Original Article

Expression of Cathepsin B and Cystatin A in Oral Lichen Planus

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ABSTRACT

Objective: Cathepsin B (Cat-B), a cysteine protease, and cystatin A (Cys-A), a protease inhibitor, are involved in the immune response. This study determined Cat-B and Cys-A expression in oral lichen planus (OLP) by immunohistochemistry. **Materials and Methods:** Thirty specimens each of OLP and healthy gingiva (HG) were included. The expression pattern, the number of positive cells, the staining intensity, and the immunoreactive score (IRS) of Cat-B and Cys-A were investigated. The data were analyzed by using unpaired *t*-test, Chi-square, and Spearman's rank correlation. **Results:** The Cat-B expression in OLP was observed as cytoplasmic staining in the epithelial cells, whereas Cys-A expression was exhibited in the nucleus and cytoplasm of the epithelium. An increase in Cat-B staining intensity was also observed in the basal cells. Conversely, the high staining intensity of Cys-A was observed in the stratum spinosum, but not the stratum basale. In HG, Cat-B expression demonstrated a relatively consistent intensity in the epithelial layer. The Cys-A expression in HG was similar to OLP with a lower staining intensity. The mean percentage of positive cells and the IRS score of Cat-B and Cys-A in OLP were significantly higher than HG ($P < 0.05$). There was no correlation between Cat-B and Cys-A levels in OLP. Interestingly, Cat-B expression in erosive OLP was greater than in non-erosive OLP ($P < 0.05$). **Conclusion:** The Cat-B and Cys-A expression in OLP was more outstanding than in HG, suggesting possible roles for the process of OLP pathogenesis. In addition, Cat-B expression may be an indicator of the disease severity.

Keywords: *Autoimmune disease, cathepsin B, cystatin A, cysteine proteases, oral lichen planus, protease inhibitors*

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Introduction

Oral lichen planus (OLP) is a chronic mucocutaneous disease with an uncertain etiology. The pathogenesis of OLP is believed to be involved in the activation of keratinocytes and antigen-presenting cells, resulting in a T-cell-mediated immune response. The T-cell activation by the antigens associated with the major histocompatibility complex (MHC) class II induced the production and secretion of cytokines, leading to inflammation of the epithelial layer and keratinocyte apoptosis.[1,2]

Cysteine cathepsins are lysosomal proteases that are found in the tissues of the human body. Cathepsin B (Cat-B) plays a role in cellular functions, such as antigen processing, inflammatory responses against antigens, and tissue apoptosis.[3-5] It participates in the processing of antigen presentation to MHC class II. Cat-B is also required for the toll-like receptor signaling cytokine production, including tumor necrosis factor alpha (TNF-α).[4,6] Abnormal activity of Cat-B has been associated with rheumatoid arthritis, systemic sclerosis, and various cancers.[7-9] A previous study also

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reported the possible role of Cat-B as a marker in OLP malignant transformation. Nevertheless, there were no data regarding the role of Cat-B in the pathogenesis of OLP.[10]

The specific cysteine protease inhibitors regulated the activities of cysteine cathepsins, such as cystatins. Cystatins A (Cys-A) can form complexes with Cat-B.[11] The relationship between Cat-B and Cys-A expression was previously investigated, particularly in human cancers. An inverse correlation between increased Cat-B expression and decreased Cys-A level has been shown in laryngeal cancer and brain tumor.[12,13] Conversely, an increase in both Cat-B and Cys-A levels was observed in hepatocellular carcinoma.[14,15] There were no previous reports of the expression of Cys-A and the relationship between Cat-B and Cys-A expression in OLP. Therefore, the role of Cat-B and Cys-A in OLP is still unclear.

Based on the data from prior studies, Cat-B and Cys-A may contribute to autoimmune inflammation of OLP. Therefore, this study aimed at investigating Cat-B and Cys-A expression in OLP compared with healthy gingiva (HG) by using immunohistochemistry (IHC). The correlation between the clinical presentation of OLP and the expression of Cat-B and Cys-A was also studied.

