Predictive significance of Charcot-Leyden Crystal mRNA levels in nasal brushing for nasal polyp recurrence*

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Abstract

Background: Tissue eosinophils have been shown to be associated with polyp recurrence in chronic rhinosinusitis with nasal polyps (CRSwNP). We addressed whether the mRNA levels of Charcot-Leyden Crystal (CLC) in nasal brushing samples, a molecule mainly released from activated eosinophils, could serve as an effective non-invasive biomarker to predict polyp recurrence.

Methods: A total of 51 patients with CRSwNP completing the postoperative follow-up over a period of 12-18 months were enrolled. Baseline CLC mRNA levels of the nasal brushings collected prior to endoscopic sinus surgery were quantified by quantitative real-time polymerase chain reaction (qRT-PCR). Polyp specimens were collected during surgery and were evaluated for inflammatory cells by histopathologic staining. The patients' baseline characteristics were reviewed and analyzed for associations with recurrence. Logistic regression analysis was performed to determine the predictive factors for polyp recurrence, and receiver operating characteristic (ROC) curves were performed to determine their predictive values.

Results: Overall, 25/51(49.02%) patients experienced polyp recurrence during the 12-18 months follow-up. The baseline relative CLC mRNA level in nasal brushing samples was significantly increased in patients with recurrence compared to those without recurrence ($p<0.001$). ROC curves demonstrated that a cut-off value of -6.419 for the relative CLC mRNA level normalized to GAPDH in nasal brushings predicted the recurrence with 92.00% sensitivities and 88.46% specificities (AUC=0.932, $p<0.001$).

Conclusions: The relative CLC mRNA levels in nasal brushings may serve as a reliable non-invasive biomarker to predict CRSwNP recurrence.

Key words: chronic rhinosinusitis with nasal polyps (CRSwNP), Charcot-Leyden Crystal (CLC), polyp recurrence, logistic regression analysis, receiver operating characteristic (ROC) curves

Introduction

Chronic rhinosinusitis with nasal polyps (CRSwNP) is a heterogeneous disease characterized by massive inflammatory cell infiltration[1, 2]. Although patients with CRSwNP benefit from the mainstay therapy of intranasal and/or oral corticosteroids and endoscopic sinus surgery (ESS)[1], the disease remains difficult to control. It has been reported that more than half of patients accepting ESS developed polyp recurrence during a minimum of 12 months of follow-up and approximately 30% of patients underwent the revision surgery[3].

Radical endoscopic sinus surgery (RESS)[4], long-term application of intranasal glucocorticoids after surgery[5] may prevent or delay polyp recurrence in CRSwNP patients. Thus identification of predictors for polyp recurrence is crucial as it may instruct the therapy and follow-up of nasal polyps. Several parameters, including tissue eosinophils, interleukin (IL)-5, preoperative

computed tomography (CT) score, nasal polyp size score, comorbid asthma, allergic status, and adhesion molecules expression, have been proposed as predictors of polyp recurrence[6-12]. Mucosal eosinophilia determined by immunopathology appears to be the most important predictor of them all[9, 11].

However, as the acquisition of the polyp tissue at baseline increases the risk of infection and bleeding[8, 11, 13], non-invasive sampling method should be developed. Nasal brushing sampling is a non-invasive nasal cytology method to detect nasal mucosal inflammation[14, 15]. Gröger et al.[16] have suggested eosinophilic granulocytes are mainly found in submucosal areas and cytobrush could gather high levels of the eosinophilic fraction in chronic nasal-sinus diseases, especially for patients with CRSwNP. Thus, molecules that could be detected in nasal brushing samples may serve as effective biomarkers to predict polyp recurrence.

Charcot-Leyden crystals (CLC), which belong to the galectin superfamily, are primary granules mainly released by activated eosinophils and basophils[17]. CLC has been identified in eosinophilic airway diseases such as asthma, allergic rhinitis and CRSwNP[17-19] and could reflect the severity of eosinophilic inflammatory airway diseases[20, 21]. Thus, we have hypothesized that CLC might be associated with outcome following nasal polyp surgery in patients with CRSwNP. In this retrospective study, we evaluated the relative mRNA levels of CLC in nasal brushings at baseline from patients with CRSwNP to assess the predictive value of CLC mRNA level for polyp recurrence. We further investigate the association between CLC mRNA level in nasal brushings and clinical characteristics of patients with CRSwNP.

