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Decreased PLUNC expression in nasal polyps is associated with multibacterial colonization in chronic rhinosinusitis patients

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Abstract PLUNC (palate, lung, and nasal epithelium clone) is an epithelium-secreted protein that plays a crucial role in the host's defense against bacterial infection. The function of PLUNC in the sinus remains poorly understood. To examine whether the expression levels of PLUNC could serve as a predictive outcome biomarker for patients with CRSwNP and bacterial colonization, we investigated the association of PLUNC expression levels with bacterial colonization in the sinuses. A total of 174 patients who underwent sinus surgery for chronic rhinosinusitis with nasal polyps (CRSwNP) were enrolled in this study. The tissue samples obtained from patients were examined using preoperative sinus computed tomography (CT) scans, postoperative bacterial cultures, and nasal polyp examinations. PLUNC mRNA and protein expression were quantified using RT-PCR and immunohistochemistry. We identified that decreased PLUNC expression is associated with multibacterial colonization ($P = 0.0001$), specifically those mediated by *Staphyloccocus aureus* ($P = 0.037$) and *Pseudomonas aeruginosa* ($P = 0.002$). The patients who required repeated sinus surgeries for recurrent or persistent sinusitis also presented much lower PLUNC expression than those who did not require repeated sinus surgery ($P = 0.001$). However, gender, age, and CT scores were not associated with PLUNC expression. These results suggest that reduced PLUNC expression is associated with bacterial colonization as well as treatment outcome in CRSwNP patients. Investigation of the association between PLUNC expressions and chronic rhinosinusitis may lead to the development of a novel biomarker for treatment outcome in CRSwNP patients.

Keywords PLUNC · Sinusitis · Nasal polyposis · Bacterial colonization · Diagnosis

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Introduction

PLUNC (short palate, lung, and nasal epithelium clone), a 25-kDa protein that is a member of the bactericidal/permeability-increasing protein (BPI) family, is found to play an important role in host defense against microbes [1]. Several reports have shown that PLUNC is expressed specifically in the nasopharyngeal and respiratory epithelium [2, 3]. Moreover, the amino acid sequence of PLUNC is highly similar to those of secretory proteins produced by salivary glands and glandular epithelium of the trachea and the parotid secretory protein [4]. The biological function of PLUNC is not well understood. However, its structure is similar to BPI family proteins; hence, PLUNC might be

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involved in defense responses in the airways [5]. PLUNC is secreted by human and mouse bronchial epithelial cells, where it acts to reduce the *Mycoplasma pneumoniae* population and decreases interleukin (IL)-8 production in airway epithelial cells [6]. Moreover, PLUNC is known to harbor a novel antibacterial protein that protects against *Pseudomonas aeruginosa* infection and likely plays a critical role in airway epithelium-mediated innate immune responses [5]. These evidences suggest that PLUNC may serve as a novel host-defense protein against bacterial infection, and reduced PLUNC may contribute to the persistent nature of bacterial colonization in the airway. A recent report has shown that PLUNC may inhibit biofilm formation by decreasing surface tension of airway secretions rather than exerting a direct antimicrobial action [7]. However, the mechanism of antimicrobial activity by PLUNC remains unclear, and the role of PLUNC in rhinosinusitis requires further investigation.

Chronic rhinosinusitis with nasal polyposis (CRSwNP) patients are more refractory to surgical cure [8, 9]. Many patients require revision sinus surgery for persistent nasal disease or rapid recurrent sinonasal infections. The high post-surgery recurrence and long-term antibiotic treatment may be due to a poor understanding of the cause of paranasal sinusitis [10]. Although a recent study has indicated a positive relationship between PLUNC and lower airway disease, such as asthma and chronic obstructive pulmonary disease (COPD) [11], the association of PLUNC expression level and chronic rhinosinusitis requires further investigations. In this study, we first assessed the expression levels of PLUNC in CRSwNP patients. Further, we carried out microbiological investigation of various bacterial infections and categorized them using the expression levels of PLUNC. The identification of the relationship between upper airway diseases and PLUNC may elucidate the underlying cause of nasal polyposis and paranasal sinusitis.