Materials and Methods

The 30 paraffin-embedded tissues of OLP were obtained from the Department of Oral Surgery and Oral Medicine, Faculty of Dentistry at Srinakharinwirot University between 2010 and 2019. Age, sex, clinical features of OLP, and biopsy sites were retrospectively investigated from the patient's records. The clinical features of OLP were classified as non-erosive OLP and erosive OLP based on clinical diagnosis from dental records. All of the OLP selected specimens were histologically reviewed and diagnosed based on modified WHO criteria.[16] Specimens that presented artifacts such as superficial biopsy or did not demonstrate characteristic features of OLP were excluded. The 30 control specimens were HG tissues, obtained during the third molar surgical removal process from the volunteers, who signed a written informed consent form. The research project was approved by the Ethical Committee for Research in Human Subjects at Srinakharinwirot University, no. DENTSWU-EC29/2563X.

Immunohistochemistry

Briefly, paraffin-embedded specimens (4 μm) were deparaffinized and rehydrated. The IHC was performed using EnVision kit (Dako Agilent, USA). The antigen retrieval was performed in 10 mM citrate buffer pH 6.0 by using a microwave (700 W). After cooling down to room temperature, endogenous peroxidase activity was blocked. The sections were washed with phosphate buffer saline (PBS), blocked with protein blocking reagent, and incubated with the primary antibody against Cat-B (sc-365558, Santa Cruz Biotechnology, USA) or Cys-A (sc-376759, Santa Cruz Biotechnology, USA). Next, the secondary antibody was added to these sections. After washing with PBS, chromogen,3,3I-Diaminobenzidine was applied. Finally, the sections were dehydrated, counterstained, and mounted in the Permount mounting medium (Bio-Optica, Italy). For negative staining control, the protein block without the primary antibody was added to specimens. The mouse liver and human esophagus tissues were taken as positive control of Cat-B and Cys-A, respectively.

Interpretation of immunostaining

Cat-B and Cys-A expression was evaluated based on the expression pattern, the number of positive cells, and the staining intensity. The immunoreactive score (IRS) was also applied for interpretation. Four photographs each of the epithelial layer and the connective tissue layer were taken from all slides at 400× magnification using a Microscope camera (Motic, China). All of the photographs were scored using ImageJ software (NIH, USA). The Cat-B and Cys-A expression showing intensities of brown color as a cytoplasmic or nuclear staining in the epithelial cells as well as the underlying stromal cells were considered positive.

The percentage of positive cells was presented as mean ± SD and also determined as follows: 0 = no positive cells, 1 = <10% of positive cells, 2 = 10%–50% of positive cells, 3 = >50%–80% of positive cells, and 4 = >80% of positive cells. The staining intensity score was graded as: 0 = no color reaction, 1 = mild, 2 = moderate, and 3 = intense. The IRS score gives a range of 0–12 as a product of multiplication between positive cells proportion score (0–4) and staining intensity score (0–3).[17] The IRS was interpreted as negative (score 0–1), mild (score 2–3), moderate (score 4–8), and strongly positive reaction (score 9–12). The specimens were analyzed and scored by two of the investigators (PB and WH). The inter-investigator calibrations were performed with intraclass correlation coefficient values equal to 0.965.

Statistical analysis

The statistical analysis was performed using SPSS software, Version 26 for Windows. The mean percentage and statistical significance difference of positive cells between groups were calculated using

an unpaired *t*-test. The differences in the scores of the positive cells, the score of staining intensity, and IRS between groups were analyzed using the Chi-square test. The correlations between variables were analyzed using Spearman's rank correlation. The statistical significance was defined as a *P* value of <0.05.

Results

The clinical data on the cases and controls are presented in Table 1. The mean ages of the OLP patients and controls were 54.97 ± 15.88 and 25.5 ± 10.78, respectively. A difference for age distribution was noted ($P < 0.001$). The male to female ratio in the OLP group and the control group was 1:1.72 and 1:1.14, respectively. The clinical presentation of OLP specimens consists of 17 cases of non-erosive OLP and 13 cases of erosive OLP. The biopsy site of the OLP specimens was taken from buccal mucosa ($n = 22$) and gingiva ($n = 8$). All of the control specimens were obtained from the gingiva ($n = 30$).

