Cd8+ expression in oral potentially malignant disorders associated with risk factors in selected population of east Indonesia

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ABSTRACT

Background: *Oral potentially malignant disorder (OPMD) is a lesion with a high potential to turn into oral squamous cell carcinoma (OSCC). The discovery of OPMD lesions precedes most cases of OSCC. Sensitive and specific biomarkers can help in the early detection of high-risk patients, one of which is CD8+, part of the cytotoxic T lymphocytes, which have a role in eliminating cancer cells. Several studies use infiltration density and CD8+ T cells' activity as biomarkers in malignancy. This study aims to observe the expression of CD8+ in OPMD lesions and correlate with risk factors*

Method: *Twelve paraffin blocks samples consist of OPMD lesions. One oral lichen planus (OLP) as the positive control. Samples were stained using HE to observe the degree of dysplasia and immunohistochemistry to observe the expression of CD8+ T cells. Observation of results using a microscope with 100x magnification to select the field of view and 400x magnification to count the number of positive CD8+ T cells with a cut-off point of 500 cells from a total of 5 fields of view and determined by weak intensity (<500 cells) and strong intensity (≥ 500 cells).*

Result: *61,5% of samples showed weak intensity CD8+ T cell, and 38.5% showed strong intensity. There is a significant correlation between risk factors and CD8+ expression (p*=0.005*).*

Conclusion: *Expression of CD8+ T cells with strong intensity was found in OPMD lesions with moderate to severe dysplasia. Patients with risk factors showed CD8+ T-cell expression at a weaker intensity than those without risk factors.*

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INTRODUCTION

Oral cancer is a problem in the oral cavity that must be considered because it may lead to death. Oral squamous cell carcinoma (OSCC) is the most common form of oral cancer.[1] There have been 149,102 new cases and 89,377 deaths from oral cancer. Reporting to GLOBOCAN 2018, OSCC in Southeast Asia is the fifth most common type of cancer.[2] OSCC is often diagnosed at an advanced stage and spreads to other body parts when detected.[3] It should be noted that most OSCC cases are preceded by a lesion of oral potentially malignant disorders (OPMD). OPMD lesions are defined by WHO as changes in tissue morphology or abnormalities in the oral epithelium that have a high risk of turning into OSCC.[4,5] The worldwide prevalence of OPMD is estimated at 4.47% and may vary in some populations and is higher in Asian populations.[3] OPMD lesions prevalence is more common in middle-aged to elderly people and is higher in the male than the female population.[6]

The appearance of OPMD lesions in the oral cavity is characterized by mutations in the genetic material of oral epithelial cells with or without clinical changes that can lead to transformation into OSCC.[7] The main risk factors for developing OPMD lesions are related to lifestyles such as tobacco products, alcohol, and areca nut.[8,9] OPMD lesions in the oral cavity are divided into several categories, namely leukoplakia, erythroplakia, oral submucous fibrosis (OSMF), oral lichen planus (OLP), actinic cheilitis (AC).[10] Locations of OPMD lesions in the oral cavity are often found on the buccal mucosa, mandibular gingiva, tongue, floor of the mouth, and palate.[11] Most OPMD lesions appear asymptomatic, so OPMD lesions are often found to have reached an advanced stage with a poor prognosis.[1,5,12,13] Therefore, sensitive and specific biomarkers can assist in detecting high-risk patients.[2] Biomarkers are biological molecules that show signs of normal or abnormal processes and disease conditions such as cancer.[14] In detecting biomarkers, immunohistochemical techniques can be used.[15,16]

CD8+ T cells are the main subpopulation of cytotoxic T lymphocytes whose task is to eliminate cancer cells; infiltration density and activity of CD8+ T cells are independent prognostic biomarkers for OSCC.[17] Changes from OPMD to OSCC are associated with decreased infiltration of immune cells, namely CD8+ T cells.[18] A high CD8+ T-cell infiltration density is significantly associated with a good prognosis in OSCC. Patients with high infiltration of CD8+ T cells are significantly associated with increased overall survival in patients with OSCC.[19] Based on this background, the authors are interested in studying the density or expression of CD8+ T cells as a diagnostic and prognostic marker in OPMD lesions.

