Distribution of T cells in Oral Leukoplakias Predict Malignant Transformation – Immunophenotype of A Potentially Malignant Oral Disorder

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**Abstract:**

Leukoplakias (LPLs) are lesions in the oral mucosa that have the potential to transform into oral squamous cell carcinoma (OSCC). The aim of this study was to determine the presence of CD1a+ Langerhans cells, CD3+ T cells, Ki-67- and p53-expressing cells in LPLs with dysplasia and the potential as prognostic markers for the risk of transformation into OSCC. Biopsies from patients with the clinical diagnosis LPL and histopathological diagnosis hyperkeratosis with dysplasia were obtained from 23 patients. The specimens were immunostained for Ki-67, p53, CD1a and CD3 positive cells. The patients were divided into two groups: LPLs with dysplasia that have not transformed into cancer (LPL-d) and LPLs with dysplasia that have transformed into cancer (LPL-c). The results showed significantly lower numbers of CD3+ cells in LPL-c than in LPL-d (Ep: p= 0,009; CT: p=0,029). No significant differences were seen between LPL-d and LPL-c in CD1a (Ep: p= 0,577; CT: p=0,533), p53 (Ep: p= 0,622; CT: p=0,502) and Ki-67 (Ep: p=0,498; CT: p=0,951) positive cells. The presence of CD3 positive cells in LPLs with dysplasia, indicates the involvement of the adaptive immune system’s presence in LPLs indicating that. The lower number of CD3+ cells in the LPL-c group supports the cancer immunoediting theory indicating that the number of T cell both intraepithelial and in the connecting tissue surrounding the dysplasia. The presence of Ki-67 and p53 supports the cell-intrinsic mechanisms for tumor development. However, it is doubtful if any of these markers could be used as prognostic biomarkers in determining which LPLs with dysplasia that will turn into OSCCs.

**Introduction**

Leukoplakias (LPLs) is the main potentially premalignant disorder in the oral mucosa. Approximately 3-17% transform into squamous cell carcinoma (OSCC) and there is an annual transformation rate of 1% [1]. The risk of malignant transformation of LPLs is
difficult to predict. Identification of molecular markers that could possibly predict disease aggressiveness and clinical outcome are needed but currently there are no specific biomolecular marker that has been found that will indicate whether a LPL will turn into OSCC or not [2]. There are no diagnostic or treatment modalities that prevent or distinguish LPLs that transform into OSCC from those LPLs that do not transform. Hence, most clinicians arbitrary regard the severity of dysplasia to be proportional to the risk of malignant transformation. The malignant transformation is considered to be a multistep process with an accumulation of genetic mutations and epigenetic changes most often in genes that regulates the cell cycle. Either there is an over expression of oncogenes that increase the cellular proliferation or suppression and mutation on the tumor suppressor genes that leads to an uncontrolled proliferation of malignant cells. During the last decades a lot of studies have been carried out on the importance of the immune system and prognosis of cancer, including OSCC [3] [4, 5] [6] [7]. However much less has been done on the connection between the immune system and prognosis of premalignant lesions in the oral mucosa. The recently introduced concept of cancer immunoediting have resulted in a view of dynamic interaction between the immune system and dysplastic cells/tumour cells, with phases of elimination, equilibrium and escape [8]. Thus, in an early phase of cell dysplasia or early tumour cell formation the immune system have capacity to eliminate cells with DNA damages that may result in, or already have resulted in cancer [9]. Immunoediting is probably of outmost importance in our defense against malignant cells that may occur in LPLs. The purpose of this study is to investigate the presence of dendritic Langerhans cells (LCs) and T cells as well as the expression of the more classical tumour associated proteins, Ki-67 and p53, in leukoplakias with dysplasia that have developed into OSCC and LPLs with dysplasia that have not transformed into OSCC.

**Patients and methods**

**Patients**

Twenty-three patients who had been biopsied for oral lesions and received the clinical diagnosis leukoplakia and histological diagnosis: epithelial dysplasia, were included in this study. Biopsy specimens with the histopathological diagnosis of hyperkeratosis with dysplasia were retrieved from the Archives of the Department of Oral medicine and Pathology and divided into two groups: Group 1: being LPLs that have not developed
into OSCC and Group 2 where the LPLs have developed into OSCC. Group 1 will be called
the LPL-d group and Group 2 the LPL-c group. All biopsies were analyzed and given a
diagnosis by the same oral pathologist. The Ethics committee at the Sahlgrenska
Academy, University of Gothenburg, approved the study and all patients signed an
informed consent before they were included in the study.