Materials and methods

Subjects and study design

This was a retrospective study that used samples and data collected prospectively from patients with bilateral nasal polyps who had undergone ESS at Beijing TongRen Hospital, from October 2016 to June 2017. Diagnosis of CRSwNP was strictly confirmed according to the European position paper on rhinosinusitis and nasal polyps (EPOS) 2012[1]. Patients suffering from fungal sinusitis, choanal polyps or cystic fibrosis were excluded. None of the patients had been treated with topical or oral glucocorticoids, or other immunomodulatory drugs within 4 weeks before the surgery. We reviewed data of patients whose nasal brushings were collected at baseline and completing at least 12-month follow-up. A total of 51 patients satisfied the criteria and the medical records of whom were reviewed for preoperative demographic characteristics, experimental data, and medical history. Patients undergoing septoplasty for anatomic variations and without any other nasal diseases were recruited as control subjects. This study was approved by the Ethics Committee of Beijing TongRen Hospital and written informed consent was provided by each patient.

The baseline demographic and clinical characteristics were reviewed, CLC mRNA levels were evaluated in nasal brushing samples acquired from each subject prior to ESS, and samples obtained during surgery of polyp tissue specimens from CRSwNP patients and inferior turbinate mucosal tissues specimens from control subjects were processed for histologic evaluation. Postoperative follow-up for CRSwNP patients with several visits over a period of 12-18 months was performed for assessment of recurrence/non-recurrence of nasal polyps.

Clinical assessment

A total of 18 demographic and clinical characteristics; including age, gender, asthma, allergic rhinitis, atopy, preoperative symptoms self-assessment score, tissue and peripheral blood inflammatory cells proportion, Lund-Mackay score, and nasal polyp size score were reviewed for each patient as shown in Table 1. Asthma was diagnosed according to the Global Initiative for Asthma Guidelines (GINA) 2014[22] and allergic rhinitis was diagnosed according to the Allergic Rhinitis and its Impact on Asthma (ARIA) 2010 guidelines[23]. Atopy was confirmed based on positive test for serum antigen-specific IgE (cut-off value, 0.35kUA/L)[24], measured by Immuno-CAP 100 system (Pharmacia, Uppsala, Sweden). Nasal symptoms, including olfactory dysfunction, nasal obstruction, rhinorrhea, and headache/facial pain, were self-assessed by each patient and scored on a visual analogue scale (VAS) from 0 to 10, in which 0 indicated the absence of any symptom and 10 signified the presence of the most severe symptom. Nasal polyp scoring was evaluated by an independent observer (CW) for both nasal cavities in all patients by nasal endoscopy, and obtained a total polyp score according to the nasal polyp scoring system[25]. All patients underwent sinus computed tomography (CT) scanning (Philips Health Care, Best, the Netherlands) and the sinus CT images were scored according to the Lund-Mackay scoring system[26] by an otolaryngologist blinded to the clinical condition of patients. The data for peripheral blood counts were obtained using routine blood tests. All endoscopic surgeries were performed by the same surgeon to complete removal of nasal polyps (NPs) along with full maxillary antrostomy, total ethmoidectomy, wide sphenoidotomy and Draf IIA frontal sinusotomy (full-house functional endoscopic sinus surgery)[27].

Follow-up, postoperative evaluation and medical treatment

Postoperative follow-up included one visit weekly in the first month, followed by one visit monthly in the second to third months, and then one visit every 3 months. The follow-up was performed by the same surgeon (CW) who was blinded to all laboratory data. Recurrence was defined as the presence of nasal polyps observed under nasal endoscopy, together with at least one symptom (nasal obstruction, rhinorrhea, headache/facial pain, reduction or loss of smell, sleep disturbance/fatigue)

Table 1. Baseline characteristics of the participators .

