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EZH2 Expression Correlates With T-Cell Infiltration in Oral Leukoplakia and Predicts Cancer Transformation

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Abstract. *Background/Aim: The EZH2 complex is involved in cellular proliferation and modulates the immune response in cancer. Less is known about the importance of EZH2 in precancerous lesions such as oral leukoplakia (OL). The aim of the study was to explore the association between EZH2 expression, immune activation, and cancer transformation in OL. Patients and Methods: Analyses were retrospectively performed on nine OL cases that had undergone transformation to oral squamous cell carcinoma (OSCC; OL-ca) and nine that had not undergone transformation (OL-non). EZH2-expressing cells, CD3+ and CD8+ T cells, and CD1a+ Langerhans cells were visualized with immunohistofluorescence and counted. Results: A moderate positive correlation between CD3- and EZH2-expressing and CD8- and EZH2-expressing cells in the epithelium was found (r=0.57, p=0.01; r=0.59, p=0.01). The number of EZH2-expressing cells in the epithelium of OL-ca was significantly higher compared to OL-non (p=0.0002). Cancer-free survival rates differed significantly between patients with EZH2^{high} compared to EZH2^{low} expression (p=0.001). EZH2^{high} expression in OL epithelium was associated with a 13-fold higher risk for developing OSCC (HR=12.8). Conclusion: EZH2 expression in oral epithelium predicts OSCC transformation of OL and correlates with the level of T-cell infiltration.*

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Oral squamous cell carcinoma (OSCC) constitutes 92%-95% of all oral cancers with a 5-year survival rate of approximately 50% (1, 2). OSCCs may occur as de novo tumors or may be preceded by precursor lesions in the oral mucosa. These precursor lesions, which have an increased risk of malignant transformation, are designated as oral potentially malignant disorders (OPMDs) (3). Early detection of precursor lesions or tumors is significantly correlated with survival (4). Oral leukoplakia (OL) (Figure 1) is one of the OPMDs that has an estimated overall mean malignant transformation rate of 9.8% (5).

Cancer transformation is considered to be a multistep process. Overexpression of oncogenes that control cellular proliferation and/or dysregulation of tumor suppressor genes can lead to uncontrolled proliferation of malignant cells (6-9). Epigenetic changes leading to altered gene expression have been attributed an important role in cancer development (10). Identifying these changes provides insights into novel epigenetic biomarkers for clinical applications (11-16). Polycomb group proteins (PcGs) influence epigenetic processes in cancer (17). PcGs comprise two core complexes: polycomb repressive complex 1 (PRC1) and polycomb repressive complex 2 (PRC2) (18, 19). PRC2 has an important core component called enhancer of zeste homolog 2 or 1

Figure 1. *Oral leukoplakia presenting as a white patch on the right border of the tongue.*

Table I. *Patient characteristics.*

	N (%)	Mean age years (Median; range)
Clinical diagnosis		
Homogenous leukoplakia	8 (44)	
Non-homogenous leukoplakia	3 (17)	
No data registered	7 (39)	
Age		67 (68; 46-78)
<60 years	5 (28)	
≥60 years	13 (72)	
Sex		
Male	13 (72)	71 (74; 58-78)
Female	5 (28)	57 (55; 46-68)
Site of lesion		
Floor of the mouth	2 (11)	
Buccal mucosa	4 (22)	
Lateral tongue	3 (17)	
Ventral tongue	3 (17)	
Dorsal tongue	2 (11)	
Gingiva	4 (22)	
Histopathological diagnosis		
Benign hyperkeratosis	5 (28)	
Hyperkeratosis with dysplasia	13 (72)	

(EZH2/1) (20, 21). EZH2 is involved in the regulation of genes associated with cellular proliferation, differentiation, and apoptosis (18, 22). With respect to OL, Cao and coworkers reported that increased numbers of EZH2-expressing cells in the epithelia of OL patients are predictive of cancer transformation (23), indicating the importance of this molecular complex also in the precancerous stage.

Another important function of EZH2 is to modulate the immune response in cases of cancer (24). EZH2 influences the differentiation of T-cell subsets, thereby regulating the differentiation and polarization of T cells and subsequent cytokine production (25). EZH2 also regulates major transcriptional factors involved in the differentiation of central memory precursor T cells (26-28), and plays a critical role in the activation of naive CD8-expressing T cells (29). Importantly, EZH2 also influences immune check-point expression, by regulating PD-L1 expression in T cells (30). In contrast, EZH2 does not influence the development of dendritic cells (DCs), neutrophils or macrophages. The exception to this is the dendritic Langerhans cells (LC), in that EZH2 impairs their capacity to migrate from the epithelial compartments (31, 32).