Methods
Blocks of paraffin-embedded biopsies were cut into 4 μm thick sections. The sections
were then mounted on electrostatically precharged slides (Superfrost Plus; Menzel-
Gläzer, Frankfurt, Germany), deparaffinised and rehydrated. Antigen retrieval was
carried out using TRIS /EDTA pH 9.0 and microwave treatment for 20 min. After cooling
at room temperature (RT) the slides were rinsed in phosphate-buffered saline (PBS).

Immunohistochemistry
Immunohistochemical staining was performed according to a previous protocol (3) to
describe the presence and phenotype type of cells in tissue specimens.
The sections were incubated with a primary antibody for 25 min at RT. The antibodies
used were Ki-67: Clone: MIB-1 (Dako, Glostrup, Denmark) diluted 1:100; p53: clone:
D07 (DAKO, Glostrup, Denmark) Diluted 1:500; CD1a: Clone: 010 (Dako, Glostrup,
Denmark) diluted 1:10 and CD3 (Clone: LN10 Novocastra, Newcastle, United Kingdom)
diluted 1:100. All subsequent washings were performed in ChemMate Buffer kit (K5006;
Dako A/S). After blocking of endogenous peroxidase using the ChemMate Peroxidase-
Blocking Solution (S 2023; Dako A/S), the sections were incubated with the ChemMate
DAKO EnVision Detection Kit, Peroxidase/DAB, Rabbit /Mouse (K5007; Dako A/S) for
25 min in RT and developed with DAB substrate. Counterstaining with ChemMate
Haematoxylin S2020 (Dako A/ S) was followed by dehydration in ethanol/xylene.
Mountex (Histolab AB, Gothenburg, Sweden) was used to permanently mount slides.
Sections from tonsils served as positive controls, while omission of primary antibodies
served as negative controls.
Quantitative analysis was performed on two compartments per biopsy; the epithelium
and the connective tissue. Digitalized images were obtained with a light microscope
(Leitz Wetzler, Leica Microsystems, Wetzlar, Germany) with an attached digital camera
(UC30; Olympus, Sweden). Photographs were taken of the epithelium and the
connective tissue in the specimens. Between one to six areas with dysplasia were
photographed. Ki-67, p53 and CD1a were photographed at a 63x magnification. CD3 was
photographed at 100x magnification because of dense inflammatory cellular infiltrate. The sections were then analysed with a computer software (CellSense, Olympus; Hamburg, Germany). In the epithelium and connective tissue, positively stained nucleated cells were counted. Cell counting was performed blinded. Results are expressed as the number of positively stained cells /mm².

The data was compiled for each patient. After that, the mean was calculated in order to identify trends in number of cells. Also the median and range were obtained.

Statistical analysis: Analyses of differences between groups were carried out by the Mann–Whitney U-test, with the statistical software programme SPSS v17 (SPSS Inc., Chicago, IL, USA). A p-value < 0.05 was considered as a significant difference.

Table 1. Patient characteristics in LPL-d and LPL-c

<table>
<thead>
<tr>
<th>Patient characteristics</th>
<th>Group 1: LPL-d</th>
<th>Group 2: LPL-c</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of patients (female / male)</td>
<td>11 (5/6)</td>
<td>12 (3/9)</td>
</tr>
<tr>
<td>No. of biopsies</td>
<td>11</td>
<td>13</td>
</tr>
<tr>
<td>Age in years at the time of biopsy: median (range)</td>
<td>70.5 (49-91)</td>
<td>67.5 (49-91)</td>
</tr>
<tr>
<td>Localization<em>1</em>2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bucca</td>
<td>4</td>
<td>2</td>
</tr>
<tr>
<td>Gingiva</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>Lateral border of the tongue</td>
<td>3</td>
<td>5</td>
</tr>
<tr>
<td>Floor of the mouth</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td>Soft palate</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Degree of dysplasia at first biopsy</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mild</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Moderate</td>
<td>5</td>
<td>4</td>
</tr>
<tr>
<td>Severe</td>
<td>3</td>
<td>7</td>
</tr>
<tr>
<td>Carcinoma in situ (CIS)</td>
<td>3</td>
<td>2</td>
</tr>
</tbody>
</table>

*1: This information was not attainable from one patient because of lack of detail in the records
*2: There are two biopsies obtained from one patient in different areas of the mouth.
Table 2. Observation-period (months) between LPL-d and LPL-c

