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Original Article

MiR-127 regulates the development of oral pemphigus by targeting IL-6

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Abstract: Aims: This study is to investigate role and mechanism of IL-6 in the development of pemphigus vulgaris (PV). Methods: Oral mucosal tissues, serum and saliva were collected from 65 patients with oral PV between February, 2013 and October, 2015. Normal samples from 38 healthy people were collected as control. The IL-6 mRNA expression in these samples was examined by quantitative Real-time PCR and its protein level was analyzed by Western Blot and ELISA. MiRNAs that regulate IL-6 expression was predicted by bioinformatics analysis, and validated by Dual luciferase reporter assay. Results: Compared with healthy controls, IL-6 was significantly upregulated at both mRNA and protein levels in oral mucosal tissues, serum and saliva samples from patients with oral PV ($P<0.05$). The 3'UTR of IL-6 mRNA was predicted to have a miR-217 binding site and IL-6 was confirmed as a direct target of miR-217 by Dual luciferase reporter assay. MiR-217 expression was significantly downregulated in samples from PV patients ($P<0.05$). Conclusion: The IL-6 expression in PV patients may be directly regulated by miR-217.

Keywords: MiRNA-217, oral, pemphigus vulgaris (PV), IL-6

Introduction

Pemphigus, characterized by blisters, is a severe autoimmune disease with high mortalities. In some cases, these blisters can cover a significant area of the skin [1]. Pemphigus greatly endangers physical and psychological health of patients and even poses threaten to life [2].

There are four types of pemphigus: pemphigus vulgaris (PV), pemphigus vegetans, pemphigus foliaceus, and paraneoplastic pemphigus [3]. PV is the most common and most severe form of the disorder [4]. The complications of PV are severe and even fatal due to the use of immunosuppressive drugs [5]. Several studies have reported the relationship between Th17 cytokines, such as IL-17, IL-6, IL-22, IL-23, and autoimmune diseases including rheumatoid arthritis, multiple sclerosis and erythematosus lupus [2, 6-9]. IL-6, as an important factor in immune responses, is secreted by activated monocytes and tissue macrophages to promote the maturation of pre-B cells. IL-6 also regulates the proliferation and differentiation of hematopoietic cells from several lineages at different maturation stages, as well as enhances the cytotoxicity of NK cells in synergy with colony stimulating factors [10-12]. However, little is known about the role and regulation of IL-6 in PV until now.

In the present study, Real-time PCR, Western blot and ELISA were used to analyze changes of IL-6 expression in oral mucosal tissues, serum and saliva from patients with PV at both mRNA and protein level. IL-6 was predicted as a target of miR-217 by bioinformatics prediction and verified by Dual luciferase report assay. The regulation mechanism of miR-217 and IL-6 in PV was discussed.

Material and methods

Patients

A total of 65 patients with PV that were first diagnosed and 38 healthy controls from Stomatological Hospital Affiliated to Sichuan Medical University between February, 2013 and October, 2015 were enrolled in the study. A total of 38 male PV patients and 27 female PV

Table 1. Primers used for Real-time PCR

Primers	Primer sequence (5'-3')
IL-6_Forward	5'-GGCACTGGCAGAAAACAACC-3'
IL-6_Reverse	5'-GCAAGTCTCCTCATTGAATCC-3'
GAPDH_Forward	5'-GGGAAACTGCGGCGTGAT-3'
GAPDH_Reverse	5'-AAAGGTGGAGGAGTGGGT-3'
U6_Forward	5'-GCTTCGGCAGCACATATACTAAAAT-3'
U6_Reverse	5'-CGCTTCACGAATTTGCGTGTCAT-3'
miR-217_Forward	5'-CGCTCTACTGCATCAGGAACTGA-3'
miR-217_Reverse	5'-GTGCAGGGTCCGAGGT-3'

patients were included, and the median age of patients was 61 (ranging from 27 to 86). A total of 28 male and 10 female healthy controls were included, and the median age of patients was 59 (ranging from 30 to 82). All subjects with complications in organs such as heart, liver and kidney, diabetes, tumors and autoimmune diseases were excluded. Healthy controls were volunteers who had no abnormalities in oral examinations. Prior written and informed consent were obtained from every patient and the study was approved by the ethics review board of Sichuan Medical University.

