Paper III

DNA content and Cyclooxygenase-2 as risk markers of cancer development in Oral lichen planus

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List of abbreviations:

Ab	Antibodies
BSA	Bovine serum albumin
Cox-2	Cyclooxygenase-2
DAB	Diaminobenzidine
E-cadherin	Epithelial-cadherin
EDTA	Ethylene-diamine-tetraacetic-acid
LP	Lichen planus
mAb	Monoclonal antibodies
OLP	Oral lichen planus
OSCC	Oral squamous cell carcinoma
PBS	Phosphate-buffered saline
TBS	Tris-buffered saline

Abstract

Oral lichen planus (OLP) is associated with an increased risk of malignant transformation. The aims of this study were to investigate candidate prognostic markers for risk of cancer development, such as DNA content, expression of Cox-2 and E-cadherin in lesions of OLP. We investigated 78 archival biopsies from; (1) 26 OLP patients with at least two biopsies, of whom seven presented OLP with epithelial dysplasia, followed by OSCC in five of them, (2) 19 OLP patients with one biopsy taken.

Image cytometry for measurement of DNA content and immunohistochemistry for visualization of Cox-2 and E-cadherin expression were performed.

All OLP biopsies investigated were classified as diploid, one OLP with epithelial dysplasia was tetraploid and all OSCC were diploid. Cox-2 was detected in the epithelium of all OLP specimens investigated, as well as in epithelial dysplasias and OSCC. Focal loss of E-cadherin expression was observed in basal keratinocytes in 88% of the OLP specimens investigated, in all epithelial dysplasias and OSCC.

In conclusion, neither ploidy, Cox-2 or E-cadherin were significant sensitive markers to select OLP lesions at risk for development of OSCC in the present patient material.

Introduction

Oral lichen planus (OLP) is a chronic inflammatory disorder, defined as a precancerous condition by the World Health Organization (1). However, the malignant potential of OLP is still controversial and has been debated for years (2-4). Previous studies indicate a risk of 0.4-6.25% for development of oral squamous cell carcinoma (OSCC) (5-6), and also a greater risk for development of a second primary OSCC in these patients (7). Accordingly, there is a great need for objective markers to select which lesions that are prone to undergo malignant transformation in OLP, as well as in other lesions considered to be premalignant. Candidate prognostic markers may include genetic and molecular changes that arise during carcinogenesis.

Aneuploidy, an unbalanced representation of chromosomes, is considered to be a primary cause of genomic instability of preneoplastic and neoplastic cells (8), and a frequent change observed in human cancers and pre-cancers (9-10). Measurements of DNA content are promising, but it is not yet clarified whether aneuploidy alone is a significant prognostic factor in oral epithelial premalignant lesions and OSCC (11-13). In OLP, a few reports of epithelial DNA content measurements including OLP biopsies and cytology samples, have presented highly variable results from none of the lesions classified as aneuploid up to 41% (14-18). Further investigation is therefore necessary for evaluation of aneuploidy as a candidate predictive marker, alone or together with other potential markers, for risk of cancer development in OLP.

Chronic inflammation has been suggested to be a cofactor for development of OSCC in OLP (19). Special attention has been paid to the expression of cyclooxygenase-2 (Cox-2) and its potential role in oral epithelial dysplasias and development of OSCC (20-22). Cox-2 is an

inducible enzyme that catalyses the synthesis of prostaglandins in most tissues and cell types including epithelial keratinocytes, fibroblasts and T cells (23-24). The presence of Cox-2 is often associated with cell activation and pathophysiological stimuli including inflammation (25). Several processes important for cancer development are influenced by Cox -2, such as cell proliferation, apoptosis and angiogenesis (21, 26). Cox-2 may inhibit apoptosis via different pathways, such as down-regulation of arachidonic acid (26), reduced cytocrome-c and caspase-3 activation (27), as well as up-regulation of the protooncogene Bcl-2 (28). Accordingly Cox-2 may contribute to increased cell survival. With additional molecular cellular changes and genetic instability, it is thus suggested that Cox-2 may promote tumour progression in chronic inflammatory conditions (29).