The concept of cancer immune-editing has generated the picture of dynamic interactions between the immune system and dysplastic cells/tumor cells, involving phases of elimination, equilibrium and escape (9). Thus, in an early phase of cell dysplasia or during early tumor cell formation, the immune system has the capacity to eliminate cells that have DNA damage that might result in (or already has resulted in) cancerous lesions (10).

The immune response in cases of OL has not been well investigated. A study conducted by Gannot and colleagues revealed the presence of higher numbers of immune cells in moderate and severe dysplasia or OSCC, compared to cases of hyperkeratosis without dysplasia or mild dysplasia (33). Xu *et al*. have recently reported more CD3- and CD8-positive T cells in OL with high-grade dysplasia as compared to cases of OL with low-grade dysplasia (34). Öhman *et al*. reported an increased number of CD8-positive T cells in OL with dysplasia compared to OL without dysplasia (35), as well as an increased number of T cells in non-cancer-transforming OL compared to cancer-transforming OL (36).

Since EZH2 has proven effects on immune mechanisms, it is reasonable to assume that this molecular complex is involved in immune response in cases of OL. In the present study, we tested the hypothesis that there are associations between EZH2 expression, immune activation, and cancer transformation in OL.

Patients and Methods

Patients. A retrospective analysis was performed on tissue samples from 23 patients retrieved from the archives of the Department of Oral Medicine and Pathology, Institute of Odontology and Department of Pathology, Sahlgrenska University Hospital, Gothenburg. Inclusion criteria were: a clinical diagnosis of OL, a histopathological diagnosis of hyperkeratosis with or without dysplasia, sufficient tissue in the samples to allow immunohistochemical analysis, and a follow-up time of ≥6 months between the primary biopsy and OSCC transformation. During the follow-up period, nine patients with OL transformed into OSCC. In addition, nine patients had a follow-up period of ≥5 years without OSCC transformation. Histopathology-based diagnoses were re-reviewed before study inclusion. After this revision, three patients were excluded due to revision of the histopathological diagnosis: one