RESEARCH METHOD

The study was conducted from August to December 2022. The aspects studied in this study were CD8+ T-cell expression in OPMD. The samples in this study used 12 blocks of OPMD lesion paraffin from the selected population in Bajawa, East Indonesia, with risk factors obtained from the archives of OPaDCORE laboratory, Faculty of Dentistry, Universitas Trisakti. This study's positive control used OLP.

Paraffin blocks of OPMD lesions were stained using hematoxylin and eosin (HE) staining to determine the degree of epithelial dysplasia and immunohistochemical staining using CD8+ T cell markers (*Clone* C8/4391R: RM0409, RM409RTU7; Meydasis Company) to observe CD8+ cell expression. Retrieval antigens (AR) are performed on the sample before entering the immunohistochemistry stage to increase the

intensity of antibodies that can be weakened due to the fixation and paraffinization process. Positive CD8+ T cells will show brown staining of the cytoplasm of cells in the connective tissue of the observed OPMD lesions. Observation of samples using the Axio Scope.1 digital microscope (100x) to select the field of view that contains the most CD8+ T cells, then using 400x magnification to count the number of cells. Calculating the number of CD8+ T cells was performed in 5 different fields of view semi-quantitatively with a cut-off value of 500 cells from a total of 5 fields of view on one sample using the ImageJ application. The expression of CD8+ T cells in OPMD lesions was classified into two categories, namely category 1 for weak intensity (< 500 cells) and category 2 for strong intensity (≥ 500 cells).

The reliability test was carried out to determine the consistency of measurement of research data conducted by inter-raters using the intraclass correlation coefficient (ICC) through the statistical package for the social sciences (SPSS) application. This study used three raters to observe the expression of CD8+ T cells. The data analysis in this study used the chi-square test through SPSS application. This research uses the Ethical Clearance issued by the Health Research Ethics Commission of the Faculty of Dentistry, Universitas Trisakti, with an Ethical Exemption letter Number: 574/S1/KEPK/FKG/7/2022.

RESULTS

Based on the socio-demographic characteristics of the study sample (Table 1), it was shown that most of the ages at which OPMD lesions were diagnosed were in the age range of 40-50 years (38.5%). The subjects of this study were dominated by female subjects (71.4%). In addition, subjects with risk factors (30.8%) encountered more than those without risk factors (23.0%).

Table 2 shows the results of calculating CD8+ T-cell expression in OPMD lesions. Most samples (61.5%) showed CD8+ T cell expression with weak intensity. Judging from the degree of dysplasia of the samples, CD8+ T cell expression with weak intensity was found more (75.0%) in OPMD lesions that did not have dysplasia (No.3) and mild dysplasia degrees (No.4,5,7,9,10). CD8+ T cell expression with strong intensity was only found in lesions with moderate dysplasia (38.5%) (No.2,8,12,13). The positive controls (OLP) used in this study did not have dysplasia and were included in the strong intensity (No.1).

Most of the risk factors that patients have, based on table 2, are betel nut chewer (83.0%). The patient's risk factors were found with a combination of several factors: consuming alcohol (16.6%) and smoking (16.6%). From these data, all samples with risk factors had low intensity CD8+ T-cell expression (No.4,5,6,7,9,10). 71.4% of the samples without risk factors showed strong intensity CD8+ T-cell expression (No.1,2,8,12,13). From these data, all samples with risk factors had low intensity CD8+ T-cell expression (No.4,5,6,7,9,10).

Based on the chi-square test in table 3, the CD8+ intensity is determined in values of minimum is 1 (weak intensity) and the maximum is 2 (strong intensity). It is known that based on clinical diagnosis, the most median value is 1, and the p value is 0.524. By gender, the most median value of 1 and p value of 0.803 are shown. The p value on both criteria is >0.05, meaning there is no significant between clinical diagnosis and gender with CD8+. Based on the degree of dysplasia, there is no significant correlation between dysplasia and the expression of CD8+ (p=0.111). The risk factor shows the median value on the sample with the risk factor is 2 and without risk factor is 1. The risk factor shows a p value with value of 0.005. Risk factors show a p<0.05, meaning there is a significant

correlation between dysplasia and the expression of CD8+. The reliability test results in 3 raters using the ICC method through the SPSS application obtained an ICC value of 0.964, indicating very good inter-rater reliability.