	Control	CRSwNP		
		Recurrence	non-Recurrence	p-value
Number, (n)	20	25	26	-
Gender, (M/F)	10/10	14/11	16/10	0.688
Age, mean (SD)	43.7 (11.0)	42.4(9.9)	46.3(13.7)	0.246
Asthma, (Y/N)	0	15/10	7/19	**0.017**
Allergic rhinitis, (Y/N)	0	6/19	4/22	0.499
Atopy, (Y/N)	0	8/17	4/22	0.162
Olfaction score, median (IQR)	0.0(0.0-0.0)	10.0(7.0-10.0)	6.0(4.0-7.8)	**0.003**
Nasal obstruction score, median (IQR)	7.0(6.0-7.0)	7.0(6.0-9.0)	7.0(5.0-8.0)	0.153
Rhinorrhea score, median (IQR)	2.0(1.0-3.0)	5.0(4.0-6.0)	4.0(3.0-6.0)	**0.044**
Head/facial pain score, median (IQR)	2.0(0.0-3.0)	0.0(0.0-4.0)	1.0(0.0-4.8)	0.387
Tissue eos %, median (IQR)	0.00(0.00-1.93)	68.00(57.63-80.10)	11.38(4.42-25.23)	**<0.001**
Tissue neu %, median (IQR)	0.00(0.00-2.44)	0.00(0.00-6.00)	1.90(0.13-6.31)	0.046
Tissue lym %, median (IQR)	66.67(59.71-77.30)	12.01(8.09-26.28)	41.52(28.99-60.46)	**<0.001**
Tissue pla %, median (IQR)	28.79(19.51-38.13)	10.34(7.05-16.07)	26.47(19.22-42.97)	**<0.001**
Peripheral blood eos %, mean (SD)	2.08(1.36)	6.96(3.03)	3.69(2.42)	**<0.001**
Peripheral blood neu %, mean (SD)	59.05(5.44)	55.51(6.32)	54.82(6.77)	0.710
Peripheral blood lym %, mean (SD)	32.87(5.31)	31.25(5.35)	33.54(6.55)	0.179
Lund-Mackay score, mean (SD)	-	18.8(3.5)	16.1(4.3)	**0.015**
Nasal polyp size score, median (IQR)	-	4.0(4.0-5.0)	5.0(4.0-6.0)	0.109

SD: standard deviation; IQR: interquartile range; eos: eosinophil; neu: neutrophil; pla: plasma cell; lym: lymphocyte.

lasting at least 1 week, despite appropriate intranasal corticosteroid treatment[1].

Medical treatment was applied according to the inflammatory status evaluated by histopathological examination, as recommended[1]. Postoperatively, intranasal budesonide nasal spray (Rhinocort Aqua, AstraZeneca, Sweden) 128 µg twice a day was routinely used for at least 6 months for patients with eosinophilic inflammation or for 3 months for patients with non-eosinophilic inflammation, until the nasal symptoms were controlled. All patients were also treated with clarithromycin 250 mg daily for 3 months following surgery[28]. When nasal polyps or mucosal edema were observed again during follow-up, intranasal steroid therapy was commenced again as above. In cases where the symptoms could not be controlled adequately, oral tapering course of glucocorticoids and/or larger doses of intranasal glucocorticoids were administered to patients with eosinophilic mucosal inflammation; or clarithromycin 250 mg daily was administered to patients with neutrophilic inflammation[29].

Nasal brushing collection

Nasal brushing specimens were obtained prior to ESS. Nasal brush (Copan, Brescia, Italy) was pressed against the surface of the nasal polyp for CRSwNP patients or the inferior turbinate for the control subjects for 30 s and then, rotated for 3-4 full turns to acquire mucosal cells. After sampling, the brush was immediately placed into corresponding RNase free collection tubes and 1 mL TRIzol® Reagent (Invitrogen, CA, USA) was added. All samples were stored at -80 °C until RNA extraction.