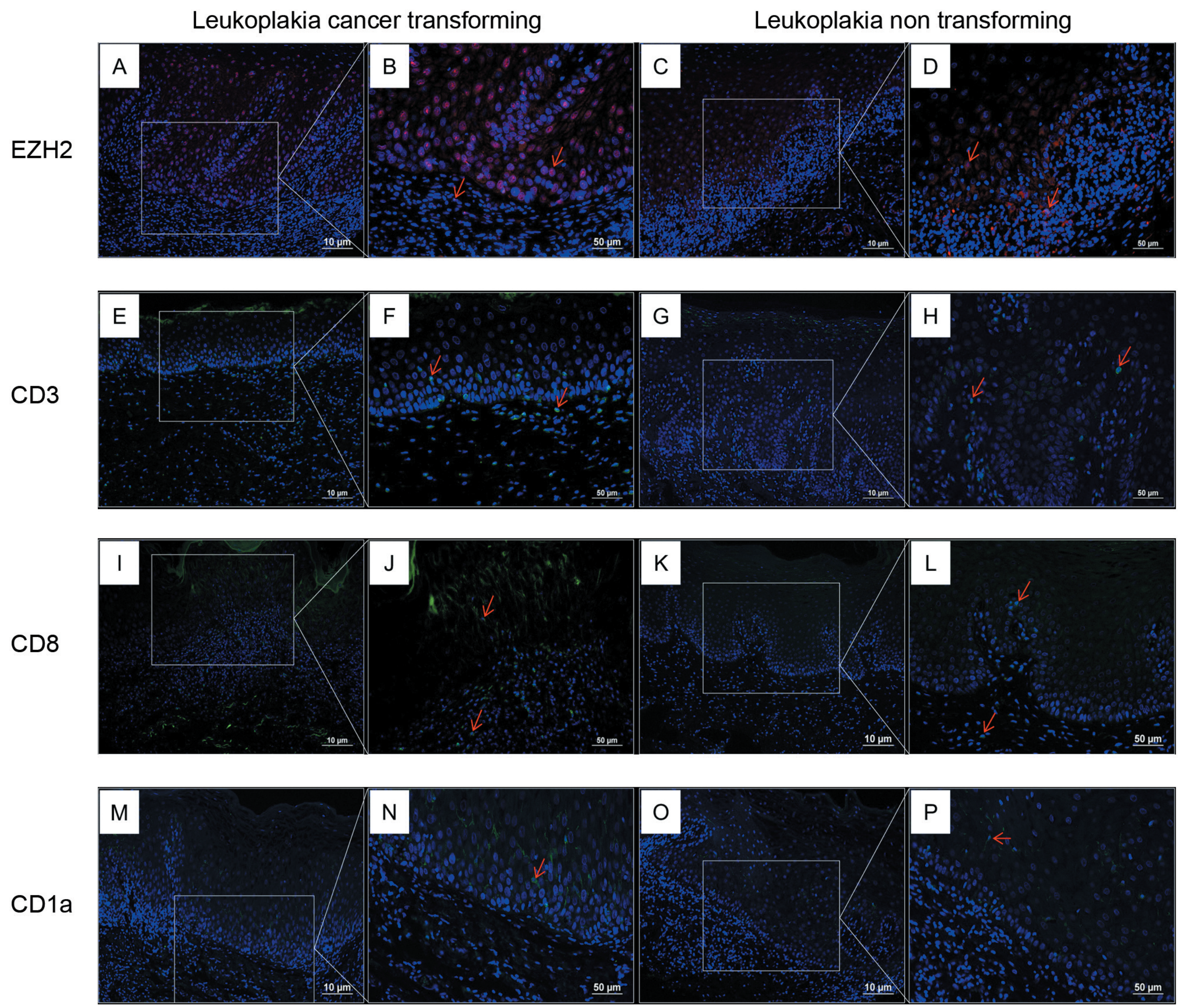

Figure 2. *EZH2 positive cells (pink) in OL-ca ×100 (A), ×200 (B) and OL-non ×100 (C), ×200 (D); CD3 positive cells (green) in OL-ca ×100 (E), ×200 (F) and OL-non ×100 (G), ×200 (H); CD8 positive cells (green) in OL-ca ×100 (I), ×200 (J) and OL-non ×100 (K), ×200 (L); CD1a positive cells (green) in OL-ca ×100 (M), ×200 (N) and OL-non ×100 (O), ×200 (P). CD1a are sparse in the connective tissue. White rectangles indicate the magnified area with a line pointing (×200). Red arrows indicate the positive cells in the epithelium and connective tissue.*

because of missing connective tissue and one because of damaged tissue samples. No signs of fungal or bacterial infection were noted in the tissue specimens. The cohort that proceeded to analysis consisted of 18 patients. The patient characteristics are listed in Table I.

Immunohistofluorescence. Immunofluoroscent staining was performed on formalin-fixed paraffin-embedded tissue specimens, to identify cells that expressed EZH2, CD3, CD8 and CD1a. Mouse monoclonal antibodies raised against EZH2 (Cat. No. 612666; BD Transduction Laboratories, Stockholm, Sweden) and rabbit monoclonal antibodies raised against CD3 and CD8 (MA5-14524 and MA5-14548; Thermo Fisher Scientific, Gothenburg, Sweden) and CD1a (Cell Marque, EP3622; Sigma Chemical Co., Stockholm, Sweden) were used. The tissue blocks were cut into 4-μm-thick sections, mounted on electrostatically precharged slides, deparaffinized using two changes of xylene, and then rehydrated in 99.5% and 95% ethanol for 5 min each, followed by washing in distilled water for 10 min, and two washes in phosphate-buffered saline (PBS) for 5 min each. Antigen retrieval was carried out by boiling the sections in citrate buffer (pH 6) for 30 min. After cooling to room temperature, the sections were rinsed in two changes of PBS, for 5 min each time. To reduce autofluorescence, sections were incubated in $CuSO_4$ (4%) for 1.5 h. The sections were then rinsed twice in PBS for 5 min each time, and non-specific background was blocked by applying PBS that contained 5% goat serum for 1 h at room temperature. Sections were then incubated

