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Original Article

Increased granulysin in the peripheral blood and tissues of patients with oral lichen planus

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Abstract: Oral lichen planus (OLP) is a chronic inflammatory disease of unclear etiology and pathogenesis. Granulysin (GNLY) participates in various immune responses and mediates various skin diseases. However, its expression in OLP has not been reported. This study was to investigate whether there was an abnormal expression of GNLY in the peripheral blood and tissues of patients with OLP. Twenty patients with non-erosive OLP, twenty patients with erosive OLP, and twenty healthy controls were enrolled. The mRNA expression of GNLY in the peripheral blood and tissues was detected using RT-qPCR. The protein expression of GNLY in the peripheral blood plasma was measured using ELISA. The GNLY in tissues was investigated using immunohistochemistry. The mRNA and protein expression of GNLY in non-erosive and erosive OLP patients, when compared with the controls, were upregulated both in the peripheral blood and tissue. Also, in non-erosive and erosive OLP lesion tissues, there was a weak positive expression of GNLY in the lymphocyte membrane of the lamina propria layer and was less expressed in the epithelial layer. In normal mucosa, GNLY was hardly expressed. Furthermore, the immunohistochemical levels of GNLY in non-erosive and erosive OLP lesion were significantly higher than that in the normal oral mucosa. In conclusions, the expression of GNLY in the peripheral blood and tissues of OLP patients were significantly higher than controls, suggesting that there exists an abnormality in the expression of GNLY in OLP which might be involved in the pathogenesis of OLP.

Keywords: Granulysin, oral lichen planus, peripheral blood, tissues, T lymphocytes

Introduction

Oral lichen planus (OLP) is a chronic inflammatory mucosal disease, which has been defined as a potentially precancerous condition, and the incidence is 0.1%-4% [1, 2]. However, little is currently known of the etiology and pathogenesis of OLP. Previous studies suggest that it may be an autoimmune disease caused by an abnormal immune response mediated by T lymphocytes [1, 3].

Granulysin (GNLY) is a protein molecule in the cytoplasm of natural killer (NK) cells and cytotoxic T lymphocytes (CTLs), located in the cytolytic granules of these two kinds of cells with perforin (PFP) and granzyme [4]. GNLY has the potential of killing microbes and tumor cells [5], and induces keratinocyte apoptosis through mitochondrial and receptor-mediated pathways [6]. At the same time, GNLY is also an immune effector molecule with chemotactic and pro-inflammatory effects [7]. Sarwal thought that if the expression of GNLY could be used as a sign of acute rejection and steroid resistance in kidney transplantation, then GNLY may also serve as a sign of steroid resistance in polymyositis (PM) [8].

Increasing studies have revealed that GNLY is involved in the occurrence and development of some immune-mediated diseases. GNLY participates in multiple illnesses such as Stevens-Johnson syndrome/toxic epidermal necrolysis (SJS/TEN), psoriasis, and graft-versus-host disease [9-11]. However, the involvement of GNLY in the immune response of OLP remains unknown.

In this study, to further clarify the pathogenesis of OLP, we proposed to explore the immunologic roles of GNLY in the pathogenesis of OLP by

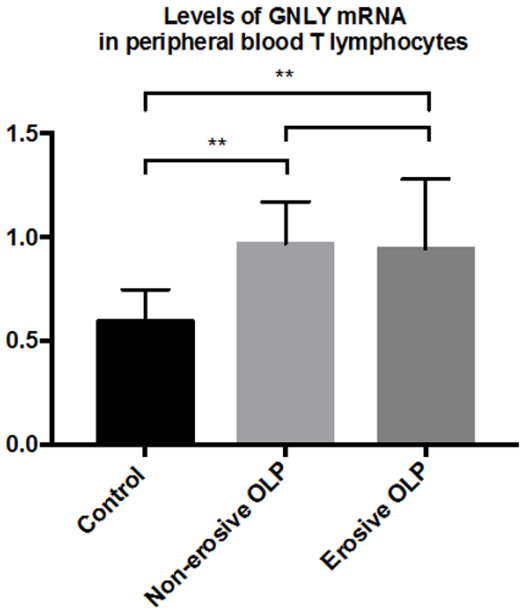

Figure 1. RT-qPCR results: the expression level of GNLY mRNA in non-erosive and erosive OLP patients was significantly higher than that in the control group (**P < 0.01).

detecting it in the peripheral blood and tissue of patients with OLP and healthy individuals.

