG-TGA-CCG-GTT-GGC-3'</td>
<td>50</td>
</tr>
<tr>
<td></td>
<td>2. 5'-GAG-AAG-AAG-CCG-ACG-GGG-CAG-3'</td>
<td>50</td>
</tr>
<tr>
<td></td>
<td>3. 5'-AGC-GAG-GTG-AGA-CCA-GGC-GGC-3'</td>
<td>52</td>
</tr>
<tr>
<td></td>
<td>4. 5'-AGC-CCC-TCA-CCC-TCA-GGC-CAC-3'</td>
<td>52</td>
</tr>
<tr>
<td>CD40L</td>
<td>1. 5'-GTA-ATG-AGG-AGT-GGG-CAG-GCT-CAG-GGC-3'</td>
<td>68</td>
</tr>
<tr>
<td></td>
<td>2. 5'-GCA-CTC-CTC-CTC-CTC-CC-3'</td>
<td>46</td>
</tr>
<tr>
<td></td>
<td>3. 5'-AGG-GGG-TGG-GCT-TAA-CCG-CTG-3'</td>
<td>52</td>
</tr>
<tr>
<td></td>
<td>4. 5'-GAG-CCT-GGC-CCC-CTC-CAA-CAA-3'</td>
<td>52</td>
</tr>
</tbody>
</table>
(55–60%) of the mononuclear cells present (Fig. 1a,b). CD40 was detected in all specimens of normal OM in most basal keratinocytes (95–97%). The two mAb against CD40 gave similar staining patterns. Staining was more distinct with the mAb compared with the pAb. In tonsils, CD40 was detected in the germinal centres, mantel zones and interfollicular regions.

Loss of CD40L in the basement membrane zone in areas of basal cell destruction in OLP
CD40L was detected in the basement membrane zone in all OM specimens and non-diseased areas of OLP. However, CD40L was lost in areas of OLP exhibiting basal cell destruction, or the expression was weak and diffuse in areas where the basement membrane zone was not well defined (Fig. 1c,f). Occasional CD40L-expressing cells were seen in the epithelium. In the subepithelial cell infiltrate, <5% of the cells expressed CD40L. In addition, a few scattered cells in the deeper connective tissue stroma expressed CD40L. The mAb against CD40L was more specific and gave less background staining compared with the pAb. In tonsils, a few lymphoid cells in the germinal centres and interfollicular zones expressed CD40L.

Intraepithelial double-labelled – CD40- and CD3-expressing cells in OLP
Scattered intraepithelial T cells (CD3+) expressing CD40 were detected in OLP samples (Fig. 1c,d). Some of the T cells were present in the basal cell region, in close proximity to CD40+ basal keratinocytes (Fig. 1c,d). In the subepithelial cell infiltrate, some
T cells (CD3⁺) expressed CD40, while others expressed CD3 only (Fig. 1c,d). In addition, cells expressing CD40 only were observed in the mononuclear cell infiltrate (Fig. 1c,d).

**Expression of CD40 and CD40L mRNA**

ISH demonstrated CD40 mRNA expression in all biopsies of OLP and OM investigated (Fig. 2a,b). In OM, CD40 mRNA was predominantly detected in basal keratinocytes (85–90%) and parabasal cells (Fig. 2a). In contrast, CD40 mRNA was detected in only a few keratinocytes (6–7%) of the basal cell layer in OLP (Fig. 2b). CD40 mRNA was widely detected in the subepithelial cell infiltrate (45–50%; Fig. 2b). Incubation with the sense probes was negative. In tonsils, CD40 mRNA was detected in almost all cells in the mantel zones of follicles, and fewer cells in the germinal centres and interfollicular zones were positive.

ISH demonstrated CD40L mRNA expression in all OLP and OM biopsies investigated (Fig. 2c,d). In OM, scattered CD40L mRNA-expressing cells were present in the epithelium and in occasional subepithelial inflammatory cells (<5%; Fig. 2c). In OLP, only a few (<5%) scattered cells demonstrated CD40L mRNA expression in the epithelium and in the mononuclear subepithelial inflammatory cell infiltrate (Fig. 2d). Incubations with the sense probes were negative. In control tonsillar tissue, CD40L mRNA was predominantly detected in germinal centres. Total mRNA detected by the ‘poly-T’ probe, was demonstrated in all sections of OLP, OM and tonsillar tissue.

---

**Figure 2** Demonstration of CD40 and CD40L mRNA expression by in situ hybridization. CD40 mRNA expression in oral mucosa (OM; a) and oral lichen planus (OLP; b). CD40L mRNA expression in OM (c) and OLP (d). Original magnification ×200.

---

**Focal loss of E-cadherin expression and maintained CD44 expression in the epithelium of OLP**

In diseased areas of OLP, there was a focal reduction of E-cadherin expression in basal keratinocytes especially in areas with intense subepithelial cell infiltrate (Fig. 3a,b). In non-diseased areas of OLP and normal OM, E-cadherin showed a marked expression in the basal cells (except for the part of the cell membrane attached to the basement membrane) and parabasal cells, and lower intensity in superficial cell layers of OLP and normal OM (Fig. 3c,d). Scattered E-cadherin-positive cells with the morphology suggestive of macrophages were detected in the subepithelial cell infiltrate (Fig. 3a,b). Salivary glands deeper in the tissues showed E-cadherin-positive ductal cells.

A marked expression of CD44 was detected in the epithelial basal cell region with a lower intensity in the spinous and superficial cell layers. However, no difference in the expression was observed in OLP compared with normal OM (Fig. 3e,f). In the subepithelial infiltrates of OLP samples, CD44 was observed in one-third of the mononuclear cells. Most fibroblasts in normal OM expressed CD44.