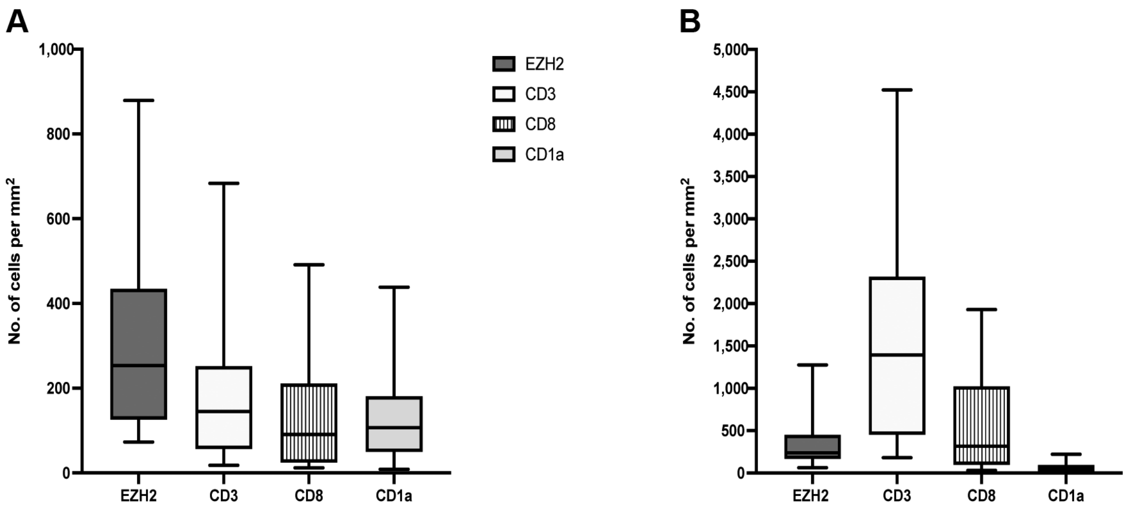

Figure 3. *Box plots depicting number of EZH2-, CD3-, CD8- and CD1a- expressing cells per mm^2 in the epithelium (A) and in the connective tissue (B). Graphs show median (horizontal bars), interquartile range, and maximum and minimum values (whiskers).*

with the primary antibody targeting EZH2 (1:400) combined with a primary antibody targeting CD3 (1:100), CD8 (1:100) or CD1a (1:100), at 4˚C overnight. Omission of the primary antibodies served as negative controls. On the second day, the sections were washed thrice with PBS for 5 min each, and then incubated for 1 h at room temperature with Texas red-X secondary goat anti-mouse antibody (T-862, 1:200; Thermo Fisher Scientific) and Alexa Fluro 488-conjugated secondary goat anti-rabbit antibody (A-11008, 1:250; Thermo Fisher Scientific). This was followed by three washes with PBS for 5 min each, rinsing with distilled water, and dehydration in 95% and 99.5% ethanol for 15 min each. Thereafter, the sections were mounted with cover glasses and Prolong™ Gold Antifade reagent with DAPI (P36931; Thermo Fisher Scientific).

Quantitative analysis. Digital images were acquired with an immunofluorescence microscope (Eclipse 90i; Nikon Inc., Tokyo, Japan). In the epithelium, images of three areas (one central and two peripherals; ×200) were saved for quantification of the cells. In the connective tissue, one image of the central area was obtained. Quantitative analyses were performed using the QuPath computer software (37). Slides were blinded, the areas of interest were manually counted, and the results are expressed as the median numbers of positive cells/mm^2 in the epithelium and in the connective tissue, respectively.

Statistical analysis. The GraphPad Prism version 9.2.0 software (GraphPad Inc., San Diego, CA, USA) was used for other analyses. The correlations between EZH2 expression and numbers of immune cells were analyzed using Spearman correlation, where a p-value <0.05 was considered to be a significant correlation. Correlation graph after log10 transformation were plotted. The Mann-Whitney U-test was used to analyze differences between groups. Statistical significance was set at a p-value <0.05. To assess the prognostic power of EZH2 expression, receiver operating characteristic (ROC) curve analyses were performed. Cancer-free survival (CFS) rates were calculated using the Kaplan-Meier survival analysis, and the outcomes for patients in the different groups were compared using a two-sided log-rank (Mantel-Cox) test and the hazard ratio were estimated using logrank (B/A). Occurrence of an OSCC was defined as an event in the Kaplan-Meier analysis.

Ethics approval. The Regional Ethics Board in Gothenburg, Sweden approved the study (no. 2019-04579).

Results

EZH2-expressing cells. EZH2-expressing cells were observed predominantly in the suprabasal region of the epithelium. A majority of the EZH2-expressing cells had the keratinocyte morphology (Figure 2B, D and Figure 3A). In the connective tissues, EZH2-expressing cells were observed predominantly in the subepithelial infiltrate (Figure 2B, D and Figure 3A).

Correlation between CD3-, CD8- and CD1a-positivity and EZH2-expressing cells. The CD3- and CD8-expressing cells had the lymphocyte morphology and were predominantly localized to the stratum basal and stratum spinosum in the epithelium (Figure 2F, H, J, L and Figure 3A), whereas CD1a-expressing cells had the dendritic morphology and were found scattered in the suprabasal region of the epithelium (Figure 2N, P and Figure 3A). In the epithelium, the mean number of CD3-positive cells that also expressed EZH2 was 15 cells/mm^2, which represented approximately 5% of all the EZH2-positive cells present within the epithelium.