Sample collection

Oral mucosal tissues from patients (pathological tissues) and healthy controls (normal tissues) were collected and stored liquid nitrogen until analysis.

Serum was isolated from sterile fasting peripheral blood. In general, 10-15 mL of peripheral blood were placed in 4°C for 1-2 hr, and serum in the upper layer were aspirated and centrifuged for 10 min at 400× g. Serum was aliquoted and stored at -70°C.

Saliva samples were collected at the day of endoscopic examination by the standard method. Briefly, sterile cotton swabs were moisture by 2% citric acid solution and put on the posterolateral side of tongue for about 5 s and saliva were collected, followed by another side of tongue. This procedure was repeated by several times until 5 mL saliva was collected. Subjects were instructed to not drink, eat or smoke and oral hygiene procedures were performed at least 2 hours before the collection.

RNA isolation and quantitative real-time PCR

Total RNA was extracted by Trizol Reagent (Yusheng Biotech, Shanghai, China). MiRNAs were extracted by miRcute miRNAisolation kit (Tiangen Biotech Co., Ltd., Beijing, China) form cells and by miRNeasy Serum/Plasma Kit (Jianlun Biotech, Guangzhou, China) form serum. Following gel electrophoresis verification of RNA integrity and quantification using UV spectrophotometer, mRNA was reverse transcribed by TIANScriptIIcDNAkit (Tiangen Biotech Co., Ltd.), and miRNA was reverse transcribed by miRcute miRNA cDNAkit (Tiangen Biotech Co., Ltd.).

Quantitative real-time PCR was performed using a miRcute miRNA qPCR detection kit (Tiangen Biotech Co., Ltd.) for miRNAs and SuperRealPreMix (SYBR Green) (Tiangen Biotech Co., Ltd.) for mRNA according to the manufacturer's instruction on IQ-5 (Bio-Rad Laboratories, Hercules, California, USA). For mRNA quantification, the reaction mixture was incubated for 1 cycle at 95 C for 5 min, followed by 45 cycles at 95°C for 5 s, 57 C for 30 s. For miRNA, the reaction mixture was incubated for 1 cycle at 95 C for 3 min, followed by 40 cycles at 95 C for 12 s, 62 C for 40 s, and 72°C for 20 s. Primers used for detection was shown in **Table 1**. The relative expression levels were evaluated using the $2^{-\Delta\Delta Ct}$ method, and the expression of GAPDH or U6 snRNA was used as internal control for mRNA and miRNA, respectively.

Western blot

Proteins were extracted by protein extraction kit (BestBio, Shanghai, China) and determined by BCA assay kit (Zhongkeruitai, Beijing, China). A total of 20 μg proteins were separated on 10% SDS-PAGE and transferred to polyvinylidene difluoride membranes (PVDF) at 100 V for 2 h. After blocking with 5% skimmed milk, the membranes were probed with the following antibodies: rabbit anti-IL-6 antibody (1:1000, ab6672, Abcam, Cambridge, UK) and rabbit anti-β-actin monoclonal antibody (1:5000, ab6276, Abcam). For detection, goat anti-rabbit (1:3000, ab6721, Abcam) secondary antibodies conjugated to HRP (Abcam) were used. Signal detection was performed using chemiluminescence reaction (ECL) (ab65623, Abcam, GA, USA). The acquired images were analyzed by Image lab 3.0 (Bio-Rad Laboratories) and the relative protein expression was expressed as the densitometric value ratio of IL-6 to β-actin.