Epithelial (E)-cadherin, a member of the cadherin superfamily of proteins, is involved in cell-to-cell adhesion and signalling in stratified squamous epithelium (30). Among its other functions, this protein may act as an invasion suppressor molecule (31). E-cadherin loss or heterogenous expression is observed in oral epithelial dysplasias, and has also been related to a more aggressive behaviour and worse prognosis in OSCC (32-33). One immunohistochemical study has recently shown focal loss of E-cadherin in basal keratinocytes in diseased areas of OLP (34).

In order to investigate if aneuploidy, Cox-2 or E-cadherin expression may be utilized as risk markers of cancer development in OLP lesions, we have investigated a unique biopsy material from OLP patients. These patients were followed over time and had several biopsies taken, and a few of them developed into epithelial dysplasia and OSCC.

Materials and methods

Tissue specimens

Seventy-eight archival biopsies from patients (n=45) with clinically and histologically verified OLP were included in this study. The biopsies were divided into two groups: (1) Fifty-nine biopsies from 26 patients (18 females and 8 males, mean age 55 yrs, and range: 31-71) who had been followed up for 6-225 (mean: 91) months, and had more than one biopsy taken. One of these patients showed epithelial dysplasia within the OLP lesion at the first biopsy, and six of the patients developed epithelial dysplasias in their lesions over time (Fig. 1). Five of the seven patients developed OSCC (Fig. 1), of whom one patient had recurrence of OSCC and another got a new primary OSCC in another area of the oral cavity with OLP. (2) Biopsies from 19 patients (11 females and 8 males, mean age 64 yrs, and range: 36-83) selected due to classical histological picture of OLP, and with a biopsy from a primary lesion taken for diagnostic purpose. The biopsies were taken (1978-2003) at Department of Oral Surgery and Oral Medicine, Faculty of Dentistry, University of Bergen, and Department of Maxillofacial Surgery, Haukeland University Hospital, Bergen, Norway.
Normal oral mucosa samples from voluntary healthy individuals (n=7) were included as controls.

Preparation of isolated nuclei and measurements of DNA content

The specimens were blinded. Three tissue sections (50µm) were deparaffinized in xylene, rehydrated through a decreasing alcohol gradient and washed in phosphate-buffered saline-(PBS). To disaggregate the cells, the sections were incubated for 1h in 0.05% protease (0.5 mg/ml diluted in PBS), and the preparation of isolated nuclei/ monolayers were performed according to established protocols (35).

The DNA content of Feulgen–Schiff stained nuclei was measured and analyzed using the Fairfield DNA Ploidity System according to established protocols (35-36). Monolayers were analyzed using a Zeiss Axioplan II microscope (Zeiss, Oberkochen, Germany) (40x/0.65) with a 546 nm green filter and modified for computer control of the stage, equipped with a high resolution digital camera (C4742-95, Hamamatsu, Photonics K. K., Hamamatsu, Japan). At least 500 cell nuclei were measured and stored in galleries for each case. The nuclei were picked manually based on morphologic appearance of keratinocyte nuclei, being aware of the large amount of mononuclear inflammatory cells in the samples. Lymphocytes were included as internal controls (calibration). Some samples were measured automatically for internal control as well (2000 cell nuclei). Staining and DNA measurements as well as classification of histograms were performed at the Department of Pathology, Rikshospitalet-Radiumhospitalet Medical Center, Oslo, Norway.

DNA Ploidy – classification

A lesion was classified as diploid if only one G0/G1 (2c) peak was present, if the number of nuclei in the G2 (4c) peak did not exceed 10% of the total, or if the number of nuclei with DNA content >5c was below 1% of the total number of nuclei (Fig. 2A). A lesion was classified as tetraploid when one G0/G1 (2c) peak was present in the diploid position together with a G2 (8c) peak, or when the number of nuclei in 4c peak exceeded 10% of the total number of nuclei (Fig. 2B). A lesion was defined as aneuploid if the peaks appeared outside the area of 2c, 4c or 8c, or if the number of nuclei with DNA content above 5c or 9c exceeded 1%. A lesion was classified as polyploid when the number of nuclei at 8c exceeded 10% of the total, or if the number of nuclei at 9c exceeded 1% (35).