A moderate positive correlation between CD3 positivity and EZH2-expressing cells in the epithelium was found (r=0.57; 95%CI=0.12-0.82; p=0.01) (Figure 4A). In addition,

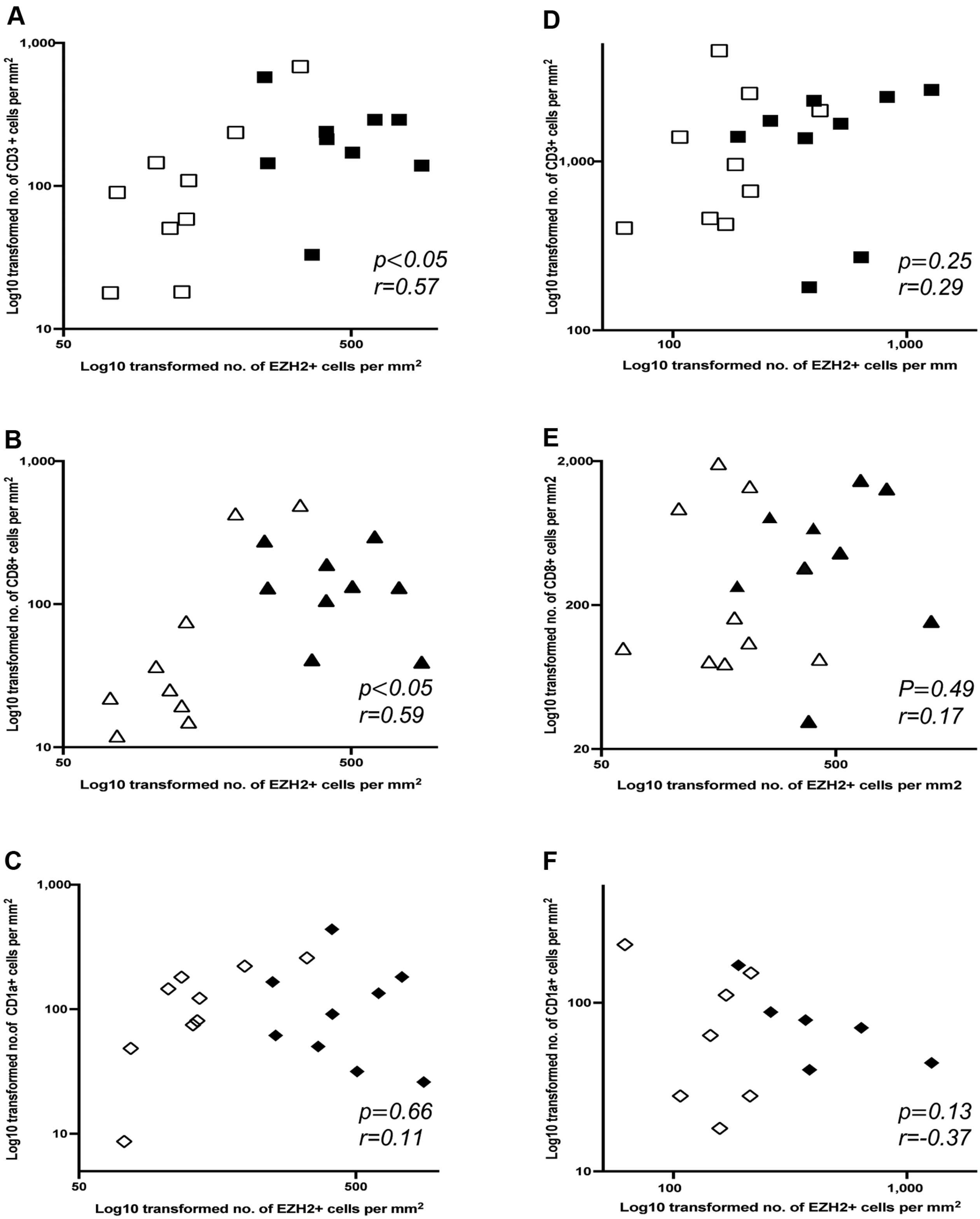

Figure 4. *Pearson correlation between Log 10 transformed no. of EZH2+ cells per mm^2 (LOG10 EZH2) and Log 10 transformed no. of CD3+ cells per mm2 in the epithelium (A), connective tissue (D) (closed squares); LOG10 EZH2 and Log 10 transformed no. of CD8+ cells per mm^2 in the epithelium (B), connective tissue (E) (closed triangles); LOG10 EZH2 and Log 10 transformed no. of CD1a+ cells per mm^2 in the epithelium (C), connective tissue (F) (closed diamonds).*