Materials and methods

Patient selection and sample collection

Nasal tissues were obtained from 174 immune-competent patients with CRSwNP who had undergone sinus surgery at China Medical University Hospital (Taichung, Taiwan) during the period from January 2008 to December 2010. This patient population included 66 female and 108 male patients. The age of the patients ranged from 19–56 years (mean age 33.3 ± 10.4 years). Patients with sinonasal polyposis who received medical treatment, including intranasal steroid and/or antibiotics for over 3 months, but still complained of nasal polyps were submitted to functional endoscopic sinus surgery. Sinus cultures were routinely obtained from the osteomeatal complex during the sinus surgery. The specimens were streaked across Tryptic soy agar (Becton–Dickinson, Franklin Lakes, NJ, USA) and incubated at 37 °C for 18–24 h. Microorganisms were identified using a BD Phoenix™ Automated Microbiology System (Becton–Dickinson) as described previously [12].

To measure the RNA and protein expression levels of PLUNC in each sample, tissue real-time PCR (RT-PCR) and immunohistochemistry, respectively, were performed. The data were analyzed to identify any potential correlation between PLUNC expression and age, gender, sinus computed tomography (CT) score, sinus cultures, or postoperative follow-up [13]. Patients who had undergone repeated sinus surgeries were included in the study if they had experienced recurrent sinonasal polyposis or persistent sinusitis disease after the initial operation, and postoperative antibiotic treatments had failed for at least 3 months. This study was approved by the Ethics Committee of the China Medical University Hospital prior to patient enrollment (DMR101-IRB1-135). All participants provided informed consent.

Immunohistochemical staining for PLUNC

A total of 17 CRSwNP patients with or without bacterial infection were randomly selected for immunohistochemical (IHC) analysis. Paraffin-embedded nasal polyp samples were prepared and sectioned as described previously [14]. The tissue sections were deparaffinized and rehydrated. After blocking with 3 % BSA, a mouse monoclonal antibody against PLUNC (Santa Cruz Biotechnology, Santa Cruz, CA, USA) was added to the tissue sections. The samples were then incubated for 24 h at 4 °C. After being washed in PBS, the samples were probed using peroxidase-labeled goat anti-mouse secondary antibodies (Santa Cruz Biotechnology) and detected with an ABC kit (Vector Laboratories, Burlingame, CA, USA). The stained tissues were then observed and scored the percentage of cells with the expression of PLUNC. The scoring system was subjected to determine the expression levels of PLUNC and read by two independent pathologists [15].

Preparation of tissue RNA and quantitative RT-PCR

Frozen samples from patient nasal polyp tissue were transferred to a tube with MagNA Lyser Green Beads (Roche, Indianapolis, IN, USA) pre-cooled on ice. Total RNA was isolated from tissues using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Samples were cooled on ice for 1 min between the two processing steps. The total RNA from homogenized samples was prepared according to the manufacturer's instructions (Invitrogen). Quantitative real-time reverse transcription PCR was performed as

described previously with slight modifications [16]. In brief, 0.5 μg of total RNA was reverse transcribed into cDNA through the use of an oligo (dT) primer. The oligonucleotide primers used corresponded to human PLUNC (forward, 5′-AGTCTGTTGAGGCTGGCTGT-3′; reverse, 5′-CAAGATCCCTGTGAGGCTGT-3′) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (forward, 5′-ATGCTGGCGCTGAGTAC-3′; reverse, 5′-TGAGTC CTTCCACGATAC-3′). All oligonucleotide primers were synthesized by Invitrogen. Quantitative RT-PCR using SYBR green I master mix and a model 7900 sequence detection system was conducted according to the manufacturer's instructions (Applied Biosystems, Foster City, CA, USA). After preincubation at 50 °C for 2 min and 95 °C for 10 min, PCR was performed with 45 cycles of 95 °C for 10 s and 60 °C for 1 min. The threshold was set above the non-template control background and within the linear phase of target gene amplification to calculate the cycle number at which the transcript was detected (i.e., the threshold cycle [C_T]).

Statistical analysis

Between-group comparisons were performed using the Chi-square test. The difference was considered significant when the P value was less than 0.05. Statistical analyses were carried out using the SPSS program (version 13.0; SPSS Inc., Chicago, IL, USA).