In this study, the relationship between histopathological samples was also observed. The degree of dysplasia and the distribution of patients based on risk factors (Table 4) shows patients with and without risk factors describing different degrees of dysplasia. Samples with no dysplasia showed the absence of risk factors and the intensity of CD8+ T cells of weak intensity (50%) and strong (50%). Samples of mild dysplasia were all risk factors (35.1%) and showed only CD8+ in weak intensity (100%). There were more moderate dysplasia samples without risk factors (35.1%) than with risk factors (7.1%). Samples with severe dysplasia were accompanied by risk factors (7.1%) and showed strong CD8+ intensity. Patients with risk factors with different degrees of dysplasia show weaker CD8+ T cell expression than those without risk factors.

Table 1. Socio-demographic characteristic

Description	**N (%)**
Age	
< 40	1 (7,7%)
40-50	5 (38,5%)
51-60	4 (30,8%)
> 60	3 (23,0%)
Gender	
Man	4 (30,7%)
Woman	9 (69,2%)
Risk Factors	
With	6 (46,1%)
Without	7 (53,8%)

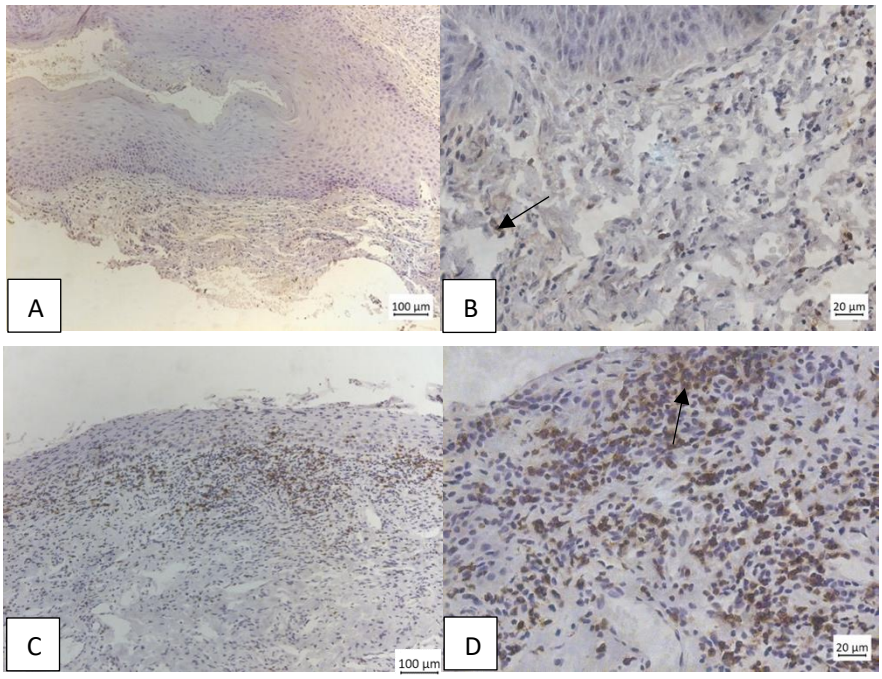

Figure 1. Expression of CD8+ T cells positive in connective tissue is indicated by brown cells (black arrows). CD8+ T cell expression in weak intensity with mild dysplasia, (a) 100x magnification, (b) 400x magnification. Expression of CD8+ T cells in strong intensity with a moderate degree of dysplasia, (c) 100x magnification, (d) 400x magnification.