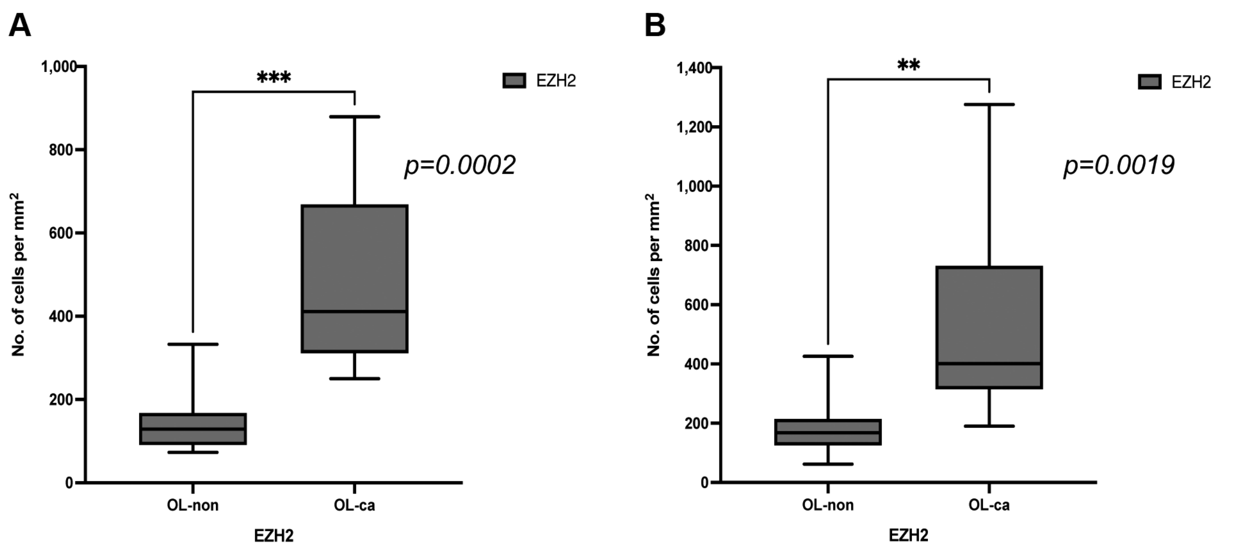

Figure 5. *Box plots depicting number EZH2-positive cells/mm^2 in epithelium (A) and connective tissue (B) in OL-non and OL-ca. Graphs show median (horizontal bars), interquartile range, and maximum and minimum values (whiskers). Statistical differences (p<0.05) between groups are indicated.*

a moderate positive correlation was observed between CD8 and EZH2 expressing cells in the epithelium (r=0.59; 95% CI=0.15-0.83; *p*=0.01) (Figure 4B). However, there was no significant correlation between CD1a-positivity and EZH2-expressing cells in the epithelium (r=0.11; 95% CI=–0.39-0.56; *p*=0.66) (Figure 4C).

In the connective tissue, CD3- and CD8- expressing cells had the lymphocyte morphology and formed a subepithelial infiltrate of cells below the epithelium (Figure 2F, H, J, L and Figure 3B). In the connective tissue, CD1a-positive cells were sparsely detected. In this tissue compartment, the numbers of CD3- and CD8-expressing cells did not correlate with the number of EZH2-expressing cells (for CD3: r=0.29; 95% CI=–0.22-0.67; *p*=0.25; Figure 4D; and for CD8: r=0.17; 95% CI=–0.33-0.60; *p*=0.49; Figure 4E). Neither did CD1a-positivity and EZH2-expressing cells show any correlation (r=–0.37; 95% CI=–0.72-0.13; *p*=0.13; Figure 4F).

EZH2-expressing cells in OSCC-transforming versus non-transforming leukoplakia. The number of EZH2-expressing cells in the epithelium was significantly higher in the OSCC-transforming OL (OL-ca) group than in the non-transforming OL (OL-non) group (*p*=0.0002) (Figure 5A). The number of EZH2-positive cells/mm2 in the connective tissue was also significantly higher in the OL-ca group than in the OL-non group (*p*=0.0019) (Figure 5B).

Patients were stratified into two groups depending on epithelial EZH2 expression level above (EZH2^{high}) or below (EZH2^{low}) the median cutoff value of 253 cells/mm^2, yielding nine patients in each group. This resulted in an area under the ROC curve of 0.97 (*p*=0.0007) (Figure 6A), with 89% sensitivity and 89% specificity. The log-rank test showed a significant difference in CFS between the patients in the EZH2^{high} group and the patients in the EZH2^{low} group (*p*=0.001) (Figure 6B). EZH2^{high} expression in OL was associated with a 12.8 higher risk for developing OSCC (HR=12.8, 95% CI=3.3-49.1).

CD3-, CD8- and CD1a-expressing cells in OSCC-transforming versus non-transforming leukoplakia. The numbers of CD3- and CD8-expressing cells detected in the epithelium tended to be higher in the OL-ca group than in the OL-non group (*p*=0.09 and *p*=0.07, respectively) (Figure 7A). The numbers of CD3- and CD8-expressing cells in the connective tissue were not significantly different between the OL-ca and OL-non groups (*p*=0.73 and *p*=0.49, respectively) (Figure 7B). The numbers of CD1a-expressing cells in the epithelium and in the connective tissue did not differ significantly between the OL-ca and OL-non groups (*p*=0.80 and *p*=0.86, respectively) (Figure 7A and B).