Materials and methods

Patients and controls

Twenty patients with non-erosive oral lichen planus (OLP) (7 males and 13 females; age, 25-79 years; mean age, 56 years) and twenty patients with erosive OLP (6 males and 14 females; age, 26-74 years; mean age, 53 years) were enrolled. OLP patients in this study were from the Department of Oral Medicine, Affiliated Hospital of Stomatology, Nanjing Medical University (Nanjing, China) diagnosed during the period of 2015-2018. All the patients were clinically and pathologically diagnosed with OLP according to the modified WHO diagnostic criteria [12, 13]. The erosive group's symptoms were characterized by painful, ulcerated and erythematous areas, which reflected a more destructive phase of the disease [14]. The non-erosive group's primary manifestations were asymptomatic Wickham striae on the oral mucosa with no painful, ulcerated and erythematous areas. Twenty healthy volunteers without systemic diseases who had undergone orthognathic surgery were recruited as the controls (7 males and 13 females; age, 27-68 years; mean age, 48 years). There were no statistically significant differences in sex and age between the three groups. None of the subjects had received antibiotics or immunologic agents within at least 3 three months before the collection of specimens. The OLP patients also had no other oral mucosal diseases and autoimmune diseases. All the subjects gave written informed consent. This study complied with the guidelines of the Declaration of Helsinki and was approved by the Ethical committee of Nanjing Medical University (Nanjing, China).

Peripheral blood T lymphocytes isolation

Peripheral blood samples (10 ml) from each subject were immediately isolated. The fresh blood was diluted with the phosphate buffered saline (PBS) solution and then collected in a centrifuge tube containing a lymphocyte isolation medium. Then the density gradient centrifugation was performed using Ficoll-Hypaque (Tianjin Haoyang Biology Manufacturing, China) to obtain peripheral blood mononuclear cells (PBMCs). We isolated T lymphocytes from peripheral blood by Lymphoprep™ separation. The blood was diluted with PBS (Sigma-Aldrich, Munich, Germany) at 1:1 ratio. We put 5 ml diluted blood on 5 ml T lymphocytes separation medium to spin for 20 min at 800 g and collected the buffy coat after centrifugation. Then, we added five times more volume of PBS, washed the cells twice at 1500 rpm for 10 minutes, and discard the supernatant. Next, we isolated T lymphocytes using EasySep™ human CD3 positive selection kit (Stemcell Technologies, Canada) and counted the viability of T lymphocytes by the cytometer (> 95%). Finally, we used RPMI 1640 complete medium (Gibco, USA) to adjust the cell concentration and then placed T lymphocytes at an incubator with 37°C, 5% CO_2.

Oral mucosa tissue

Specimens from the oral mucosa of patients with OLP were collected, in which the area lesion was observed. All the biopsies were taken before an initial topical or systemic therapy. The specimens of the control group were taken from the normal mucosa of healthy volunteers. This study divided all samples into two parts: one was used for immunohistochemistry whereas the other part was snap-frozen and stored at -80°C for RT-qPCR.

Table 1. mRNA expression levels of GNLY in the peripheral blood T lymphocytes of the three groups (mean ± SD)

	Control group	Non-erosive OLP	Erosive OLP
n	20	20	20
GNLY mRNA	0.59 ± 0.15	0.97 ± 0.20	0.94 ± 0.37

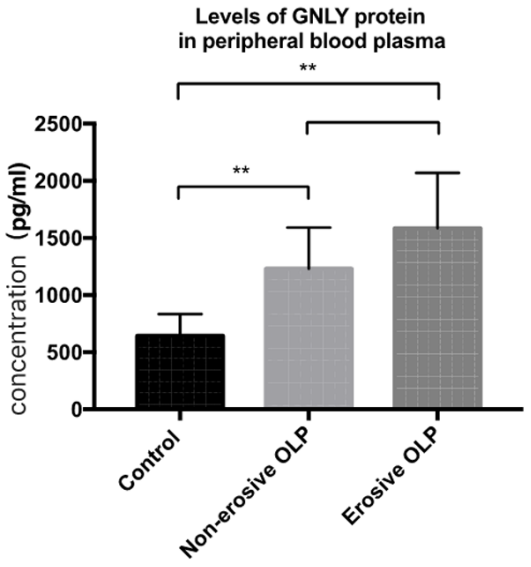

Figure 2. ELISA results: the protein expression level of GNLY in non-erosive and erosive OLP patients was significantly higher than that in the control group (**P < 0.01).