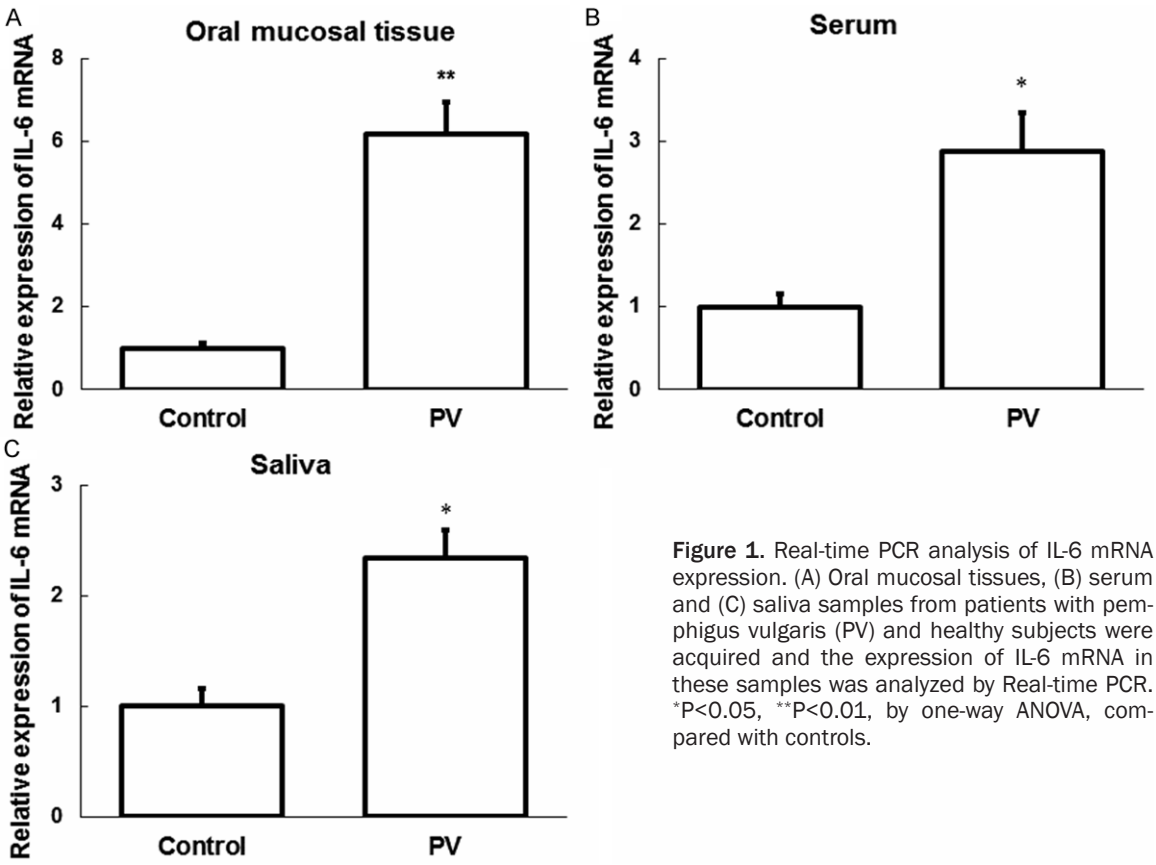

Figure 1. Real-time PCR analysis of IL-6 mRNA expression. (A) Oral mucosal tissues, (B) serum and (C) saliva samples from patients with pemphigus vulgaris (PV) and healthy subjects were acquired and the expression of IL-6 mRNA in these samples was analyzed by Real-time PCR. *P<0.05, **P<0.01, by one-way ANOVA, compared with controls.

ELISA

IL-6 protein levels in serum were measured by IL-6 ELISA kit (ab178013, Abcam, GA, USA). In brief, 1:4 dilution of serum samples and eight serial dilutions of standard substrate at the volume of 50 μl were incubated overnight at 4°C. After horseradish-peroxidase (HRP) conjugated secondary antibody incubation for 1 hr followed by washing for 5 times, the chromogenic substrate solution was added and the reaction was stopped with H_2SO_4 and read at 450 nm.