RNA extraction, reverse transcription and quantitative real-time polymerase chain reaction (qRT-PCR) amplification

Total RNA in nasal brushing sample was extracted by the TRIzol® method following the manufacturer's instructions[30]. The quality of total RNA was assessed with the Nanodrop-2000 (Thermo Fisher Scientific, Waltham, MA, USA). Single-strand cDNA was synthesized with PrimeScript™ RT Master Mix (TaKaRa Biotechnology, Dalian, China), and aliquots of cDNA equivalent to 10 ng of total RNA in each well were used for qRT-PCR. The qRT-PCR was performed as described previously[31], briefly, with SYBR® Premix Ex Taq (TaKaRa Biotechnology) using an Applied Biosystems ViiA 7 Dx System (Applied Biosystems, Foster City, CA, USA) in 10 µL reactions. The primer sequences used were as follows: for GAPDH: forward primer, 5'-CTCCTCCTGTTCGACAGTCAGC-3', reverse primer, 5'-CCCAATACGACCAAATCCGTT-3'; for CLC: forward primer, 5'-CTACCCGTGCCATACACAGA-3', reverse primer, 5'-GTTCATGACCACACGACGAC-3'. The CLC mRNA levels in the

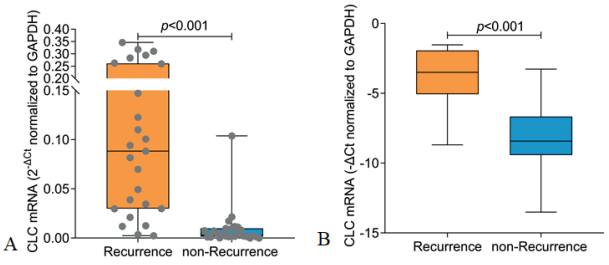

Figure 1. The relative Charcot-Leyden Crystal (CLC) mRNA levels between recurrence and non-recurrence group. (A) The relative CLC mRNA levels ($2^{-\Delta Ct}$) normalized to GAPDH. (B) The linearized value by log2 for CLC mRNA levels normalized to GAPDH (-ΔCt).

recurrence group and non-recurrence group relative to GAPDH levels were calculated as $2^{-\Delta Ct}$, where $\Delta Ct = (Ct^{CLC} - Ct^{GAPDH})$. For the purpose of statistical analysis, the $2^{-\Delta Ct}$ values were log2 transformed (i.e. equal to -ΔCt) to linearize and present the relative level of CLC mRNA normalized to GAPDH mRNA. The higher –ΔCt represents the higher level of CLC mRNA.

Histological evaluation

Polyp tissue samples were processed using standardized procedures for histologic evaluation. Dehydrated and paraffin-embedded tissue samples were sectioned at 4 μm thickness, stained with hematoxylin and eosin (H&E) stain and observed by light microscopy at 400× magnification. Five non-overlapping high-power fields (HPF) were selected randomly and analyzed to count the infiltrating inflammatory cells, including eosinophils, neutrophils, plasma cells and lymphocytes, and to calculate the percentages of each type of inflammatory cells.

Statistical analysis

Continuous variable differences were analyzed by the Student's t-test or Mann-Whitney U test when assumptions of a t-test may not be met. Categorical variables were compared using the Chi-square test or Fisher's exact test, where appropriate. Correlations between mRNA level of CLC and continuous variables were assessed by Spearman's rank correlation analysis. The variables that were found to be associated with polyp recurrence based on the univariate analysis ($p<0.05$), were included in multivariate binary logistic regression analysis using a stepwise forward method to identify predictive factors associated with polyp recurrence. Receiver operating characteristic (ROC) curves analysis was performed to determine the predictive values of special parameters, based on the area under the curves (AUCs) for a particular characteristic. Statistical analysis was performed by using SPSS version 25.0 (IBM Corp., Armonk, NY). Comparisons of AUCs were performed by MedCalc statistics software package (version 15.2, Ostend, Belgium) using the referenced method[32], and a maximum Youden index was determined as the best cut-off point with optimal sensitivity and specificity. All significance levels were 2-tailed, and p-values less than 0.05 were considered significant.

Table 2. Logistic regression analysis to identify risk factors for polyp recurrence.

Variables	OR	95% CI	p-value
CLC mRNA levels (-ΔCt)	2.880	1.214-6.832	0.016
Tissue eosinophils %	1.069	1.015-1.126	0.011

OR: odds ratio; CI: confidence interval

Table 3. The area under the curves (AUCs) of predictors for polyp recurrence.