Discussion

The main finding of the present study was that EZH2 expression in OL may serve as a biomarker for OSCC transformation, with a 97% chance that EZH2 expressing cells will correctly distinguish between non-cancer and cancer transforming OL patients based on the number of EZH2 expressing cells in the epithelium. The study also shows moderate positive correlation between the numbers of T cells, and EZH2-expressing cells in the epithelium of patients with OL, indicating a correlation between EZH2 expression and immune activation.

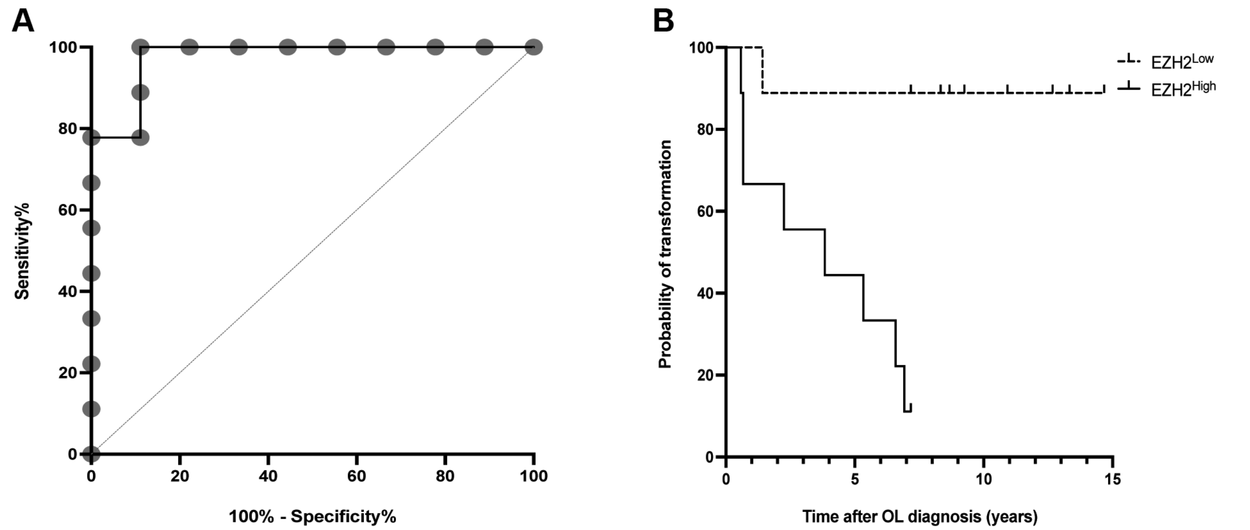

Figure 6. *(A) ROC curve analysis of EZH2 in the epithelium. (B) Kaplan-Meier curves of probability of cancer transformation after OL diagnosis (censored cases are depicted as -).*