Immunohistochemical analysis

Four μm sections from formalin fixed, paraffin embedded tissue were processed by DAKO autostainer (DAKO-USA). Sections were deparaffinized in Xylene, hydrated through a graded alcohol series and rehydrated in distilled water. For target retrieval, samples were treated with Tris-Ethylene-diamine-tetraacetic-acid (EDTA) (pH: 9.0) at 97^0C (in a boiling hot tub) for 25 min, or 10 mM Citrate buffer (pH: 6.0), and left for 25 min to cool in room temperature. Endogenous peroxidase activity was quenched by 0.03% H_2O_2 diluted in Tris-buffered-saline (TBS; 0.05M Tris-HCl, 0.15M NaCl, pH 7.6, with Tween 0.001%) for 15 min. Incubations were performed with different primary antibodies (Ab): Mouse monoclonal Ab (mAb) Cox-2, clone 229 (Zymed laboratories, Invitrogen immunodetection, South San Franscisco, CA 94080) 1:600, mAb Cox-2, Clone 294 (DAKO) ready to use, rabbit mAb Cox-2 (NeoMarkers, Lab Vision UK, Ltd. The Pines, Suffolk, UK) ready to use, Anti-human E-cadherin, clone HECD-1, 1:1500 (R&D systems Inc., Minneapolis, MN 55413, USA) as used in a previous study (34), for 1 hour in humidified chamber. All Ab were diluted in antibody diluent (DAKO). Secondary Ab, conjugated with horseradish peroxidase labelled polymer (EnVision$^+$ System, DAKO), were applied for 30 min. After each step, sections were washed twice (10 min) in TBS. Development with 3,3'diaminobenzidine (DAB, DAKO) in buffered substrate solution (pH 7.5) was performed for 10 min. After dehydration to Xylene, sections were counterstained with Hematoxylin (DAKO) and mounted with Eukitt (O. Kindler GmbH&Co, Germany). Sections from OSCC served as positive controls for Cox-2, and normal oral mucosa for E-cadherin. Specimens incubated with antibody diluent (DAKO) served as negative controls. The various mAb of the same isotype served as negative control for each other.

Evaluation of tissue sections

Tissues were evaluated morphologically under light microscope (Leica DMLM, GmbH, Munster, Germany). Histologically, most OLP lesions (n=40) presented regions with both epithelial atrophy and acanthosis within the same specimen. Eighteen lesions showed acanthosis only and five had epithelial atrophy. Five lesions had minor ulcerations within the OLP lesion. Special attention was given to evaluate the epithelium, basal keratinocytes and subepithelial inflammation in diseased areas compared to non-diseased areas of OLP. Biopsies with epithelial dysplasia (n=7) and OSCC (n=8) were carefully evaluated separately and together with the previous OLP biopsies from the same patients to verify the diagnosis. Semi-quantitative registration of Cox-2 expression in the epithelium of OLP was performed. OLP lesions were divided into three groups according to Cox-2 expression; (1) in the basal cells only with a perinuclear localization, (2) in both basal and parabasal cell layers with perinuclear and cytoplasmic localization, or (3) in focal areas also including more superficial cell layers, with both perinuclear and cytoplasmic localization. In addition, cell counts were performed for Cox-2 in the subepithelial cell infiltrate for each slide at x 400 magnification and presented as percent positive cells of total. Expression of E-cadherin in keratinocytes of OLP was registered. Results are presented as percent of biopsies with observed focal E-cadherin loss in basal keratinocytes of total biopsies investigated.

Results

All OLP lesions investigated were diploid, and one epithelial dysplasia was tetraploid

In all biopsies of OLP (n=58) investigated the keratinocyte nuclei were classified as diploid (Fig. 2A). One patient with epithelial dysplasia within the OLP lesion in the first biopsy was also classified as diploid, and later biopsies from this patient showed OLP without signs of epithelial dysplasia (Fig. 1, patient no: 6). Of the six epithelial dysplastic lesions which had developed in areas of OLP over time (Fig. 1), one lesion was classified as tetraploid (Fig. 2B), and this patient presented with OSCC 2 years after (Fig. 1, patient no: 1). All the OSCC were classified as diploid (n=7), and were developed in 5 patients with previous diploid epithelial dysplasia, except for the one tetraploid epithelial dysplasia. None of the biopsies investigated were classified as aneuploid.