<table>
<thead>
<tr>
<th>Group</th>
<th>Months</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group 1: Lpl-d median(range)</td>
<td>95 (21-170)</td>
</tr>
<tr>
<td>Group 2: Lpl-c median(range)</td>
<td>20 (0.5-138)</td>
</tr>
</tbody>
</table>

Results

T cells

The CD3+ cells were stained in order to validate the presence of T-cells in the tissue, as CD3 is a component of the T-cell receptor. T cells were present in preferentially stratum basale and stratum spinosum within the epithelium. In the connective tissue, T cells formed a subepithelial infiltrate in tight junction to the basement membrane in the epithelium (Fig 1). The number of CD3 positive cells per area unit were significantly higher in the LPL-d group compared to the LPL-c group, both in the epithelium and the connective tissue (Fig. 2 and Fig. 3). In the epithelium a marked increase in the number of CD3 positive cells is observed in LPL-d compared to LPL-c. In the connective tissue the same increase in positively stained CD3 cells is shown.

Langerhans cells

CD1a is marker which stains for the presence of the dendritic Langerhans cells which are antigenpresenting cells (APC) residing in the skin and mucosa. LCs were found foremost in the epithelium but also in the connective tissue mostly scattered in the T cell infiltrate (Fig. 1). Fewer positive cells were found in the LPL-c group than in the LPL-d group in both epithelium and connective tissue (Fig. 2 and Fig. 3). However, no significant differences were determined.

Ki-67

The proliferation marker Ki-67 is present in both epithelium and connective tissue in both LPL-d and LPL-c. In the dysplastic cells you could see a clear morphological difference with abnormal size an atypical apperance and also an difference in the intensity of staining (Fig. 1). Ki-67 positive cells are present in higher amounts in the epithelium than in the connective tissue. However, when looking within the oral compartments, there seems to be no statistically significant difference between LPL-d and LPL-c (Fig. 2 and Fig. 3). When solely comparing the median values, LPL-d is slightly higher than in LPL-c.
p53, which is a protein product from the tumor suppressor gene TP53 that regulates the cell cycle and is also called “the guardian of the genome”. p53 showed a clear difference in the presence between epithelium and connective tissue (Fig. 2 and Fig. 3). The density of positively cells was higher in the epithelium than in the connective tissue. However, in the connective tissue there were very few positively stained cells and sometimes there were none at all (Fig. 1.)

Figure 1.
TCD1a positive cells in leukoplakia with dysplasia without transformation into OSCC (LPL-d) (A) and leukoplakia with dysplasia which later transformed into OSCC (LPL-c) (B). CD3 positive cells in LPL-d (C) and in LPL-c (D). p 53 positive cells in LPL-d (E) and in LPL-c (F). Ki67 positive cells in LPL-d (G) and in LPL-c (H).
Results for the presence of Ki-67, p53, CD1a and CD3 in epithelium and connective tissue:

![Graph showing distribution of mean number of positively stained cells](image1)

**Figure 2:** The distribution of the mean number of positively stained cells (cells/mm²) of Ki-67, p53, CD1a and CD3 in the epithelium of leukoplakia with dysplasia without transformation into OSCC (LPL-d) and leukoplakia with dysplasia which later transformed into OSCC (LPL-c).

![Graph showing distribution of mean number of positively stained cells](image2)

**Figure 3:** The distribution of the mean number of positively stained cells (cells/mm²) of Ki-67, p53, CD1a and CD3 in the connective tissue of leukoplakia with dysplasia without transformation into OSCC (LPL-d) and leukoplakia with dysplasia which later transformed into OSCC (LPL-c).
Discussion

The human body is a complex entity with several useful systems that help to perform many necessary functions required of a living organism. One of the most important is the immune system, that functions as a defense mechanism against pathogenic microorganisms and cancer. It can generate a variety of different cells and molecules that act in a dynamic network in recognizing and eliminating specific foreign antigens [45].

In recent years, an additional role of the immune system has been in focus, that the immunological response could have an impact on tumor development [10]. The constitution and the effectiveness of the immunological response can also affect the outcome of the patient in several different cancers including oral cancer. Several epidemiological studies has shown that immunosuppressed patients have an increased risk of developing malignancies compared to non-immunosuppressed patients [11, 12] [13]. There are also an increased prevalence of leukoplakias with dysplasia and oral squamous cells carcinomas in renal transplanted patients compared to non-transplanted patients [14] and a four-fold increased risk of developing oral squamous cell carcinoma [15].