Table 2. CD8+ T Cell Expression of OPMD based of Risk Factors and Dysplasia Status

No.	Clinical Diagnosis	Age	Gender	Degree of Dysplasia	Risk Factors	CD8+ T Cell Intensity
1	OLP	72	Female	None	None	2
2	Leukoplakia	67	Male	Moderate	None	2
3	Leukoplakia	40	Male	None	None	1
4	Leukoplakia	40	Female	Mild	Betel nut chewing	1
5	Leukoplakia	56	Male	Mild	Betel nut chewing	1
6	OLP Erosive	57	Female	Moderate	Betel nut chewing and alcohol	1
7	OLP Erosive	57	Female	Mild	Smoking and alcohol	1
8	OLP Erosive	50	Female	Moderate	None	2
9	Eritroleukoplakia	50	Female	Mild	Betel nut chewing	1
10	Eritroleukoplakia	63	Male	Mild	Betel nut chewing	1
11	Eritroleukoplakia	59	Female	Moderate	None	1
12	Eritroleukoplakia	48	Female	Moderate	None	2
13	Oral Lichenoid Lesion (OLL)	34	Male	Moderate	None	2

Note :
1 = weak intensity (< 500 CD8+ T cell)
2 = strong intensity (≥ 500 CD8+ T cell)

Table 3. Correlation of CD8+ in diagnosis, gender, dysplasia and risk factor

Description	CD8+ T Cell Expression	
	Med (min-max)	p-value
Clinical Diagnosis		0.524
OLP/OLL	2 (1-2)	
Leukoplakia	1 (1-2)	
Erythroleukoplakia	1 (1-2)	
Gender		0.803
Male	1 (1-2)	
Female	1 (1-2)	
Displasia		0.111
None	1.5 (1-2)	
Mild	1 (1)	
Moderate	2 (1-2)	
Severe	0	
Risk Factor		0.005**
Without	2 (1-2)	
With	1 (1)	

Table 4. Histopathological result and distribution based on risk factor

Degree of Dysplasia	Risk factors		CD8+ T Cell Intensity	
	With N (%)	Without N (%)	1(%)	2(%)
None	-	2 (14.2%)	50%	50%
Mild	5 (35.1%)	-	100%	
Moderate	1 (7.1%)	5 (35.1%)	33.3%	66.6%
Severe	1 (7.1%)	-	100%	

DISCUSSION

In the sample used in this study, most OPMD lesions were found at an average age of 53 years, similar to several studies showing that the prevalence of OPMD lesions was found in the age range above 50 years.[6,18,20,21,22] The prevalence of OPMD lesions in women (71.4%) from the results of this study was higher than in men (28.6%). In line with several studies that found a higher prevalence of OPMD lesions in women.[18,21,23,24] In contrast with the research of Mello *et al*. and Surendran *et al*. found a higher prevalence in men.[6,22] This can be associated with risk factors in patients (Table 2), namely tobacco products, betel nut chewing, alcohol use, and HPV, which can cause the appearance and development of OPMD lesions and can worsen the prognosis of these lesions.[8,23] In addition, this study used more female samples (N = 10) than men (N = 4).

The positive control used in this study was OLP, a lesion caused by an immune response from cytotoxic autoimmune T cells with CD8+ T cells, as the majority of lymphocytes with a characteristic histopathological picture band of lymphocytes on the surface.[25] CD8+ can be detected on the band of lymphocytes from OLP.[21] Therefore, OLP was used as a control to determine whether the CD8+ T cell markers were used to function properly. In this study, OLP as a control showed strong intensity CD8+ T cell expression.

Based on the observations (Table 2), it was found that the expression of positive CD8+ T cells with weak intensity was found in the majority (66.6%) of OMPD lesions with mild and no dysplasia. Other OPMD lesions that have strong CD8+ T-cell expression show some similarities. That is, it only appears with a moderate degree of dysplasia. This indicates that in this study, the number of CD8+ T cells was higher in the epithelium of OPMD lesions with moderate to severe dysplasia than without dysplasia and mild dysplasia.