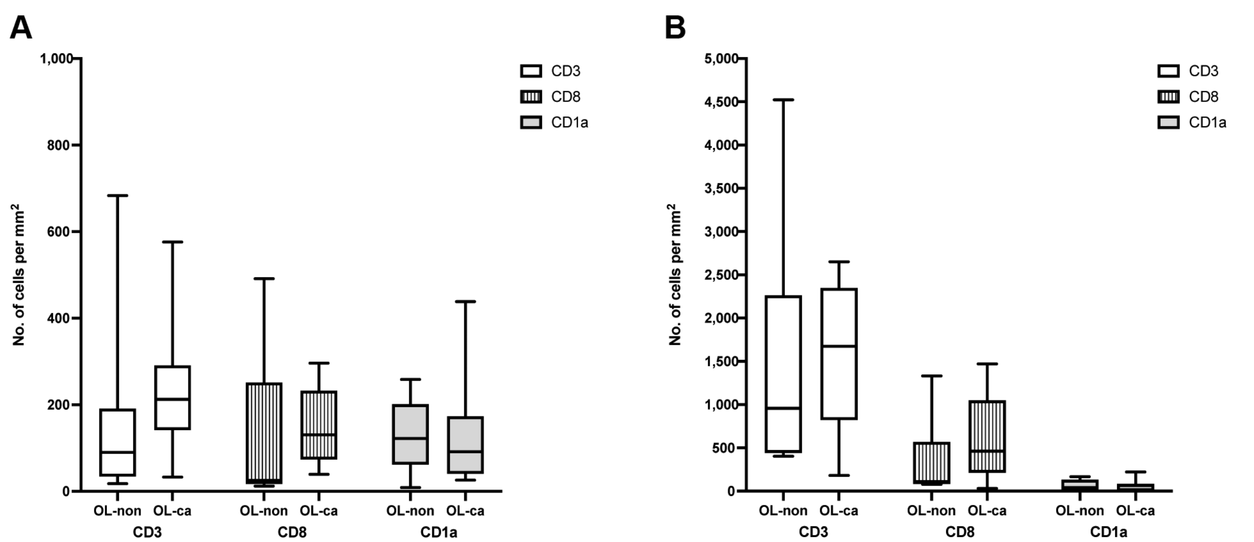

Figure 7. *Box plots depicting the number of CD3-, CD8- and CD1a-expressing cells/mm^2 in epithelium (A) and connective tissue (B) in OL-non and OL-ca. Graphs show median (horizontal bars), interquartile range, and maximum and minimum values (whiskers).*

In this study, we showed that the EZH2 molecular complex – an epigenetic modulator – influences the immune response already in a premalignant disorder such as OL. A moderate positive correlation was observed between the numbers of EZH2-positive cells and the numbers of CD3- and CD8-expressing T cells in the oral epithelium of patients with OL. The findings indicate that cytotoxic T cells are recruited to a potentially malignant disorder like OL. CD8 molecules are predominantly expressed on cytotoxic T cells, although they can also, to some extent, be found on NK cells and DCs (38). Consequently, it is possible that some of the CD8 molecule-expressing cells detected in tissue specimens from our OL patients are NK cells or DCs. However, this does not alter the interpretation of the results, since both DCs and NK cells are known to play roles in immune response to dysplastic or cancer cells (39, 40). In established cancers, evidence for a correlation between EZH2-expressing and CD8- expressing cells and prognosis has been reported (41). The presence of cytotoxic T cells shown in the present study points to the possibility that the EZH2 molecule complex plays an important role in precancerous lesions.

Comparing EZH2 expression in OSCC-transforming and non-transforming OLs, we show that EZH2-expressing cells are significantly more abundant in both the epithelium and the connective tissues of patients with OL that transformed into OSCC than in patients with OL that did not transform into OSCC. This observation is in line with a previous study by Cao *et al*. in a Chinese patient cohort (23). An important finding of this study was the high positive predictive value of EZH2 for OSCC transformation, which may be used in risk assessments of patients with OL. This was further supported by the Kaplan-Meier survival analysis, which reveals that when the cohort was divided into $EZH2^{high}$- and $EZH2^{low}$-expressing OL, that the $EZH2^{high}$ OL cases have a significantly lower CFS. Furthermore, the hazard ratio for developing OSCC is 13-times higher in the EZH2high-expressing group than in the $EZH2^{low}$-expressing group. These findings need further validation in order to enter clinical use as a risk assessment tool for OL.

In the present study, patient samples consisted of OL with a histopathological diagnosis of both hyperkeratosis with or without dysplasia. Of importance was that no signs of presence of fungi or bacteria that could cause inflammation were noted in the biopsy specimens.

A limitation of the study is the relatively small sample size and its retrospective design, which in the latter case constrains the collection of clinical data. The histopathological diagnoses consisted of both hyperkeratosis with or without dysplasia. However, both diagnoses were represented in the OL-ca as well as in the OL-non group, therefore a major influence on the results is not likely. Interestingly, as mentioned above, EZH2-expressing cells were detected also in tissues without dysplasia. Assessment of the presence of dysplasia is associated with a certain degree of uncertainty (42), depending on the subjective evaluation of a pathologist. Also, an increase in EZH2 expression may precede visual signs of dysplasia that can be detected in routine histopathology. Analysis of EZH2-expressing cells may reduce this uncertainty, although confirmatory studies are needed.

In conclusion, the findings of the present study point to a role for the interaction of EZH2-expressing keratinocytes with immune cells, and this warrants further exploration. Of special interest is the high predictive value of EZH2 expression for OL that transforms to OSCC.

Conflicts of Interest

The Authors declare no conflicts of interests regarding this study.

Authors' Contributions

Divya Ganesh: Study design, planning, immunohistochemical stainings, analyzed and interpreted data, and drafted the manuscript; Amal Dafar: study design, analyzed and interpreted data, critically reviewed the manuscript; Julia Niklasson, Ingrid Sandberg: study design, immunohistochemical stainings and evaluation, critically reviewed the manuscript; Paulo Braz-Silva, Dipak Sapkota: evaluation of histopathological data, design, analyses and interpretation of data, critically reviewed the manuscript; Jenny Öhman: study design, planning, evaluation of clinical and histopathological data, analyses and interpretation of data, co-drafted the manuscript; Daniel Giglio: study design, planning, analyzed and interpreted data and co-drafted the manuscript; Bengt Hasséus: study design, planning, analyzed and interpreted data and co-drafted the manuscript.

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