In this study we found that it was significantly higher numbers of T cells in the LPL-d group compared to the LPL-c group. This might indicate that the T cell infiltration both intra-lesionally and peri-lesionally could influence the outcome of the LPL. A lower presence of certain subsets of T-cells in LPLs that eventually turn out to be malignant gives evidence for the immunoediting theory, in that these tissues might show lower immunogenicity. This could explain the reason why T-cells are less present in this tissue. However, one must notice that the ranges in both epithelium and connective tissue are quite widely distributed in both groups. For example, the lowest value for CD3 positive staining in the epithelium of a LPL-c biopsy, may be so low that in practice it might be mistaken for being a leukoplakia that would not transform into a malignant tumor. Therefore, it might be quite unreliable in certain cases to use it as a significant biomarker. There has been some work done earlier on dendritic cells and T cells and their importance in prognosis of oral squamous cell carcinoma. The imbalance between the different subsets of T cells seems to play a great role in tumorigenesis [3]. In this study we have not subdivided the T cells to investigate if there are a shift in the normal
distribution. There can be a difference in the distribution of the phenotype and also function of T cells regarding cytotoxic T cells, regulatory T cells and T helper cells. The difference in the mean median value of LCs of LPL-d and of LPL-c, although not significant, could indicate that fewer antigen presenting cells recognize the dysplastic transformation. This would then confirm the equilibrium hypothesis of dysplastic cells showing lower immunogenicity.

Until now most studies have addressed the function of dendritic cells (DCs) and T cells in already established tumour diseases [16-18]. In contrast, studies designed to delineate the role of these leukocytes in premalignant disorders like LPLs that transform into OSCC are sparse. In LPLs a few studies have addressed the number of DCs [19-21].

Another hallmark of cancer is the unrestrained proliferation of cells. In oral cancer, it has been reported a significant increase of the expression of Ki-67 and p53 ref. However in the case of leukoplakias with dysplasia researchers are not unanimous in the use of Ki-67 as a prognostic marker. Both p53 and Ki-67 were shown to have higher mean scores in leukoplakias with dysplasias [22] [23]. In another study there was no correlation between LPLs with dysplasia that turned into OSCC [24]. In our study we showed that there were no statistical significant difference between the two groups but the fact that Ki-67 is present in both groups shows that there definitely is a proliferation process going on in both compartments. This is in line with Hanahan’s and Weinberg’s theory of the hallmarks of cancer [10]. However, with this marker alone, it is unfortunately impossible to determine which of the dysplastic tissues would eventually turn out to become malignant.

In some oral premalignant lesions the expression of p53-positive cells in the suprabasal layers of the epithelium has been seen as an indication of a developing malignancy [25]. In a study by Angiero et al, a high expression p53 was seen in epithelium with increasing dysplasia[26]. In another study a high presence of p53 showed a high correlation in OSCC epithelia compared to normal mucosa. This indicates that an increase in p53 expression would lead to a tumor progression [27]. Alterations in chromosome region 17p and mutation of the p53 gene are genetic aberrations that seem to be especially important in the progression from dysplasia to OSCC [28]. There seems to be no statistically significant difference in p53 expression in neither the epithelium nor in the
connective tissue in this study. As p53-molecules are present in these specimens, it reinforces the fact that there is an on-going abnormal process. This is in line with Hanahan’s and Weinberg’s theory about suppressing growth inhibitory factors. However, as there is no significant difference between group LPL-d and group LPL-c, there is little evidence to show which one would become malignant.

To the best of our knowledge there are no studies done where patient groups with leukoplakia with dysplasia concerning the expression on Ki-67 and p53. In our study we could not see any correlation between the expression of these two traditional cancer markers and the transformation of dysplastic to malignant lesion.

In conclusion, the key finding in this study was that there seems to be a significant increase in CD3+ T cells in LPLs that have not transformed into OSCC compared to those that had transformed. This is in line with the immunoeediting theory originally proposed by Dunn et al. [9]. This finding should be further investigated and may lead to characterisation of an "immunological signature" in high risk LPLs.

Impaired immunosurveillance may be of importance in the malignant transformation process. Thus, so far presence of CD3+ T cells seem to be a marker that can be used as a potential prognostic biomarker in both epithelium and connective tissue. Ki-67, p53 and CD1a do not give any salient information as to whether a LPL with dysplasia will transform to a malignant disease or not.

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