Variables	OR	95% CI	p-value
CLC mRNA levels (-ΔCt)	0.932	0.862-1.000	<0.001
Tissue eosinophils %	0.928	0.855-1.000	<0.001

OR: odds ratio; CI: confidence interval

Results

Clinical characteristics of nasal polyp recurrence

Follow-up data demonstrated that a total of 25 patients experienced polyp recurrence, whereas 26 patients did not have a recurrence after systematic medication. Detailed demographic and clinical characteristics at baseline for these patients and control subjects are available in Table 1. Patients who had experienced polyp recurrence had a significantly higher rate of comorbid asthma compared with those without recurrence ($p=0.017$, odds ratio (OR)=4.071, 95% confidence interval (CI)=1.252-13.243). Similarly, the baseline Lund-Mackay score ($p=0.015$), olfaction scores ($p=0.003$) and rhinorrhea score ($p=0.044$) were significantly higher in the recurrence group compared with the non-recurrence group. Higher tissue and peripheral blood eosinophil proportion (both $p<0.001$) was observed in the recurrence group, whereas the percentage of tissue neutrophils ($p=0.046$), plasma cells ($p<0.001$), and lymphocytes ($p<0.001$) were significantly lower compared with the non-recurrence group. No significant differences were observed between the two groups for the other characteristics.

The relative mRNA levels of CLC in nasal brushing samples

Real-time PCR was performed to quantify the mRNA levels of CLC in nasal brushing from CRSwNP patients and controls. The relative CLC mRNA level was significantly higher in nasal brushings from CRSwNP patients with polyp recurrence compared to those without recurrence ($p<0.001$) and control subjects ($p<0.001$). No significant difference was seen between the latter

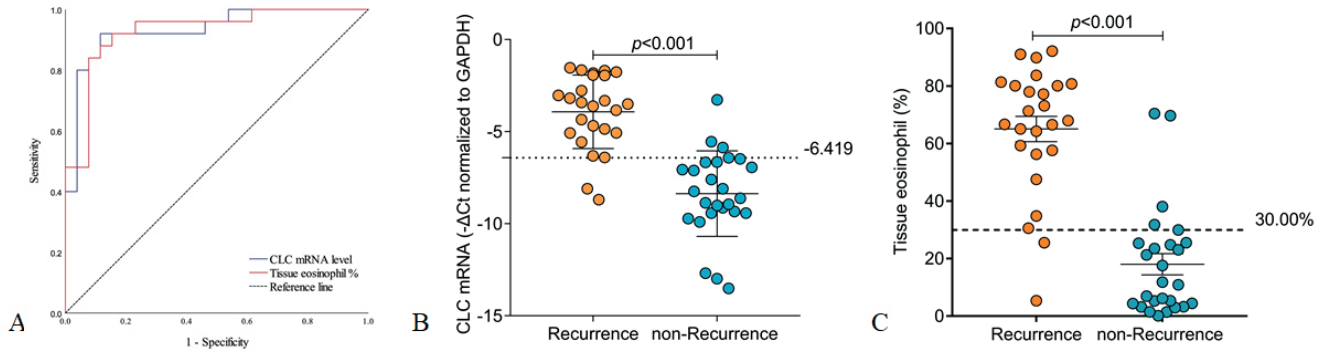

Figure 2. Receiver operating characteristic (ROC) curves of predictive factors. (A) The area under the curves (AUCs) for the percentage of tissue eosinophils and the relative CLC mRNA level; (B) The optimal cut-off point for CLC level to predict recurrence. (C) The optimal cut-off point for tissue eosinophils percentage to predict recurrence.

two groups (p>0.999) (Figure 1 and Figure S1). The median values of the relative CLC mRNA levels normalized to GAPDH in the recurrence group and the non-recurrence group were 0.088 and 0.003, respectively (Figure 1A). To satisfy the criteria of the following statistical analyses, relative CLC mRNA levels normalized to GAPDH was linearized by log2 transformed (Figure S1B and Figure 1B).