Cox-2 is expressed in the epithelium of OLP, dysplasia and OSCC

Cox-2 was expressed in all OLP specimens investigated (n=63). In the epithelium of OLP, Cox-2 was expressed in different regions and with varying intensity; (1) in the basal cells only with a perinuclear localization (16%), (2) in both basal and parabasal cell layers with perinuclear and cytoplasmic localization (44%), or (3) in focal areas with both perinuclear and cytoplasmic localization of expression, also including more superficial cell layers (Fig. 3A, B) (40%). No obvious correlation was observed between Cox-2 expression and epithelial thickness in OLP. In patients of whom there had been taken several biopsies over time, the later biopsies with probably longer standing lesions, did not show any obvious differences (like more abundant expression of Cox-2) compared with the previous biopsies. In the subepithelial cell infiltrate of OLP, a varying degree of Cox-2 expression was observed, from no Cox-2 expression in some regions up to 50% of the mononuclear cells in other regions. In

epithelial dysplasias investigated (n=4), Cox-2 expression was observed in focal areas of the epithelium with both perinuclear and cytoplasmic localization in basal, parabasal and/or spinous cell layers. In the OSCC investigated (n=5), a focal Cox-2 expression was observed with both perinuclear and cytoplasmic localization in the tumour islands of infiltrating epithelial cells and in some infiltrating mononuclear inflammatory cells. The expression had no obvious relation to the tumour front.

Striated muscles deeper in the tissue expressed Cox-2. In salivary glands present in the connective tissue stroma, Cox-2 expression was detected in acini and myoepithelial cells. In tonsils, Cox-2 expression was observed in intrafollicular central regions and interfollicular regions with perinuclear localization. There was no Cox-2 expression within the epithelium or in the connective tissue stroma of the normal OM controls (n=7). Similar expression pattern was seen with the different antibodies.

Focal loss of E-cadherin in epithelial basal cells in OLP, epithelial dysplasia and OSCC

E-cadherin was detected in all normal OM (n=7), OLP (n=56), epithelial dysplasias (n=5) and OSCC (n=7) investigated. In 88% of the biopsies there was a focal reduction of E-cadherin expression in the epithelial basal cells in areas with subepithelial mononuclear cell infiltrate (Fig. 3C, D). In the subepithelial cell infiltrate, scattered cells expressing E-cadherin with the morphology suggestive of macrophages were detected. In all lesions with epithelial dysplasia, focal loss of E-cadherin was observed in the basal cell region, but mostly maintained in the spinous cell layers. In OSCC, E-cadherin expression was lost in some regions of the epithelial invasive tumour islands, but maintained in other regions. In normal OM and non-diseased areas of OLP, E-cadherin showed a marked expression in the basal cells and parabasal cells and lower intensity in superficial cell layers.

Discussion

In the present study, we could not detect any aneuploid OLP lesions, nor did the lesions that developed into epithelial dysplasias or OSCC show aneuploidy. This may indicate that aneuploidy is not a sensitive enough marker to select OLP patients at risk for developing OSCC. We are aware that the present patient material is limited with respect to development of epithelial dysplasia and OSCC in OLP, and that careful consideration must accomplish interpretation of the results. On the other hand, the availability of larger patient materials as such is limited, since the percentage of OLP lesions that undergo malignant transformation is so low. If aneuploidy was to be used as a prognostic marker for risk of cancer development in patients with OLP, we would in our case not have been able to select those that developed epithelial dysplasia or OSCC over time.

If tetraploidy were included as a risk factor indicating an unstable lesion, one OLP patient's lesion with epithelial dysplasia would have been sorted out, and this patient developed OSCC two years later. In normal cells, tetraploidy occurs temporarily in the synthesis phase of the cell cycle prior to cell division, when the cell nucleus contains double amount of the DNA compared to a normal non-dividing cell. Polyploidy as such (any situation in which whole integer multiples of the haploid chromosomes are found) may arise as a protective mechanism upon stress or maladaptive response (37). Previous reports indicate that there is a high incidence of tetraploid endothelial cells and fibroblasts in tissue regeneration and repair processes such as human wound healing (38-39). Tetraploidy may thus arise in OLP due to tissue regeneration processes and repair, especially in areas of basal cell destruction. This may explain the reported high incidence of polyploidization in one previous study of OLP lesions (14). The generation of tetraploid and polyploid cells can however be hazardous in long

standing chronic inflammatory lesions, due to increased risk of generating aneuploid cells (40-41).