Bioinformatics analysis

To further identify the miRNAs that may regulate the expression of IL-6, five bioinformatics software (miRanda, TargetSean, PicTar, MiRanda and BibiServ) were used.

Dual-luciferase reporter gene assay

According to the results of bioinformatics prediction, the conservative miR-217 binding sequence on 3'UTR of IL-6 mRNA was cloned. Luciferase reporter plasmids were generated by insertion of wildtype or mutant binding sequences of IL-6 into the multiple cloning site (Spe-1 and HindIII) downstream of the luciferase reporter gene in the pMIR-REPORT™ Luciferase (ThermoFisher Scientific, Vilnius, Lithuania, USA). HEK293T cells were transfected with 0.8 μg constructed luciferase reporters and 100 nM miR-217 mimics or negative control RNA (NC). A total of 10 ng pMIR-REPORT™ β-gal Control Plasmid was transfected as an internal control for transfection efficiency. Luminescence was measured 24 h after transfection using Dual-luciferase detection kit (Promega, Madison, WI, USA) according to the manufacturer's instructions. Measurements of luminescence were performed on the luminometer (Glomax 20/20, Promega).

Statistical analysis

Data analysis was carried out using the SPSS 18.0 (IBM Corp, Chicago, IL, USA) and expressed as mean ± SD. Normal test was performed. Differences between groups were evaluated for significance using one-way ANOVA. LSD or SNK methods were used when there was homoge-

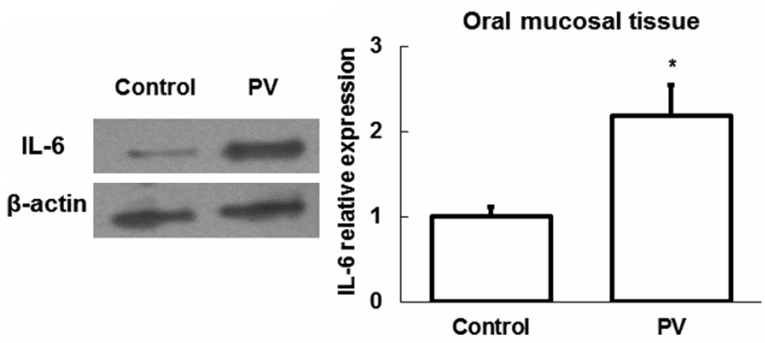

Figure 2. Western blot analysis of IL-6 protein expression. Oral mucosal tissues from patients with PV and healthy controls were acquired and the expression of IL-6 protein was analyzed by Western blot. Quantification of Western blot was shown in right panel. *P<0.05 by one-way ANOVA, compared with controls.

neity of variance; otherwise, Tamhane's T2 or Dunnett's T3 methods were used. P<0.05 was considered as significant.

Results

IL-6 mRNA expression is upregulated in patients with PV

Oral mucosal tissues, serum and saliva were collected from patients with PV and healthy subjects, and the expression of IL-6 mRNA was analyzed by quantitative Real-time PCR. Compared with that in normal controls, IL-6 mRNA expression in the three samples was significantly upregulated (P<0.05, **Figure 1**) in PV patients, suggesting a regulatory role of IL-6 in the development of PV.

IL-6 protein is upregulated in patients with PV

Expression of IL-6 was next analyzed at the protein level by Western blot in oral mucosal tissues from PV patients and healthy subjects. IL-6 protein level was also upregulated in PV patients compared with healthy controls (P< 0.05, **Figure 2**), indicating that upregulated IL-6 expression may regulate the development of PV. We further investigated the expression of IL-6 protein in serum and saliva by ELISA and found that samples from PV patients had higher levels of IL-6 (P<0.05, **Figure 3**).