The expression pattern of Cat-B and Cys-A

The Cat-B expression was found in all cases (30 cases) in the OLP specimens, whereas in HG, Cat-B expression was detected in 29 cases. The immunostaining Cat-B pattern in OLP was predominantly found in the epithelium with granular, dot-like characteristics in the cytoplasm. An increase in staining intensity was also observed in the basal cells [Figure 1A and B]. In contrast, Cat-B expression in HG demonstrated a relatively consistent staining intensity in the epithelial layer [Figure 1D and E]. Cat-B expression was also detected in the inflammatory cells in the connective tissue layer of both groups [Figure 1C and F].

Cys-A expression was also found in all cases of OLP and in 28 cases of HG. The pattern of Cys-A immunostaining in OLP was mainly distributed in the nucleus and cytoplasm of the epithelial cells. A strong staining intensity was found in the stratum spinosum, whereas a lower staining intensity was observed in the stratum basale [Figure 2A and B]. The expression pattern of Cys-A in HG was similar to OLP with a lower staining intensity [Figure 2C and D]. Cys-A expression was also found in the inflammatory cells in the connective tissue layer of both groups [Figure 2C and F].

The percentage of positive cells, the staining intensity, and the IRS score of Cat-B and Cys-A

The mean percentage of Cat-B positive cells in OLP (53.58% ± 16.24%) was significantly higher than in HG (29.47% ± 25.77%) ($P < 0.001$). The 23 cases (76.7%) of OLP showed moderate to intense Cat-B staining intensity, whereas most cases (53.3%) of HG showed mild staining intensity. However, there was no difference in Cat-B staining intensity between these groups ($P = 0.254$). Besides, the IRS score of Cat-B in OLP was significantly greater than in HG ($P = 0.014$). The majority of OLP cases (76.7%) showed a moderately to strongly positive IRS score, whereas in HG, 18 cases (60.0%) showed a negative to mild IRS score [Table 2].

The mean percentage of Cys-A positive cells in OLP (63.43% ± 19.87%) was significantly greater than HG (56.85% ± 28.35%) ($P < 0.001$). There were 22 cases of OLP (73.3%) that showed intense Cys-A staining intensity, whereas only 14 cases (46.7%) were observed in HG. The number of cases with a strongly positive IRS score was higher in OLP (70%) than in HG (40%). There was a statistically significant increase in the staining intensity and IRS scores of Cys-A in OLP ($P = 0.018$ and $P = 0.008$, respectively) [Table 2]. The data suggested that Cat-B and Cys-A expression in OLP was more significant than in HG.

Table 1: Characteristics of the study population

Variables	Group				*P* value
	Oral lichen planus			Human gingival ($n = 30$)	
	Erosive ($n = 13$)	Non-erosive ($n = 17$)	Total ($n = 30$)		
Sex, *n* (%)					0.407
Female	7 (23.33)	12 (40.0)	19 (63.33)	16 (53.3)	
Male	6 (20.0)	5 (16.7)	11 (36.7)	14 (46.7)	
Age					<0.001
Min–max (years)	41–84	22–72	22–84	18–61	
Mean ± SD (years)	64.54 ± 14.37	47.65 ± 13.06	54.97 ± 15.88	25.5 ± 10.78	
Duration					
Min–max (months)	0–72	0–60	0–72	N/A	N/A
Mean ± SD (months)	22.87 ± 24.78	14.59 ± 16.95	18.16 ± 20.74		
Biopsy site, *n* (%)					
Buccal mucosa	10 (33.33)	12 (40.0)	22 (73.33)	0 (0)	N/A
Gingiva	3 (10.0)	5 (16.77)	8 (26.77)	30 (100)	