Quantitative real-time RT-polymerase chain reaction (RT-qPCR)

The expression levels of granulysin (GNLY) mRNA were detected both in the peripheral blood T lymphocytes and in oral mucosal tissues. According to the manufacturer's instructions, total cellular RNA was extracted with Total RNA kit I (OMEGA, USA). The RNA concentration was determined using spectrophotometer (GE, USA). Then, the RNA was reverse-transcribed into cDNA using PrimeScriptTMRT Master Mix SYBR green (Takara, Japan). At the end of the PCR cycles, the melting curve analysis was performed to validate the specific generation of the expected PCR product. The primer sequences were designed in the laboratory and synthesized by General Biotech (General, PRC) based on the mRNA sequences obtained from the NCBI. For GNLY, the sequences of the primers used are: forward primer-CTCTCGTCTGAGCCCTGAGT and reverse primer-TGGGCTTATCCACCATCTTC. The $2^{-\Delta\Delta Ct}$ method was used to calculate the relative quantitative results to determine the mRNA expression of the target gene. Each RT-qPCR experiment was done in duplicates.

Enzyme-linked immunosorbent assay (ELISA)

To detect the protein expression of GNLY, we collected the peripheral blood plasma and centrifuged it at 5000 × g for 20 min. According to the ELISA kit instructions (Boster, USA), the O.D. values of GNLY were measured at 450 nm using a microplate reader at the end of 10 min of chromotherapy.

Immunohistochemistry

Immunohistochemistry was used to detect the expression of GNLY in the oral mucosa tissue specimens of the OLP group and the control group. The paraffin sections (4 μm) were dewaxed, hydrated, and then rinsed with tap water. The peroxidase blocking reagent was added to the slices and incubated for 10 minutes. The primary antibody was added dropwise to the slice and incubated at room temperature for 60 minutes, and then rinsed with PBS 3 times for 3 min each. The sections were incubated at room temperature with ready-to-use MaxVision™/HRP reagent for 15 mins. Colorimetric detection was developed with a freshly equipped diaminobenzidine (DAB) color reagent. Finally, the slides were counterstained with hematoxylin, dehydrated, and sealed. Antibody for GNLY (rabbit polyclonal antibody, ab204594, Abcam, UK) was used at a dilution 1:200 in PBS with 1% bovine serum albumin (BSA).

Histopathologic evaluation

A positive signal showed the brownish yellow staining in the cell nucleus or cytoplasm. All the slides were evaluated by the two senior oral pathologists independently. The result of staining was semi-quantitatively evaluated, which was based on the ratio of staining intensity and proportion of positive cells. The intensity score was defined as 0, negative; 1, weak; 2, moderate; or 3, strong brown. The proportion score was defined as 0, negative; 1, < 10%; 2, 11-50%; 3, 51-80%; or 4, > 80% positive cells. The immunoreactive score was calculated as intensity score × proportion score, which was

Table 2. Expression levels of GNLY in the plasma of the three groups (pg/ml) (mean ± SD)

	Control group	Non-erosive OLP	Erosive OLP
n	20	20	20
GNLY	645.12 ± 190.28	1231.46 ± 358.50	1585.93 ± 484.28

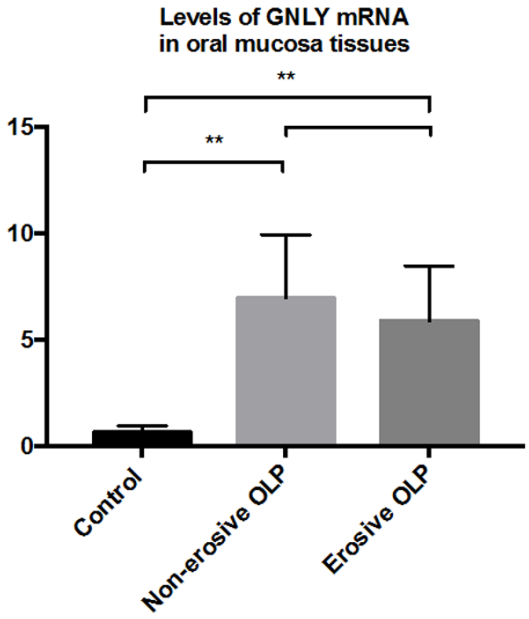

Figure 3. RT-qPCR results: the expression level of GNLY mRNA in non-erosive and erosive OLP patients was significantly higher than that in the control group (**P < 0.01).

divided into three groups based on the final score: 0, negative; 1-4, weak positive expression; > 4, strong positive expression [15, 16].