These results showed that in all three samples, IL-6 was upregulated at both mRNA and protein level. Since IL-6 was mainly produced by blood cells [13], upregulated IL-6 expression in serum was likely induced by PV. In addition, ectopic IL-6 expression in saliva of patients indicated that IL-6 may be used as a potential biomarker for PV.

IL-6 is a target of miR-217

To investigate the possible mechanism that regulating IL-6 expression, miRNAs that may bind to the 3'UTR of IL-6 was predicted using bioinformatics methods. A miR-217 binding site was predicted at the 3'UTR of IL-6 mRNA (**Figure 4A**). To confirm this prediction, dual luciferase reporter assay was performed. Co-transfection of miR-217 mimic and pMIR-REPORT-IL-6 wildtype construct significantly decreased luciferase activity (P<0.05) compared with co-transfection of NC and pMIR-REPORT-IL-6 wildtype construct. However, miR-217 mimic and pMIR-REPORT-IL-6 mutant construct co-transfection did not reduce luciferase activity (**Figure 4B**). These results demonstrate that IL-6 is a target of miR-217 and the expression of IL-6 is likely regulated by miR-217.

MiR-217 was downregulated in patients with PV

As IL-6 mRNA was confirmed to be regulated by miR-217, expression of this miRNA was investigated by Real-time PCR in oral mucosal tissues, serum and saliva from PV patients and normal controls. We found that miR-217 expression was significantly downregulated in samples from PV patients (**Figure 5**). Collectively, miR-217 may contribute to the regulation of PV by targeting IL-6 mRNA and subsequent modulation of IL-6 protein level.

Discussion

In this study, we observed expression of IL-6 mRNA and IL-6 protein in oral mucosal tissues, serum and saliva from patients with PV. We also observed expression of the IL-6 upstream miRNA, miR-217, in these samples and confirmed the binding of miR-217 to IL-6 by Dual luciferase reporter assay.

PV is a complicated oral disease mediated by circulating autoantibodies directing against keratinocyte cell surfaces, but the precise

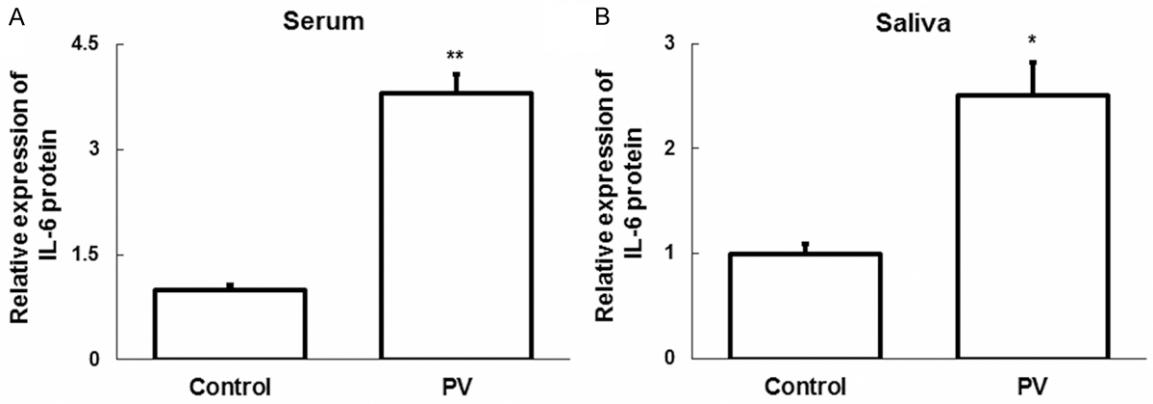

Figure 3. ELISA analysis of IL-6 protein expression. (A) Serum and (B) saliva samples from patients with PV and healthy controls were acquired and the expression of IL-6 protein in these samples was analyzed by ELISA. *P<0.05, **P<0.01, by one-way ANOVA, compared with controls.