**Laminin-5 and Collagen IV expression along the basement membrane region in OLP**

Expression of Laminin-5 and Collagen IV was detected along the basement membrane zone in all OLP investigated. However, a variation in the intensity and thickness was seen between samples. Laminin-5 expression was observed to be more intense and labelled a thicker
band along the basement membrane region in OLP compared with OM. Regions with minor breaks and thickening of polarized Collagen IV expression were seen in focal areas of basal cell destruction in OLP.

**Discussion**

In this study, we report loss of CD40 and CD40L in basal cell region of diseased areas in OLP. We suggest that the downregulation of CD40 on basal keratinocytes observed in OLP may be a mechanism of escape from apoptosis by CD40–CD40L interaction. Thus, other mechanisms are more likely to be directly involved in direct triggering of apoptosis in OLP. It is worth considering that the observed loss of both CD40 on basal keratinocytes and CD40L in basement membrane region may be signals to promote keratinocyte renewal compensating cell loss in areas of basal cell destruction in OLP. This suggestion is based on previous *in vitro* studies on skin monolayers, showing that CD40–CD40L interaction inhibits epidermal keratinocyte renewal by modulating the cell cycle (28). Furthermore, actively proliferating cells display a decreased CD40 expression (10).

In order to further characterize the CD40-expressing cells observed within the epithelium of OLP, a double-labelling with the T-cell marker CD3 was performed. We found that T cells in the epithelium and subepithelial cell infiltrate may express CD40. Accordingly, these cells may interact with other cells via the CD40–CD40L system,
which may be of importance in cell inflammatory responses in OLP. The intraepithelial T cells in OLP may further amplify inflammation as well as induce keratinocyte apoptosis, and the scattered CD40L-expressing cells observed in the epithelium of OLP are likely to be activated (29, 30). T-cell communication in the epithelium of OLP may occur via CD40–CD40L interactions with keratinocytes or Langerhans cells. Some of the observed intraepithelial CD40-expressing cells may be Langerhans cells, which are important powerful antigen-presenting cells and provide survival signals for cytotoxic T cells, allowing them to develop into powerful effectors (8).

The subepithelial cell infiltrate serves as a constant pool of T cells that may be recruited as potential apoptotic executioners of the keratinocytes in OLP. Lymphocytes expressing CD40, CD3 or both were observed, but only a few cells expressed CD40L in the subepithelial cell infiltrate. This may be explained by transient expression of CD40L (8, 29), and that only a minor fraction of the T cells (<5%) are in an activated state in the subepithelial cell infiltrate of OLP (31–33). However, this fraction of T cells may be especially potent in amplifying the inflammation via CD40–CD40L crosslinking on antigen-presenting cells such as macrophages, other T cells, endothelial cells and fibroblasts (8, 34, 35). The outcome of such crosslinking may be upregulation of different types of adhesion molecules and increased cytokine synthesis, as seen in OLP (33–37). Accordingly, the activated CD40L-expressing T cells in the subepithelial cell infiltrate of OLP may be important as orchestrators of chronicity in OLP.

As E-cadherin is important in maintaining the structural integrity of normal stratified squamous epithelium (15, 16), its observed focal loss of expression in basal keratinocytes may contribute to loss of cell-to-cell contacts and destruction of basal keratinocytes. Although, the precise function of E-cadherin in oral epithelial biology has not yet been thoroughly investigated, a non-functional E-cadherin has been shown to cause leaky epithelium in mouse skin and intestine in vivo (38, 39). Reduced E-cadherin expression has also been reported in basal keratinocytes of psoriatic lesions, and reduced levels of functional E-cadherin has been related to enhanced transmigration of human neutrophils in epithelial cell monolayers (40). Thus, loss of E-cadherin compromises epithelial integrity and may play a role in the pathogenesis of OLP by allowing cytokines as well as T cells to penetrate more easily into the epithelial compartment. Moreover, it is worth considering that this E-cadherin loss observed may be involved in apoptosis of basal keratinocytes in OLP. This suggestion is supported by studies indicating that apoptosis may be prevented by E-cadherin-mediated cell contact (18, 19). To the best of our knowledge, focal reduction of E-cadherin expression in OLP has not been previously reported.

CD44 expression was observed to be maintained in OLP, with no obvious differences compared with normal OM. This observation is consistent with previous immunohistochemical reports with pan CD44 Ab in skin biopsies of LP (41, 42). One of these studies indicated, although the material only comprised a few lesions of skin LP, that the expression of some isoforms may be reduced in the spinous cell layers (41). As we found no difference in the expression of CD44 in OLP and normal OM, CD44 is most probably not directly involved in apoptosis of basal keratinocytes in OLP. Other changes in the epithelial basement membrane constituents observed, such as Laminin-5 and breaks in Collagen IV expression (43, 44), may together with E-cadherin loss contribute to a compromised epithelial integrity facilitating T cells to more easily migrate into the epithelium.

Taken together, the CD40–CD40L system may orchestrate inflammatory T-cell responses both in the epithelium and subepithelial cell infiltrate of OLP. As basal keratinocytes downregulate CD40, they may escape CD40–CD40L-induced apoptosis in OLP. Thus, other mechanisms of triggering apoptosis are most likely involved. On the other hand, loss of E-cadherin in basal keratinocytes may contribute to reduced basal cell structural integrity allowing enhanced T-cell migration into the epithelial compartment in OLP.

References


Acknowledgements

We thank Gudveig Fjell for skilful technical assistance and Søren Nielsen (DAKO, Denmark) for good technical advices. We also wish to thank Knut Tornes at Department of Oral and Maxillofacial Surgery, Haukeland University Hospital and the staff at Department of Oral Surgery and Oral Medicine, Dental Faculty for help in patient sampling.