Results

Association of PLUNC expression levels with various clinical factors

A total of 174 patients (66 women and 108 men) were enrolled in this study. The ages of the patients ranged from 19 to 56 years. Patients with CRSwNP were diagnosed based on the observation of clinical symptoms and a CT evaluation of the paranasal sinuses. Nasal polyps were identified by sinoscopy during nasal examinations. A comparison of higher (upper quartile of population) and lower (lower quartile of population) PLUNC mRNA expression with clinical factors is presented in Table 1. Our data showed that lower as compared to higher PLUNC expression was associated with an increased incidence of bacterial colonization ($P = 0.002$). This correlation was strongest for multibacterial colonization ($P = 0.0001$). The patients who required repeated sinus surgeries for recurrent or persistent sinusitis presented much lower PLUNC expression than those who did not require repeated sinus surgery ($P = 0.001$). However, PLUNC expression levels were not associated with Lund-Mackay CT scores, age, or gender.

Table 1 PLUNC expression in CRSwNP patients

Clinical studies	Higher PLUNC expression (upper quartile, $n = 43$)[a]	Lower PLUNC expression (lower quartile, $n = 43$)[a]	P value[b]
Age			
>45	23	22	1.000
≤45	20	21	
Gender			
Male	27	28	1.000
Female	16	15	
Bacterial culture >1			
Yes	18	33	**0.002**
No	25	10	
Bacterial culture >2			
Yes	12	30	**0.0001**
No	31	13	
Lund-Mackay CT score ≤12			
Yes	30	34	0.607
No	13	9	
Repeated sinus surgery[c]			
Yes	2	16	**0.001**
No	41	27	

[a] Higher expression: PLUNC mRNA expression in the upper quartile of all patients. Lower expression: PLUNC mRNA expression in the lower quartile of all patients

[b] Significant differences are represented in boldface

[c] Repeated sinus surgery was defined as revision for recurrent or persistent disease within 6 months

Reduced PLUNC expression levels is associated with bacterial colonization in patients with sinusitis

We investigated the association between PLUNC expression levels and various bacterial colonizations. Our data showed that lower PLUNC expression levels are associated with colonization by *Staphyloccocus aureus* ($P = 0.037$) and *Pseudomonas aeruginosa* ($P = 0.002$) (Table 2). However, no significant relation with other bacterial colonization could be identified. We then performed the immunohistochemical analysis to measure PLUNC expression in sinonasal tissues from 17 CRSwNP patients who were infected or un-infected with either *S. aureus* or *P. aeruginosa*. Our data showed that the group of CRSwNP without bacterial colonization had 0, 1, 2, and 6 patients at grades 0, 1, 2, and 3, respectively (Fig. 1; Table 3). However, the CRSwNP with bacterial colonization group had 3, 3, 2, and 0 patients, at grades 0, 1, 2, and 3, respectively. The results from these studies indicate that PLUNC expression levels were significantly higher in the respiratory epithelial cells of patients without bacterial

Table 2 The association of bacterial isolates with PLUNC expression

Bacterial isolates	Higher PLUNC expression (upper quartile, $n = 43$)[a]	Lower PLUNC expression (lower quartile, $n = 43$)[a]	P value[b]
Gram positive			
Staphylococcus aureus	9	19	**0.037**
Streptococcus pneumoniae	2	8	0.089
Gram negative			
Klebsiella pneumoniae	5	7	0.757
Moraxella catarrhalis	4	3	1.000
Haemophilus influenzae	2	4	0.676
Pseudomonas aeruginosa	2	14	**0.002**

[a] Higher expression: PLUNC mRNA expression in the upper quartile of all patients. Lower expression: PLUNC mRNA expression in the lower quartile of all patients

[b] Significant differences are represented in boldface

colonization as compared to those with bacterial colonization ($P = 0.00047$).