That all OLP lesions were classified as diploid, may support the opinion that the lesion itself is not precancerous. The DNA nuclear ploidy analysis is however a gross measurement of genetic damage, and may thus not be sensitive enough as a marker in OLP. In the present study, all OSCC developed in patients with previous epithelial dysplasia in their OLP lesions. This fact supports the general opinion that these lesions, which present with epithelial dysplasia are the ones with increased risk for developing OSCC (2, 6). That the only tetraploid OLP lesion detected also presented with epithelial dysplasia, may add further evidence to this opinion. These findings also confirm the importance of careful histologic evaluation in OLP patients. One study has reported an alarming high incidence (41%) of aneuploid lesions in OLP, in contrast to our findings (15). Such a high incidence is not compatible with the potential risk for cancer development (0.4-6.25%) in OLP, and one may speculate if this may reflect methodological differences such as selection of patients or lack of calibration between laboratories. Our observations are however, comparable with a recent study where all reticular OLP were classified as diploid, and a few erosive lesions (2/25) only were classified as aneuploid (16). In the present study, most lesions presented histological regions of acanthosis and epithelial atrophy within the same specimens, but also the few atrophic lesions were classified as diploid. Also, another recent study of cytology samples from OLP lesions found that all samples were diploid (18).

That epithelial dysplasias and OSCC developed in patients with diploid OLP lesions, illustrates the need for other risk markers in OLP patients. The wide expression of Cox-2, not only in epithelial dysplasia and OSCC, but also in epithelial keratinocytes of OLP, appears to

be a novel observation. Accordingly this may rule out Cox-2 as a sensitive marker to select OLP patients at risk. However, the presence of Cox-2 may indicate a biological function in chronic inflammation and potential cancer development in OLP lesions. According to the varying expression observed (from expression on the nuclear membrane only to focal areas also including the cytoplasm of epithelial keratinocytes), this enzyme seems to be strictly regulated in OLP. The Cox-2 expression observed only on the nuclear membrane region of keratinocytes and T cells may be explained by the localization in the endoplasmatic reticulum and nuclear membrane (25, 42-43). With a further up-regulation, Cox-2 is also expressed in the cytoplasm (44). Since Cox-2 may inhibit apoptosis (21, 28, 45), an up-regulation as such may increase keratinocyte cell survival in OLP. This fact may be important in tissue repair processes especially in areas of basal cell destruction in OLP. However, longer cell survival itself increases the risk of molecular biological changes and genetic instability, and may cause harmful effects especially in already genetically altered cells.

Focal loss of E-cadherin in basal keratinocytes was observed in the majority of OLP lesions as well as in epithelial dysplasia, also indicating that this factor is not a significant prognostic marker candidate for risk of cancer development in OLP patients. This observed E-cadherin loss may play roles in different aspects of the pathogenesis of OLP including apoptosis of basal keratinocytes and migration of T cells into the epithelial compartment (34).

Taken together, all OLP lesions in the present patient material were diploid, including those that underwent malignant transformation. Epithelial cells in OLP lesions also expressed Cox-2 and the majority of basal keratinocytes lost E-cadherin expression in areas of basal cell destruction. Accordingly the present patient material indicates that aneuploidy is not

sensitive enough as a marker to select OLP lesions at risk, neither alone nor together with Cox-2 expression and E-cadherin loss.

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Figure legends

Figure 1

Chart demonstrating follow-up (months) of seven patients with oral lichen planus, development of epithelial dysplasia and oral squamous cell carcinoma.