Next, we stratified patients into groups by factors demonstrating differences between the recurrence and non-recurrence cases (Table 1), including asthma, olfaction score, rhinorrhea score, and Lund-Mackay score. The relative CLC mRNA level was significantly higher in the recurrence group compared with the non-recurrence group, regardless of symptoms severity, CT score, and the comorbidity of asthma (Tables S1-4).

Identification of the predictive factors for CRSwNP recurrence

To determine the specific factors associated with polyp recurrence, the logistic regression analysis was conducted. Variables introduced were based on between-group comparison analysis, including the linearized value of CLC mRNA levels, tissue and peripheral blood eosinophil percentages, percentages of tissue neutrophil, plasma cells and lymphocytes, olfaction score and rhinorrhea score, Lund-Mackay score, and comorbid asthma. The analysis demonstrated that two variables; the relative mRNA levels of CLC in nasal brushing samples (p=0.016, OR=2.880, 95% CI=1.214-6.832) and tissue eosinophil percentages (p=0.011, OR=1.069, 95% CI=1.015-1.126); were significantly associated with nasal polyp recurrence (Table 2).

Evaluation of the predictive values of parameters for CRSwNP recurrence

ROC curves for the two parameters associated with polyp recurrence are shown in Figure 2A, and the corresponding area under the curves (AUCs) are shown in Table 3. AUC value indicated that both the relative mRNA levels of CLC in nasal brushings and the percentages of eosinophils in tissue samples presented high predictive values for polyp recurrence (AUC=0.932, 95% CI=0.862-1.000, p<0.001; AUC=0.928, 95% CI=0.855-1.000, p<0.001, respectively) (Table 3). Comparison of ROC curves for the two variables further indicated that there was no significant difference between the AUCs for CLC mRNA levels and percentages of tissue eosinophils (p=0.925).

The optimal cut-off point was identified by the maximal Youden index (sensitivity + specificity – 1), to discriminate recurrence and non-recurrence of nasal polyps. The relative CLC mRNA level of -6.419 (Youden index=0.805) was identified as an optimal cut-off point for prediction of polyp recurrence with a sensitivity of 92.00% and a specificity of 88.46% (Figure 2B, Table 4). Similarly, the tissue eosinophil percentage of 30.00% (Youden index = 0.766) (Figure 2C) was identified as an optimal cut-off point for prediction of polyp recurrence with a sensitivity of 92.00% and a specificity of 84.62% (Table 4). Further using the cut-off values of CLC mRNA level and tissue eosinophil percentage, the positive

Table 4. Sensitivity, specificity, Youden index, positive predictive value and negative predictive value of predictors at the optimal cut-off point.

Variables	Cut-off value	Youden index	Sensitivity	Specificity	PPV	NPV
CLC mRNA levels (-ΔCt)	-6.419	0.805	92.00%	88.46%	88.46%	92.00%
Tissue eosinophils %	30.00%	0.766	92.00%	84.62%	85.19%	91.67%

PPV: positive predictive value; NPV: negative predictive value.

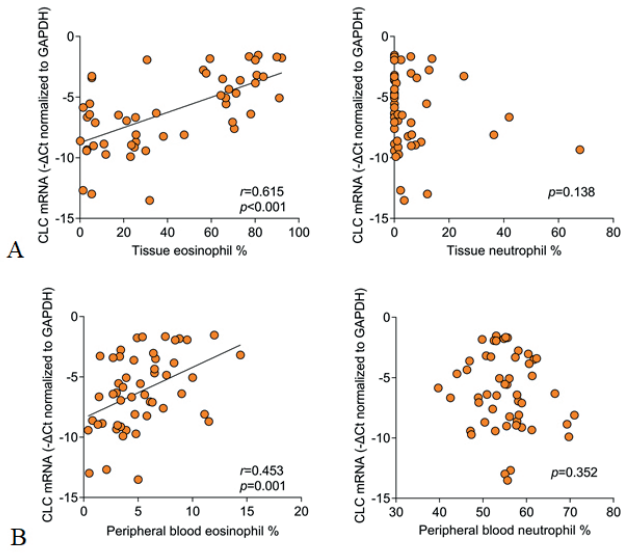

Figure 3. The relationship between clinical characteristics and the relative CLC mRNA levels. (A) Correlation between percentages of tissue eosinophils/neutrophils and the relative CLC mRNA level. (B) Correlation between percentages of peripheral blood eosinophils/neutrophils and the relative CLC mRNA level.

predictive value (PPV) for recurrence was found to be 88.46%, 85.19%, and the negative predictive value (NPV) was 92.00% and 91.67%, respectively (Table 4).