Statistics

All data were analyzed using a statistical software package (SPSS version 24.0; SPSS, Chicago, IL, USA). The results were expressed as mean ± standard deviation (SD). The differences between the three groups differences were evaluated using ANOVA analysis. Results were considered significant if P < 0.05.

Results

mRNA expression level of GNLY in the peripheral blood T lymphocytes of non-erosive and erosive OLP patients and control group

The results of RT-qPCR indicated that the mRNA expression level of granulysin (GNLY) in the peripheral blood T lymphocytes of non-erosive and erosive oral lichen planus (OLP) patients was significantly higher than that in the control group (*P < 0.05). The differences between non-erosive and erosive OLP were not significant (P > 0.05) (**Figure 1**). The data are shown in **Table 1**.

The protein expression level of GNLY in the peripheral blood plasma of non-erosive and erosive OLP patients and control group

The results of ELISA indicated that the protein expression level of GNLY in the peripheral blood plasma of non-erosive and erosive OLP patients was significantly higher than that in the control group (*P < 0.05, **P < 0.01). The differences between non-erosive and erosive OLP were not significant (P > 0.05) (**Figure 2**). The data are shown in **Table 2**.

mRNA expression level of GNLY in the oral mucosal tissues of OLP patients and control group

We detected the mRNA expression level of GNLY by RT-qPCR. The results indicated that the mRNA expression level of GNLY in the oral mucosal tissues of non-erosive and erosive OLP patients was significantly higher than that in the healthy controls (**P < 0.01). The differences between non-erosive and erosive OLP were not significant (P > 0.05) (**Figure 3**). The data are shown in **Table 3**.

Immunohistochemical detection of GNLY in non-erosive and erosive OLP lesions and normal oral mucosa

The results of immunohistochemistry showed that GNLY was hardly expressed in the normal oral mucosa (**Figure 4A**). However, in non-erosive and erosive OLP lesion tissues, GNLY showed weak positive expression mainly in the lymphocyte membrane of lamina propria layer and was less expressed in the epithelial layer (**Figure 4B**, **4C**). The negative control group showed no staining (**Figure 4D**).

The immunoreactive score of GNLY

The results of immunohistochemistry showed that the immunoreactive score of GNLY in non-

Table 3. mRNA expression levels of GNLY in the oral mucosal tissues of the three groups (mean ± SD)

	Control group	Non-erosive OLP	Erosive OLP
n	20	20	20
GNLY mRNA	0.63 ± 0.31	6.96 ± 2.98	5.88 ± 2.58

erosive and erosive OLP lesion was significantly higher than that in the normal oral mucosa (**P < 0.01) (**Figure 5**). The data of the immunoreactive score (mean ± SD) of GNLY in non-erosive and erosive OLP lesion and normal oral mucosa are shown in **Table 4**.

Discussion

Oral lichen planus (OLP) is a common inflammatory disease of unknown etiology. A large body of evidence supports the role of T-cell-mediated immune dysregulation in the pathogenesis. The main pathological characteristics of OLP are hyperkeratosis of epithelium, hyperplasia of stratum spinosum, colloid body, liquefaction degeneration of basal layer, and banded infiltration of T lymphocytes in lamina propria, which are mainly activated T lymphocytes. It is hypothesized that the apoptosis of keratinocytes is associated with the pathogenesis of OLP and the different levels of apoptosis are involved in erosive and non-erosive switching of OLP to determine different clinical manifestations [2, 17]. The colloid bodies has been identified to be apoptotic bodies. However, it remains elusive how lymphocytes induce the apoptosis of keratinocytes and the infiltration into the oral submucosa [18].