Figure 2

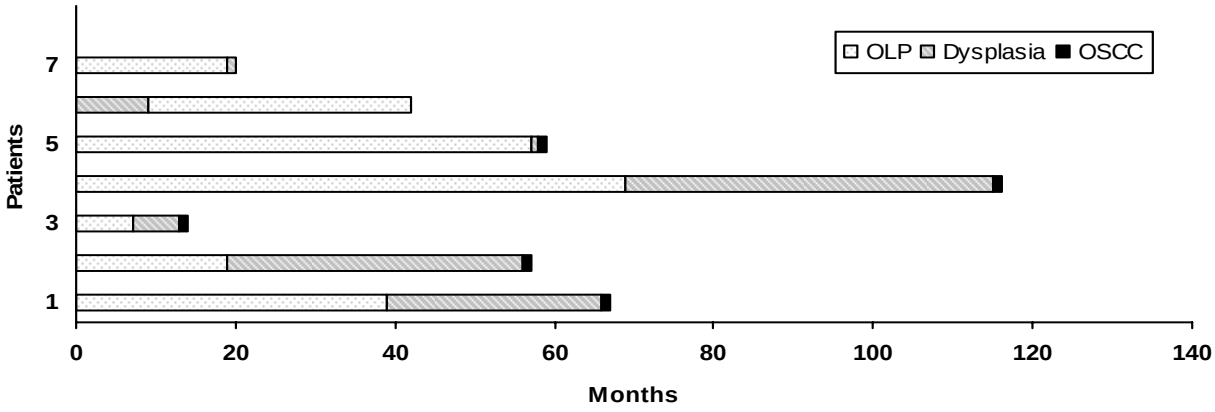
Histograms of one diploid oral lichen planus (A), and tetraploid oral lichen planus with epithelial dysplasia (B).

Figure 3

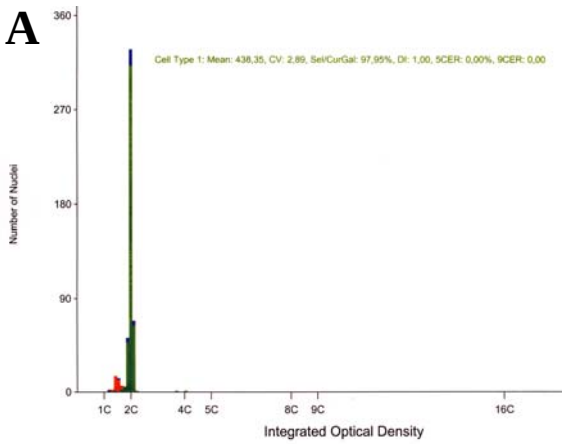
Immunohistochemical localization of Cox-2 in oral lichen planus (A), and at higher magnification (B)

Immunohistochemical E-cadherin expression in oral lichen planus (C), and at higher magnification (D).

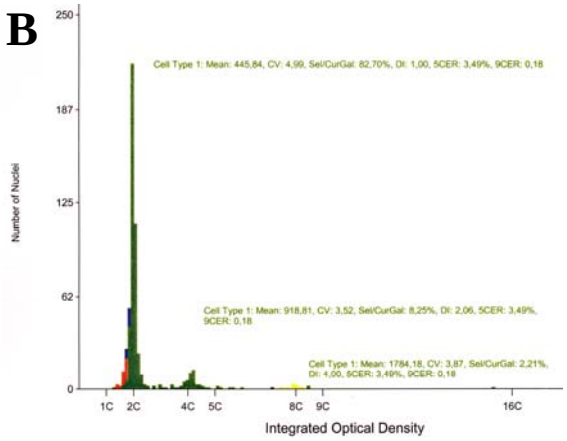
Parallel sections showing corresponding regions. Bar 100 μm.