In line with research by Öhman et al., their results found more CD8+ T cells in connective tissue in OPMD lesions with moderate and severe dysplasia than those without dysplasia and mild dysplasia. The presence of dysplasia leads to increased inflammation and increased interaction between dendritic Langerhans cells and CD8+ T cells. The body's immune system recognizes tumor cells. It secretes the tumor-associate antigens (TAAs) produced by dendritic Langerhans cells via the MHC class I pathway. It presents tumor peptides to CD8+ T cells that recognize and kill cancer cells.[26] In line with research by Surendran et al., the presence of CD8+ cells is positively correlated with the development of dysplasia. The presence of CD8+ T cells in dysplastic lesions and OSCC is a good prognostic indicator.[22]

Samples of leukoplakia (No.2,3,4,5) with varying degrees of dysplasia showed CD8+ T cell intensity in no dysplasia and mild dysplasia in weak while in moderate dysplasia, appeared strong intensity. This is to the existing theory and signals a good prognosis. Oral Lichenoid Reaction (OLL) samples in this study also showed moderate dysplasia. Previous studies show that strong CD8+ T cell intensity indicated a good prognosis. [22,26]

In this study, interesting data were found samples with moderate degrees of dysplasia, namely OLP erosive (No.6) and erythroleukoplakia (No.11), which showed weak intensity, in contrast to previous studies that showed CD8+ T cell expression at moderate to severe dysplasia in strong intensity.[22,26] In OLP erosive samples and erythroleukoplakia with moderate degrees of dysplasia both showed CD8+ T cell expression in weak intensity, in line with several studies showing a decrease in CD8+ T cells in OPMD lesions with severe dysplasia. The development of

abnormalities in the epithelium from dysplasia to OSCC is associated with a decrease in the number of CD8+ T cells. A decrease in CD8+ T cell count based on several studies is associated with possible changes to malignancies and higher stages as well as metastases. Increased infiltration of CD8+ T cells in OSCC showed improved overall survival in OSCC patients. [18,20,27] It is likely that erosive OLP and erythroleukoplakia in this study turned malignant compared to lesions with strong intensity. This study supports other studies, CD8+ with weak intensity at moderate and severe dysplasia, indicating a poor prognosis.

The chi-square statistical test data on risk factors (Table 3) shows a relationship between risk factors and CD8+ (p=0.005). The median value of CD8+ in samples with a risk factor is 1, which means the weak intensity of CD8+. In samples without risk factors, a median value of CD8+ is 2, which means the strong intensity of CD8+, which means that patients with risk factors show weaker CD8+ expression. Based on patient risk factor data in this study, OLP lesions (P6, P8) show interesting data, OLP with a moderate degree of dysplasia with risk factors has low intensity CD8+ T cell expression, while OLP with the same degree of dysplasia but without risk factors shows CD8+ T cell expression in strong intensity. OLP with CD8+ that shows weak intensity has a higher potential to become malignancy.

The patient's risk factors; betel nut, smoking, consuming alcohol, and human papiloma virus (HPV), affect the development of OPMD lesions, developing the degree of dysplasia in the epithelium and worsening the prognosis.[8,28,23,29]Several studies have shown that a decrease CD8+ T cells in OPMD lesions is associated with the possibility of these lesions turning into malignancy.[18,20,27] CD8+ T-cell expression can be a prognostic marker for OPMD lesions because, in this study, patients with risk factors showed CD8+ T-cell expression at weak intensity. Expression of CD8+ T cells in OPMD lesions meets the criteria of an ideal biomarker in line with Zhong's research et al., which can provide an adequate diagnosis, indicate the stage of cancer and a certain level of severity, and can be used in assessing the prognosis because it plays an important role in identifying the extent of cancer.[30]

CONCLUSION

CD8+ T cell expression in OPMD lesions varies depending on the degree of dysplasia and risk factors. CD8+ -cell expression showed strong intensity in OPMD lesions with moderate to severe dysplasia, whereas CD8+ T-cell expression in OPMD lesions with no dysplasia or mild dysplasia in epithelium showed weak intensity. Patients with risk factors showed CD8+ T-cell expression at a weaker intensity than those without risk factors. This study supports the results of other studies that the expression of CD8+ T cells observed using the immunohistochemical method can be a prognostic marker of OPMD lesions.

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