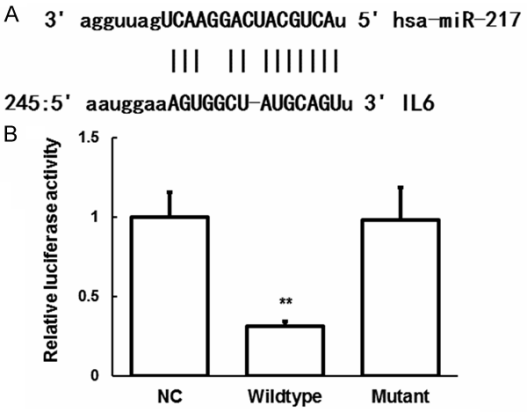

Figure 4. IL-6 is a possible target of miR-217. A. The results of bioinformatics software showing the binding of miR-217 to the 3'UTR of IL-6 mRNA. B. The wildtype *IL-6* 3'UTR luciferase reporter construct (wild type) or mutant *Otx2* 3'UTR luciferase reporter construct (mutant) was co-transfected with miR-217 mimic, and wild type *IL-6* 3'UTR luciferase reporter construct was co-transfected with NC (NC). Luciferase activities were assayed 24 h post transfection. **P<0.01, by one-way ANOVA compared with NC.

cause of the attack by the immune system is unknown [14]. High dose of glucocorticoids in synergy with other adjuvant treatment is a potentially effective therapy at early stage to reduce mortality rate to under 5% [15]. However, adverse reactions and complications may occur due to the high dose of drugs as well as long term of treatment. In addition, blistering can lead to life-threatening fluid loss, infection, and disfigurement, which further cause severe immunodeficiency in patients with PV. Glucocorticoids and immunosuppressive drugs may further suppress immunity in patients, leading to the most common complication namely infection that severely influences treatment and life quality of patients [16]. Therefore, investigation the development of PV in the molecular level has profound meaning for clinical treatment.

IL-6 is a multi-functional cytokine that is upregulated in immune responses by bacteria, endotoxin and molten dust particle [17]. IL-6 has important functions in immune responses, inflammation, cell differentiation, coagulation, and the development of tumor and it is significantly upregulated in inflammatory responses induced by damage, trauma, stress, and infection [18]. IL-6 may induce C-reactive protein and fibrinogen, etc., and may promote thrombosis in inflammation [19]. Increased IL-6 in body could cause the occurrence of inflammatory diseases, such as rheumatoid arthritis and Crohn's disease, by binding to IL-6 receptor [20]. In rheumatoid arthritis, IL-6 can stimulate T and B lymphocytes to secrete inflammatory mediators, and may promote maturation of B lymphocytes in addition to the effect of IL-1β and TNF-α [21]. IL-6 also has chemotactic effect to other inflammatory cells, such as neutral lymphocytes and monocytes macrophages during inflammatory responses [22]. These reports illustrated the important role of IL-6 in inflammatory responses. Here in this study, we found that IL-6 was upregulated in oral mucosal tissues of PV patients compared with that in normal subjects. The change of IL-6 gene expression was also observed in serum and saliva, two samples usually called body fluid specimen. Therefore, upregulation of IL-6 in