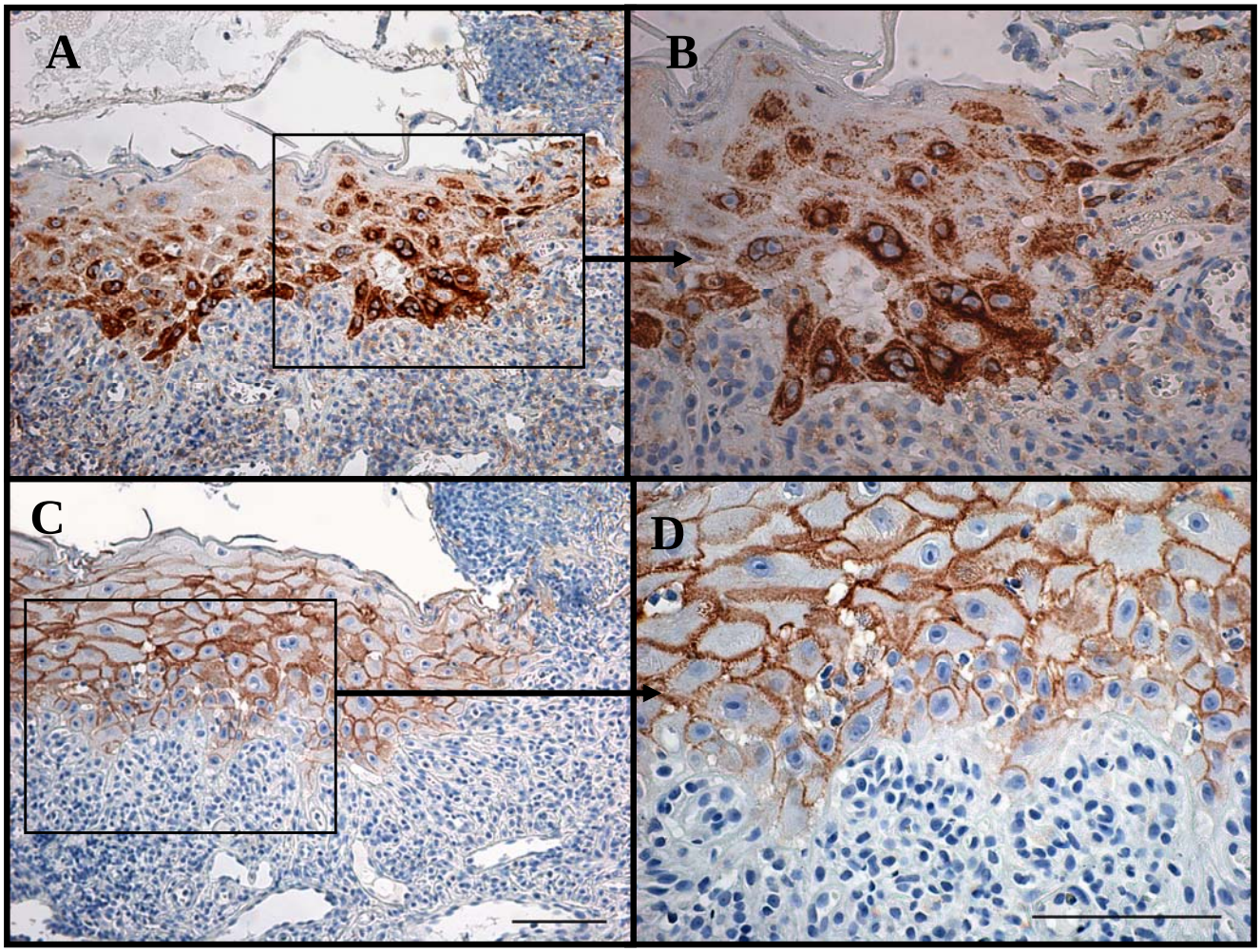

OLP
Dysplasia
OSCC
Patients
7
5
3
1
0
20
40
60
80
100
120
140
Months

A


Cell Type 1: Mean: 438,35, CV: 2,89, Sel/CurGal: 97,95%, DI: 1,00, 5CER: 0,00%, 9CER: 0,00
Number of Nuclei
360
270
180
90
0
1C
2C
4C
5C
8C
9C
16C
Integrated Optical Density

B


Cell Type 1: Mean: 445,84, CV: 4,99, Sel/CurGal: 82,70%, DI: 1,00, 5CER: 3,49%, 9CER: 0,18
Cell Type 1: Mean: 918,81, CV: 3,52, Sel/CurGal: 8,25%, DI: 2,06, 5CER: 3,49%, 9CER: 0,18
Cell Type 1: Mean: 1784,18, CV: 3,87, Sel/CurGal: 2,21%, DI: 4,00, 5CER: 3,49%, 9CER: 0,18
Number of Nuclei
250
187
125
62
0
1C
2C
4C
5C
8C
9C
16C
Integrated Optical Density


A
B
C
D