Max = maximum, Min = minimum, N/A = not applicable, SD = standard deviation

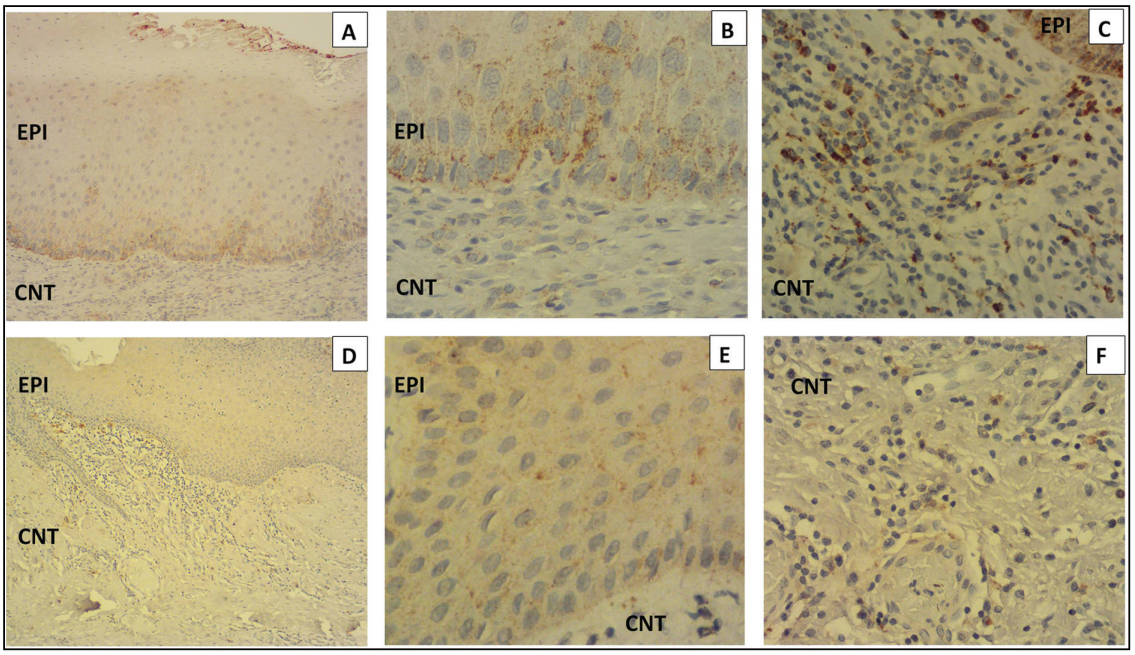

Figure 1: Immunostaining of cathepsin B in oral lichen planus (A, B, C) and healthy gingiva (D, E, F). Original magnification: 100× (A, D) and 400× (B, C, E, F). CNT = connective tissue, EPI = epithelium

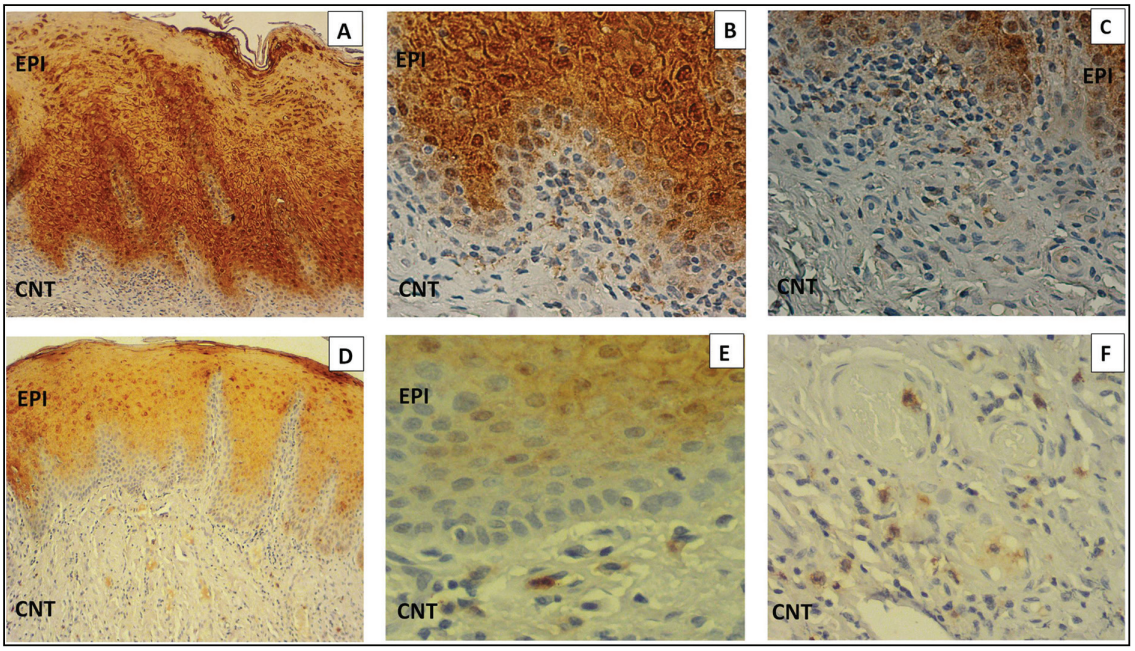

Figure 2: Immunostaining of cystatin A in oral lichen planus (A, B, C) and healthy gingiva (D, E, F). Original magnification: 100× (A, D) and 400× (B, C, E, F). CNT = connective tissue, EPI = epithelium