Correlation between CLC level and clinical characteristics

To better understand the relationship between CLC level and recurrence of CRSwNP, we further explored the correlations between CLC level and clinical characteristics. Assessment of association of the relative CLC mRNA levels with percentage of immune cells in polyp tissue and peripheral blood by Spearman rank correlation analysis demonstrated that CLC mRNA level was positively correlated with the percentages of tissue and peripheral blood eosinophils ($p<0.001$, $r=0.615$, $p=0.001$, $r=0.453$, respectively); but not with the percentages of tissue or peripheral blood neutrophils ($p=0.138$, $p=0.352$, respectively) (Figure3A, 3B).

Additional analysis of the result for CLC mRNA levels in nasal brushing samples indicated that patients with high-level CLC (higher than or equal to the cut-off value) (n=27) displayed significantly higher percentage of eosinophils in both polyp tissues and peripheral blood compared with the patients with low-level CLC (lower than the cut-off value) (n=24) ($p<0.001$, $p=0.020$, respectively). Whereas patients with high-level CLC show less infiltration of tissue neutrophils ($p=0.025$) compared with patients with low-level CLC (Figure S2A). No significant differences have been observed in terms of peripheral blood neutrophils ($p=0.426$) and lymphocytes ($p=0.604$), comorbid rates of asthma ($p=0.443$), allergic rhinitis ($p=0.731$), atopy ($p=0.276$), olfaction score ($p=0.161$), nasal obstruction score ($p=0.256$), rhinorrhea score ($p=0.052$), head and facial pain score ($p=0.636$), Lund-Mackay score ($p=0.368$) and nasal polyp size score ($p=0.055$) (Figure S2B, 2C, 2D), gender ($p=0.283$), and age ($p=0.782$).

Discussion

Although current medical and ESS interventions for CRSwNP have facilitated significant improvement in clinical outcomes [33, 34], CRSwNP still has a high propensity for recurrence[35]. The recurrence rate of 49.02% of polyps for patients enrolled is consistent with the recurrence rates of 40% to 55.3% observed in several recent studies[11, 35, 36]. Our findings for tissue eosinophils are in accordance with the findings of other studies, which have reported tissue eosinophils to be an important factor for polyp recurrence[8, 9, 11], and to be present in large numbers in patients with refractory nasal polyps[37, 38].

In this study, we have evaluated the determination of CLC level in nasal brushing as a non-invasive method to predict the recurrence of CRSwNP. Our study has demonstrated the relative CLC mRNA levels were significantly higher in nasal brushing samples obtained from patients with polyp recurrence compared with patients without recurrence. Comparable predictive capability for polyp recurrence was acquired between levels of both CLC mRNA in nasal brushing samples and the percentage of eosinophils in tissue specimens indicated by ROC curves analysis and comparison of AUC values. The optimal cut-off point of CLC relative level was determined to be -6.419 according to the Youden index with a sensitivity of 92.00% and a specificity of 88.46%.

Although the mRNA levels of CLC and tissue eosinophils both have similar capacities for predicting CRSwNP recurrence following surgery, the determination of the mRNA level of CLC provides an advantage over the determination of the percentage of tissue eosinophils. Since the determination of nasal brushing CLC mRNA is not only a non-invasive sampling of the nasal mucosa prior to surgery, but also a quantitative method providing automatic readout. In contrast, counting tissue eosinophils is semi-quantitative and depends on subjective measurement. Furthermore, the quantification of CLC mRNA is recommended mostly as the time-saving, automatic and high-throughput screening compared with ELISA and directly counting the eosinophils in nasal brushings. Firstly, qPCR can determine a single sample without enhancing the cost compared with ELISA assay. The estimation of the readout of ELISA assay depends upon the construction of a standard curve, thus enough samples should be collected and performed simultaneously to reduce the cost. For clinics without a large number of patients, waiting times arise as the result could not be acquired timely. However, the relative expression of target genes is normalized by that of housekeeping genes of the same sample, thus the qPCR can be performed for a single sample without cost enhancement. Se-

condly, the advantage of measuring mRNA over directly looking at eosinophil counts in nasal brushings is that the numerical readout by qPCR makes it easy for result judgement, friendly for the machine learning, and labor-saving.