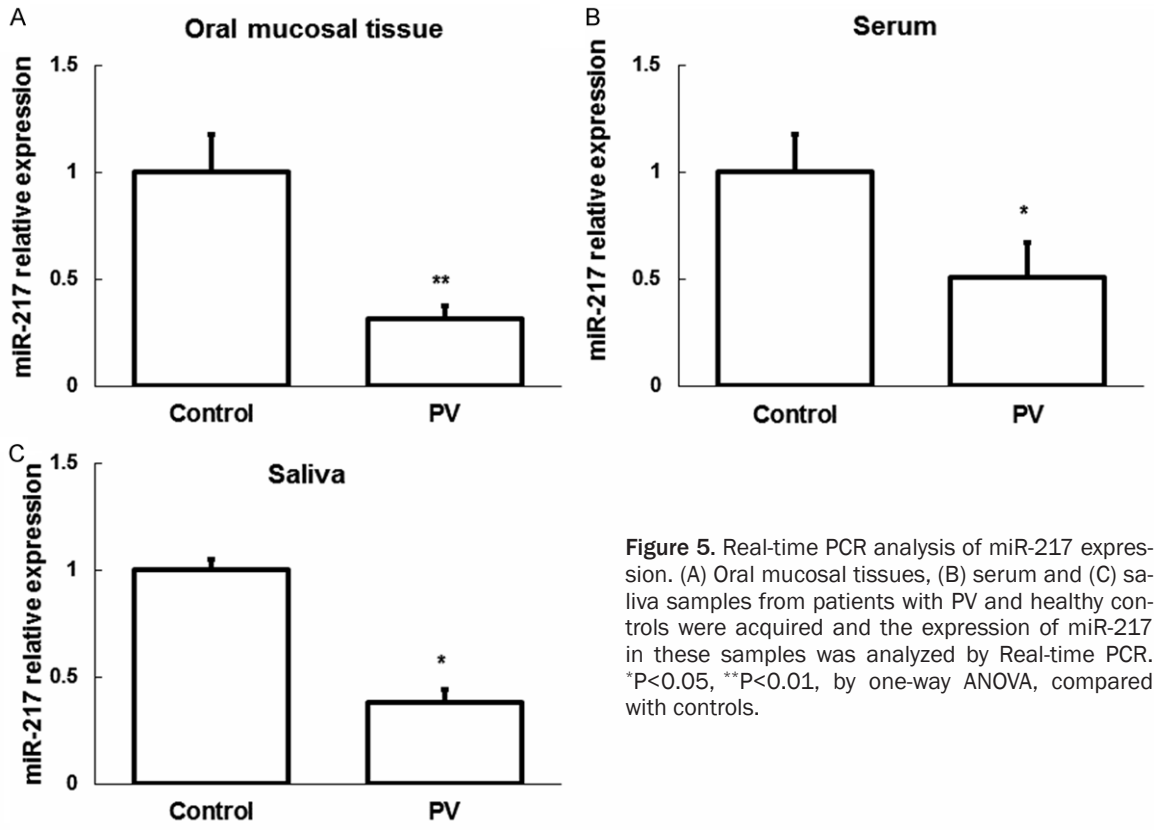

Figure 5. Real-time PCR analysis of miR-217 expression. (A) Oral mucosal tissues, (B) serum and (C) saliva samples from patients with PV and healthy controls were acquired and the expression of miR-217 in these samples was analyzed by Real-time PCR. $^{*}P<0.05$, $^{**}P<0.01$, by one-way ANOVA, compared with controls.

these samples may have the potential to be used as biomarker for PV, especially at the early stage of PV.

MiRNA is a class of non-coding small RNA molecules ubiquitously expressed in eukaryotes at the length of 18-22nt that regulate protein expression at the mRNA level [23-25]. Several miRNAs were reported to regulate the expression of IL-6. For instance, miR-365 negatively regulated IL-6 expression in HEK293 and Hela cells [26]. By bioinformatics prediction we found a miR-217 binding site at the 3'UTR of IL-6 mRNA, indicating miR-217 as a regulator of IL-6 expression. MiR-217 was found to be downregulated in PV patients with the upregulation of IL-6 mRNA and protein, suggesting a role of miR-217 in the development of PV. Results of In vitro dual luciferase report assay showed that miR-217 could directly bind to the 3'UTR of IL-6 mRNA. These results together indicate that miR-217 may affect the pathology of PV by regulating IL-6 expression.

Taken together, we believe that IL-6 and miR-217 had important biological functions in the development of PV. Further studies are needed to investigate the function of miR-217 and IL-6 in PV, providing new ideas for the prevention and treatment of this disease clinically.

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Disclosure of conflict of interest

None.

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