The expression of Cat-B and Cys-A in erosive and non-erosive OLP

Because we observed that Cat-B and Cys-A expression in OLP was more significant than in HG, the Cat-B and Cys-A expression was further analyzed in erosive and non-erosive type of OLP. The mean percentage of Cat-B positive cells in erosive OLP (59.39 ± 14.95) was higher than that in non-erosive OLP (49.14 ± 16.19), but it was not statistically significant (P = 0.087). The majority of the non-erosive OLP specimens showed the moderate staining intensity of Cat-B expression (52.94%), whereas most of the erosive OLP specimens showed intense Cat-B staining intensity (46.15%). There were no significant differences between the number of stained cells and the staining intensity between the groups (P = 0.087 and P = 0.120, respectively).

Table 2: Expression of cathepsin B and cystatin A

	Cathepsin B			Cystatin A		
	Oral lichen planus (n = 30)	Healthy gingival (n = 30)	P value	Oral lichen planus (n = 30)	Healthy gingival (n = 30)	P value
Percentage of positive cells						
Mean ± SD (%)	53.58 ± 16.24	29.47 ± 25.77	<0.001	63.43 ± 19.87	56.85 ± 28.35	<0.001
Score A (score of percentage of positive cells), *n* (%)						
0 = no positive cells	0 (0)	1 (3.3)	<0.001	0 (0)	2 (6.7)	0.182
1 = <10%	0 (0)	9 (30.0)		0 (0)	2 (6.7)	
2 = 10%–50%	10 (33.3)	13 (43.3)		9 (30.0)	8 (26.7)	
3 = >50%–80%	19 (63.3)	6 (20.0)		14 (46.7)	10 (33.3)	
4 = >80%	1 (3.3)	1 (3.3)		7 (23.3)	8 (26.6)	
Score B (intensity of staining), *n* (%)						
0 = no color	0 (0)	1 (3.3)	0.254	0 (0)	2 (6.7)	0.018
1= mild	7 (23.3)	16 (53.3)		2 (6.7)	4 (13.3)	
2 = moderate	14 (46.7)	8 (26.7)		6 (20.0)	10 (33.3)	
3 = intense	9 (30.0)	5 (16.7)		22 (73.3)	14 (46.7)	
IRS score (A × B), *n* (%)						
0–1 = negative	0 (0)	9 (30.0)	0.014	0 (0)	4 (13.3)	0.008
2–3 = mild	7 (23.3)	9 (30.0)		2 (6.7)	2 (6.7)	
4–8 = moderate	14 (46.7)	8 (26.7)		7 (23.3)	12 (40.0)	
9–12 = strongly positive	9 (30.0)	4 (13.3)		21 (70.0)	12 (40.0)	

IRS = immunoreactive score, SD = standard deviation

However, a moderate to strongly positive IRS score of Cat-B expression was greater in erosive OLP (84.61%) than in non-erosive OLP (70.59%) (P = 0.031) [Table 3].

The mean percentage of Cys-A positive cells in non-erosive OLP (67.56% ± 18.10%) was greater than that in erosive OLP (58.02% ± 21.52%), but it was not statistically significant (P = 0.198). The intense Cys-A staining intensity was mostly found in both erosive (61.54%) and non-erosive OLP (82.35%). The number of cases with a strongly positive IRS score was higher in non-erosive OLP (76.47%) than erosive OLP (61.54%). However, there were no significant differences in the intensity of stained cells and IRS score of Cys-A expression between the two groups (P = 0.163 and P = 0.201, respectively) [Table 3]. These data indicated that Cat-B expression may be related to OLP severity.

Correlation between Cat-B and Cys-A in HG and OLP

Next, the associations between Cat-B and Cys-A expression in HG and OLP were evaluated by Spearman's rank correlation analysis. The result suggested that there was a high positive correlation between Cat-B and Cys-A expression in HG (r = 0.691, P < 0.001), whereas no correlation between Cat-B and Cys-A expression in OLP was detected (r = −0.003, P = 0.986). Based on the type of OLP, there was a weak negative correlation between the expression of Cat-B and Cys-A in erosive OLP (r = −0.294, P = 0.330). However, a weak positive correlation between the two markers was observed in non-erosive OLP (r = 0.337, P = 0.186) [Table 4].