Discussion

PLUNC, a glycoprotein secreted by the surface epithelium and submucosal glands of the upper airway, is prominently expressed in the nasal secretions of healthy individuals [3, 17, 18]. In this study, we measured PLUNC expression in patients with CRSwNP. Our data showed that PLUNC expression was significantly reduced in the mucosal epithelia and submucosal glands in the patients with multibacterial colonization, particularly those mediated by *S. aureus* and *P. aeruginosa*. The patients who required repeated sinus surgeries for recurrent or persistent sinusitis also presented much lower PLUNC expression than those who did not require repeated sinus surgery. These results suggest that CRSwNP patients with reduced PLUNC expression might have immune defect in defeating bacterial infection, thus reduced PLUNC expression might facilitate recurrent *S. aureus* and *P. aeruginosa* infections in patients with CRSwNP.

In our study, we have observed that the patients who required repeated sinus surgeries for recurrent or persistent sinusitis presented much lower PLUNC expression. However, PLUNC expression levels were not associated with Lund-Mackay CT scores. Although Lund-Mackay score is widely used in assessment of chronic rhinisinusitis [19], however, whether CT scan stage alone can predict symptom outcomes or revision surgery is still controversial [19, 20]. Hopkins et al. has found no association between Lund-Mackay score and revision rates at 12 months, and can only found small but significant association at 36 months. However, because of our small sample size and retrospective cohort study that might lead to no significant result in our study. Hence, more cases are needed to correctly conclude the correlation of Lund Mackay scores and sinus revision rates in the future.

The reason for the repeated surgery is multifactorial. Surgery itself might contribute to the need of revision surgery. For example, the preserving or enlarging maxillary sinus ostium surgery will affect the surgery outcome [21]. In addition, the surgery itself in different extent will lead to different mucosa effects to sinus mucosa that will also possible causing different revision rates. Recently, "inflammatory load hypothesis" is considered to be the most important predictor of long-term outcome. Patients with a more extensive radical sinus surgery to reduce the inflammation load will lead to less revision rate and better outcome [9]. Microbial colonization, such as *S. areus*, has been suggested to work as a super-antigen that leads to nasal polyposis [22]. Furthermore, it has been indicated that the biofilm formation containing *S. areus* and *P. aeruginosa* is associated with an unfavorable outcome after sinus surgery [23]. Thus, the low tissue levels of PLUNC expression could be considered as a mucosa inflammatory measurement to assess the inflammatory load of sinus mucosa that might be due to *S. aureus* or *P. aeruginosa* colonization. Hence, patients with decreased PLUNC expression are more likely to experience poor outcomes and frequent recurrence, which often lead to repeated sinus surgeries. However, so far there is no related research has been conducted to investigate whether repeated surgery itself might actually contribute to the reduction of PLUNC expression level; thus further investigation is need to elucidate the possibility.

Numerous mechanisms underlie the interaction between PLUNC expression and inflammation in patients with microbial infections. Recent research suggested that PLUNC expression could be induced by microbial infection. There is evidence that PLUNC is secreted by neutrophils upon bacterial stimulation [24–26] Recently, PLUNC has been identified as an antimicrobial host-defense peptide that may contribute to airway health through both bactericidal and non-bactericidal mechanisms [27] PLUNC then alleviates inflammation by reducing the production of MIP2, IL-8, IL-6, and IL-1β [5, 6]. Notably, treatment with IL-13 also reduces the expression of PLUNC, which suggests that the allergic milieu or chronic inflammation may affect the host's innate immunity response by downregulating the secretion of PLUNC in