Granulysin (GNLY), in the process of the body immunity, participates in various immune responses including antibacterial, antiviral, and killing cancer cells, with granular components such as perforin and granzyme [6]. GNLY expressed in cytotoxic T lymphocytes (CTLs) and natural killer (NK) cells [19, 20] can cause apoptosis and cytotoxic activity [21-23]. GNLY is the mediator of various skin diseases, including folliculitis [24], psoriasis [10, 11], acne, lichen planus, and viral vesicles [25]. It has been demonstrated that GNLY was involved of in the worsening of psoriatic arthritis and mediated the development of joint lesions [10]. In addition, the levels of serum GNLY are significantly associated with the severity of adverse skin events [26]. It has been reported that GNLY is a key mediator for disseminated keratinocyte death in the skin lesions of SJS/TEN, suggesting that highly expressed GNLY might promote apoptosis in TEN lesions [9]. Deng et al. found that a low concentration of GNLY has a pro-inflammatory role through CCL5, while a high concentration of GNLY has a cytotoxic effect [7].

In this study, we found that the mRNA expression level of GNLY in the peripheral blood T lymphocytes and the concentration of GNLY in the plasma of OLP patients were significantly higher than those in the control group, showing that GNLY might be involved in the occurrence and development of OLP. In addition, our findings showed that there was no significant difference between GNLY in non-erosive OLP and GNLY in erosive OLP. We suspected that this might be due to the simple division of the OLP patients into non-erosion and erosion groups simply according to their clinical manifestations. However, OLP is a chronic disease, so the same patient may manifest the non-erosive or erosive type at different time periods, and the patient may be at various stages in the course of OLP. Although GNLY might participate in the immune pathogenesis of OLP, it might not be related to the severity of the disease.

Also, we found that the mRNA and protein expression levels of GNLY in non-erosive and erosive OLP tissues were significantly higher than those in the control group. The results were consistent with those in the peripheral blood of OLP, further demonstrating that GNLY might be involved in the pathogenesis of OLP process. The same outcomes were reported in the study of psoriatic arthritis: the percentage of GNLY cells in peripheral blood had no significant difference between patients of psoriatic arthritis in the acute phase and remittent stage; but it was higher than in healthy examinees [10]. Moreover, GNLY was highly expressed in the skin lesions of SJS/TEN, which was also concordant with our findings [9]. Immunohistochemical results showed that GNLY was expressed positively in the lamina propria lymphocytes and expressed weakly in the epithelial layer. However, GNLY scarcely existed in the normal mucosa. As CD8+ T cells and CD4+ T cells are densely infiltrated in OLP lesion, and GNLY is a small molecule found in CTL and NK cells, these indicate that with an increase in