Discussion

The present study evaluated the expression of Cat-B and Cys-A in OLP by IHC. Our results showed that in OLP, Cat-B expression was observed as cytoplasmic staining in the epithelial cells, whereas Cys-A expression was shown in the nucleus and cytoplasm of the epithelium. An increase in Cat-B staining intensity was exhibited in the basal cells. Contrarily, the high staining intensity of Cys-A was observed in the stratum spinosum. In HG, Cat-B expression showed a relatively consistent intensity in the epithelial layer. The Cys-A expression in HG was similar to OLP with a lower staining intensity. In addition, the mean percentage of positive cells and the IRS score of Cat-B and Cys-A in OLP were significantly higher than in HG. These results are consistent with the studies of Satelur *et al.*,[10] which was the only study conducted on Cat-B expression in OLP. They found that Cat-B was expressed in moderate intensity and located in the cytoplasm of epithelial cells, mainly in the basal and stroma cells closest to the basement membrane. The Cat-B expression was also found in normal mucosa, but with a lower number of cases (six in 10 cases) in comparison with this study (29 in 30 cases).[10]

Table 3: Expression of cathepsin B and cystatin A in erosive and non-erosive OLP

	Variables	Type of OLP		*P* value
		Erosive OLP, *n* = 13 (100%)	Non-erosive OLP, *n* = 17 (100%)	
Cathepsin B	Percentage of positive cells			
	Mean ± SD (%)	59.39 ± 14.95	49.14 ± 16.19	0.087[a]
	Intensity of staining			
	1 = mild	2 (15.39)	5 (29.41)	0.120[b]
	2 = moderate	5 (38.46)	9 (52.94)	
	3 = intense	6 (46.15)	3 (17.65)	
	IRS score			
	Score 0–1 = negative	0 (0)	0 (0)	0.031[b]
	Score 2–3 = mild	2 (15.39)	5 (29.41)	
	Score 4–8 = moderate	5 (38.46)	9 (52.94)	
	Score 9–12 = strongly positive	6 (46.15)	3 (17.65)	
Cystatin A	Percentage of positive cells			
	Mean ± SD (%)	58.02 ± 21.52	67.56 ± 18.10	0.198[a]
	Intensity of staining			
	1 = mild	2 (15.39)	0 (0)	0.163[b]
	2 = moderate	3 (23.07)	3 (17.65)	
	3 = intense	8 (61.54)	14 (82.35)	
	IRS score			
	Score 0–1 = negative	0 (0)	0 (0)	0.201[b]
	Score 2–3 = mild	2 (15.38)	0 (0)	
	Score 4–8 = moderate	3 (23.08)	4 (23.53)	
	Score 9–12 = strongly positive	8 (61.54)	13 (76.47)	

IRS = immunoreactive score, OLP = oral lichen planus, SD = standard deviation

[a]Data were analyzed using unpaired *t*-test

[b]Data were analyzed using Spearman's rank correlation

Table 4: Correlation between the expression of cathepsin B and cystatin A in each test group

Markers	Cystatin A		
Cathepsin B	Healthy gingiva	R	0.691
		P value	<0.001
	Oral lichen planus	R	−0.003
		P value	0.986
	Erosive oral lichen planus	R	−0.294
		P value	0.330
	Non-erosive oral lichen planus	R	0.337
		P value	0.186

Even though there was only one prior study of Cat-B conducted in OLP, the expression of Cathepsin K (Cat-K) and Cathepsin L (Cat-L) in OLP was previously reported and suggested to be involved in OLP pathogenesis. Kitkhajornkiat *et al.* showed that Cat-L expression in OLP was more significant than in HG. The Cat-L expression pattern was reported as dot-like staining appearances, increased density in the basal cells, and intense staining in the inflammatory cells near the basement membrane.[18] Cat-K was also expressed in OLP with a small, granule staining pattern in the cytoplasm of melanocytes, macrophages, fibroblasts, and endothelial cells. Besides, intense staining was also found in the basal cells.[19] In this study, Cat-B exhibited a similar expression pattern to Cat-L and Cat-K. Thus, Cat-B may also play a role in OLP pathogenesis as well as Cat-L and Cat-K.