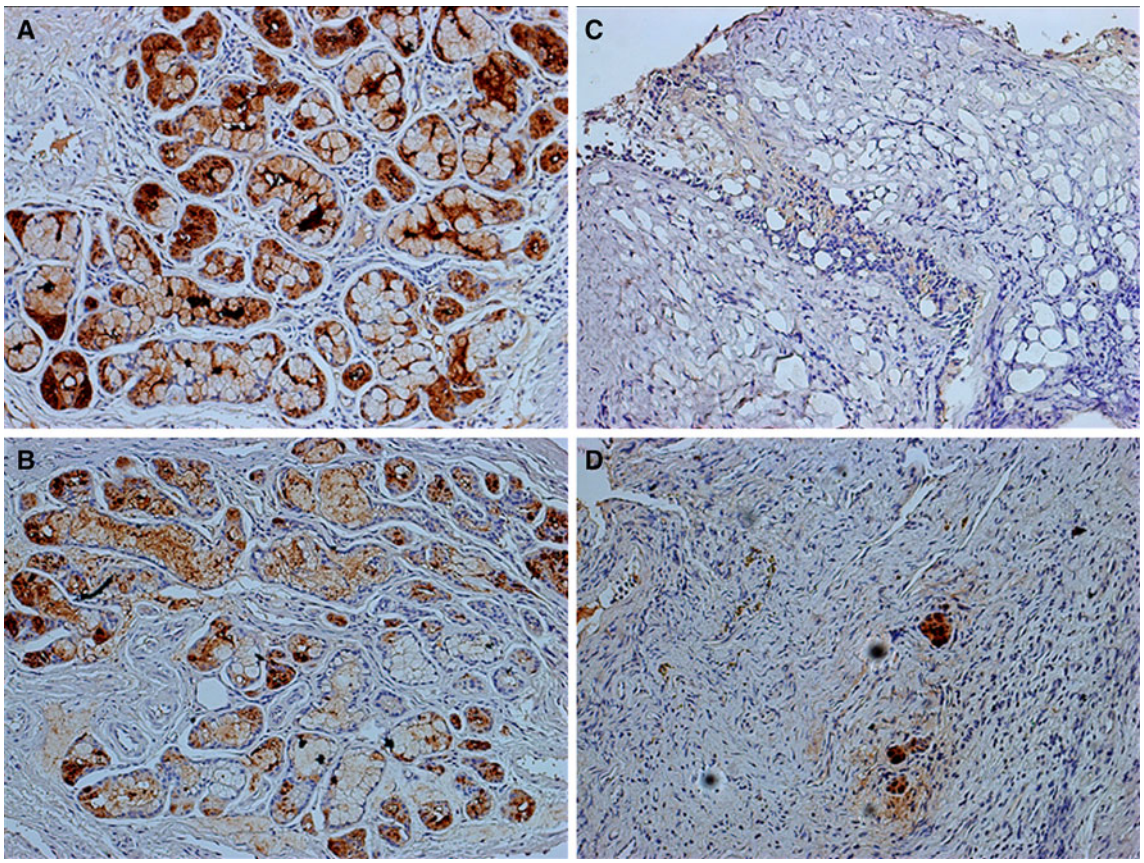

Fig. 1 Bacterial colonization is associated with reduced PLUNC expression in respiratory epithelium. Representative immunohistochemical staining of PLUNC expression in patients without bacterial colonization (**a**, **b**) and with bacterial colonization (**c**, **d**). The figures were photographed at ×200 magnification

Table 3 Distribution of immunohistochemical staining of PLUNC in CRSwNP biopsies

Biopsies[a]	% of positive cells (score)[b]				Total[c]
	0	1+	2+	3+	
CRSwNP without bacterial infection	0	1	2	6	9
CRSwNP with bacterial infection	3	3	2	0	8

[a] Tissues from 17 patients with CRSwNP were stained for PLUNC using IHC analysis

[b] The case with less than 10 % positive staining was determined as negative (grade 0); 10–30 % as grade 1+, 30–50 % as grade 2+, and more than 50 % as grade 3+

[c] $P = 0.00047$

bronchial and nasal polyp epithelial cells [6, 28] It has also be pointed out that a reduced number of submucosal glands may lead to reduced PLUNC expression in CRSwNP patients [29] thus it might be worth studying the relationship of the concentration of IL-13 or the number of submucosal glands with the expression levels of PLUNC in CRSwNP patients. In addition, one cannot rule out the possibility that low tissue levels of PLUNC is in fact related to infection by *S. aureus* and *P. aeruginosa*, that is *S. aureus* and *P. aeruginosa* might have evolved a mechanism to protect themselves from immune attack by downregulating the expression of PLUNC in the tissue, thus facilitate their colonization.

In conclusion, PLUNC might represent a novel predictive outcome biomarker for patients with CRSwNP and bacterial colonization. Continuous postoperative antibiotic use based on microbial culture reports and intensive local sinus treatment or sinus irrigation should be performed in patients with reduced PLUNC expression. Further research is warranted to elucidate the clinical utility of PLUNC for the treatment of patients with CRSwNP and sinus infections.

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Conflict of interest The authors have no conflict of interest and no financial disclosures.

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