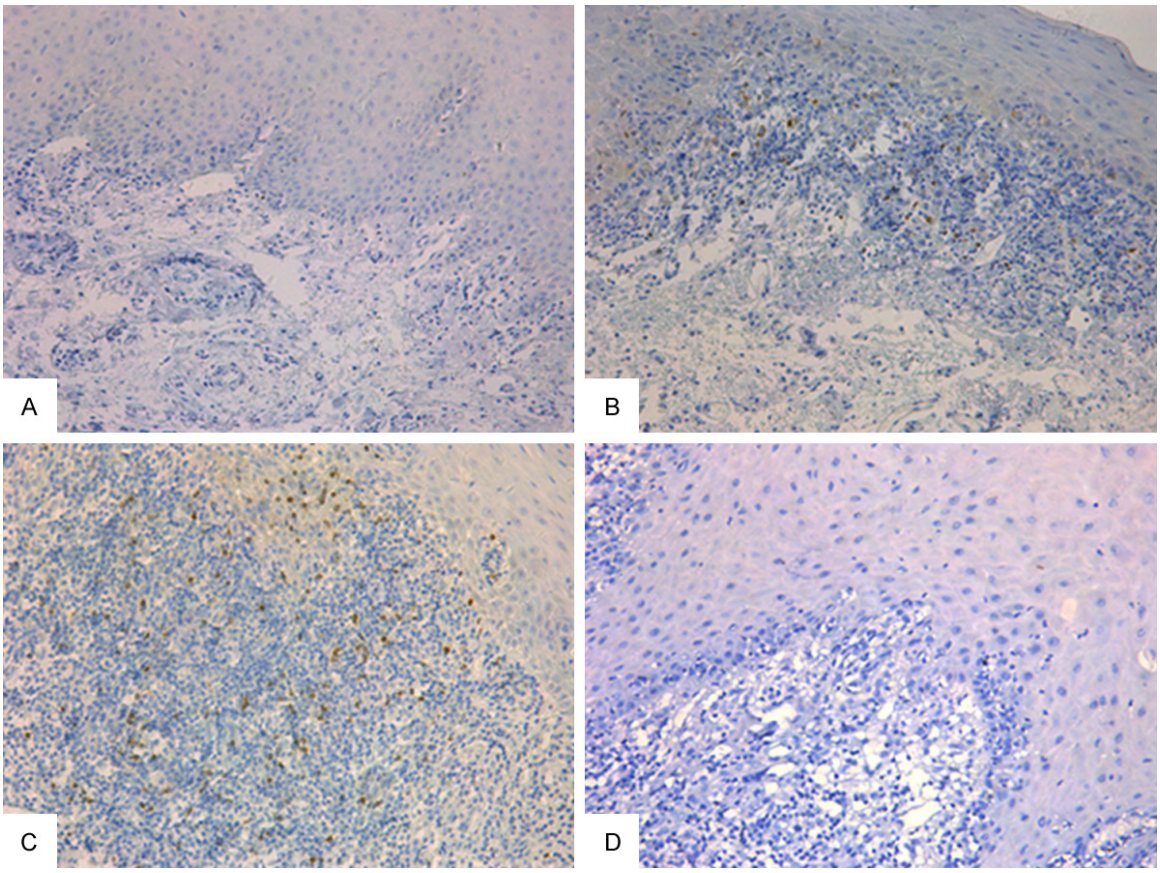

Figure 4. Immunohistochemistry: (A) Non-erosive OLP lesion, (B) Erosive OLP lesion, (C) Normal oral mucosa, (D) Negative control (× 200).

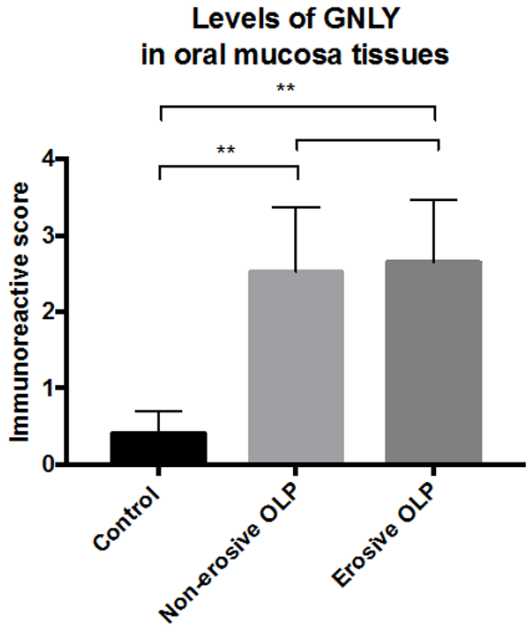

Figure 5. Immunoreactive score: the expression level of GNLY in non-erosive and erosive OLP lesion was significantly higher that than in the normal oral mucosa (**P < 0.01).

Table 4. Immunoreactive score of GNLY (mean ± SD)

	Control group	Non-erosive OLP	Erosive OLP
n	20	20	20
GNLY	0.41 ± 0.28	2.52 ± 0.85	2.65 ± 0.81

the activated lymphocytes of an OLP lesion, more GNLY are secreted which may be associated with the pathologic changes of OLP. OLP is a chronic disease of the mucous membranes, and colloid bodies is one crucial histologic feature of OLP. Ultrastructural studies have confirmed that the colloid bodies are apoptotic keratinocytes, but the specific pathway of keratinocyte apoptosis in OLP has not been clear. Our study found that GNLY had higher expression in the oral mucosal tissue of OLP, which concurred with the result of skin lichen planus. Granzyme B and GNLY were significantly highly expressed in OLP lesions compared with normal skin [27]. We hypothesized that the activated T lymphocytes release GNLY and other

cytokines and mediate the apoptosis of keratinocytes, which might partly explain the pathogenesis of OLP.

In this paper, we studied the expression of GNLY in OLP. According to the experiments, we draw the following conclusions: the increasing GNLY in the peripheral blood and tissues of OLP suggested that GNLY might be involved in the pathogenesis of OLP. The microcirculation in the local microenvironment of OLP might cause the disease in a state of chronic inflammation, which resultsin a prolonged course and incurability of this disease.

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Disclosure of conflict of interest

None.

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