The MHC class II molecules are associated with antigen presentation, expressed by Langerhans cells or keratinocytes, which is one of the antigen-specific mechanisms involved in OLP pathogenesis.[1,2] Cat-B plays a role in the invariant chain cleavage and antigen processing in the immune system. The degradation of the invariant chain results in MHC class II binding of antigens and presenting them to the immune system.[20] A study of rat hepatocytes also showed that Cat-B was involved in cell death through TNF-α mediated apoptosis.[21] Also, the function of Cat-B is probably related to the OLP histopathological features, showing an accumulation of lymphocytes, the apoptosis of keratinocytes, and the degeneration of basal cells. The data from prior studies may explain the significance of Cat-B immuno-expression in basal cells of OLP compared with HG in this study.

We also observed that Cat-B expression in erosive OLP was greater than in non-erosive OLP. A previous study reported that Cat-B could regulate the manifestations and severity of mercury-induced inflammation and autoimmunity. An increase in Cat-B activity has been observed in mercury-induced autoimmunity

(mHgIA)-sensitive mice in comparison with mHgIA-resistant mice. Further, treatment with a Cat-B inhibitor resulted in transient reduction of local induration and the expression of inflammatory cytokines.[22] These findings may help explain the reason why the immuno-expression of Cat-B was significantly outstanding in erosive OLP than in non-erosive OLP. The data may also suggest that Cat-B expression might be linked to the OLP severity.

However, the imbalance between Cathepsins and their inhibitors seemed to play a role in inflammatory diseases.[23] There has been some controversy over the correlation between the expression of Cat-B and Cys-A, especially in human cancers. Some studies reported an inverse relationship between Cat-B and Cys-A, whereas some reported an upregulation of both Cat-B and Cys-A levels.[12-15] In this study, Cat-B and Cys-A immuno-expression was increased in OLP. One possible explanation could be an increase in Cat-B expression resulting in an increase in Cys-A expression. However, Cys-A expression may not be sufficient to prevent the Cat-B-induced keratinocyte apoptosis in OLP. It is worth mentioning that a high positive correlation between Cat-B and Cys-A immuno-expression was detected in HG, suggesting that an increase in Cat-B would correspond to an increase in Cys-A expression. Interestingly, we did not find a correlation between Cat-B and Cys-A expression in OLP in this study. The data may indicate that the activities of Cat-B and Cys-A are altered in OLP.

Nevertheless, this study has limitations. Some clinical parameters of OLP that are based on chart reviews maybe missing and cannot be analyzed, such as the presence of symptoms and pain score. In addition, the sample size in this study is limited. Thus, the study of Cat-B and Cys-A expression in a larger sample size is required for more accurate results and interpretations. However, to the best of our knowledge, there is no previous report on the relationship between Cat-B and Cys-A in OLP. Therefore, the results from this study could provide a better understanding of the roles of Cat-B and Cys-A in the pathogenesis of OLP.

Conclusion

In conclusion, this study showed an increase in the expression of Cat-B and Cys-A in OLP, suggesting that Cat-B and Cys-A may play a role in OLP pathogenesis. Besides, Cat-B expression may be useful to determine the severity of OLP.

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Conflicts of interest

There are no conflicts of interest.

Authors' contributions

PB contributed to study conception, methodology, data collection, data analysis and interpretation, article writing—original draft preparation, critical revision of the article, and final approval of the article. WH contributed to methodology, data analysis and interpretation, and final approval of the article. PJ contributed to study conception, methodology, critical revision of the article, and final approval of the article. ST contributed to study conception, methodology, and final approval of the article. PT contributed to study conception, methodology, data collection, data analysis and interpretation, article writing—original draft preparation, critical revision of the article, supervision, funding acquisition, and final approval of the article.

Ethical policy and institutional review board statement

The research project was approved by the Ethical Committee for Research in Human Subjects at Srinakharinwirot University, no. DENTSWU-EC29/2563X.

Patient declaration of consent

Not applicable.

Data availability statement

The data set used in the current study is available on request from Patrayu Taebunpakul, e-mail: pathraya@g.swu.ac.th

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