CLCs have long been known as an eosinophil biomarker and associated with diseases with prominent eosinophilic inflammation, and might help to perpetuate the inflammatory process[17, 39-47]. CLCs contain the lysolecithin acyl hydrolase, which is involved in the eosinophil-mediated immune functions[48], causing damage to the airway epithelium and increasing vascular permeability[49]. In addition, CLC can co-localize with eosinophil-derived neurotoxin (EDN) induced by interferon activation[50] and function as a carrier for the vesicular transport of EDN ribonucleases during granulogenesis and degranulation in activated eosinophil, as well as enable the extracellular functions of eosinophil proteins involved in host defense and eosinophil inflammation without causing intracellular damage to the eosinophils[17]. Ueki et al.[51, 52] demonstrated that CLCs were also associated with extracellular trap cell death (ETosis), a regulated cell death program of activated eosinophils leading to CLCs being externalized from the cytoplasm. Another recent study has demonstrated that CLCs can be recognized by the nucleotide-binding oligomerization domain-like receptor protein3 (NLRP3) inflammasome, which may possibly sustain inflammation following the eosinophilic inflammatory processes[53]. In addition to its considerable expression in eosinophils and basophils, it has been reported that CLC may essentially be involved in the suppressive activity of CD25+ Treg cells and affect immune tolerance during inflammatory activities[54]. Recently, some studies have indicated that CLC could be targeted therapeutically to treat eosinophilic airway disease[21, 55]. Our investigation of the associations between CLC level and clinical characteristics of CRSwNP patients demonstrated that CLC levels were associated with percentages of eosinophil in both polyp tissue and peripheral blood. Patients with high-level CLC had higher tissue and peripheral blood eosinophil percentages compared with those with low-level CLC. These results were consistent with a previous study that CLC protein (galectin-10) in sputum was positively corrected with the percentage of sputum eosinophils and accurately determined sputum eosinophilia[43]. In addition, the low CLC in non-recurrence group indicates the non-ECRSwNP, which is a dominant subtype of CRSwNP in China. Collectively, these studies support CLC as a potential biomarker of eosinophilic and Th2 inflammatory process. Furthermore, previous observation that CRSwNP with higher degree of eosinophil infiltration (i.e. eosinophilic (E) CRSwNP) has a higher recurrence rate compared to CRSwNP with higher infiltration of other inflammatory cell types supports our hypothesis that CLC might be associated with outcome following nasal polyp surgery, and is therefore likely to be a reliable marker for predicting recurrence in CRSwNP.

This study has several implications. First, from the viewpoint of the investigation, these results demonstrate an up-expressed CLC that possibly contributes to the poor surgical outcome. Second, from the viewpoint of clinical therapy, CLC mRNA level in preoperative nasal brushing obtained by a non-invasive method may serve as a potential factor to help stratify risk patients regarding polyp recurrence before surgery. It has been reported that RESS approach[4], long-term application of intranasal glucocorticoids after surgery[9] could be used for the patients with high recurrence risk to prevent or delay the polyp recurrence. Thus, identification of potential patients with high risk for recurrence before sinus surgery by a non-invasive method may guide medication therapy, choice of surgical options and postoperative follow-up, and further help to prevent the recurrence of polyps. Furthermore, the determination of the biomarker such as CLC may shed light on the efficiency of targeting treatment such as monoclonal antibodies against CLC for CRSwNP patients.

The findings of our current study, however, are also somewhat limited due to the small sample size, the relatively short follow-up period, and the possible bias for the acquirement of immunohistological data during the follow-up. Thus, a prospective study with larger sample size and extended follow-up period are required to confirm the findings of the present study and evaluate more accurate predictive values for CLC gene expression in the future.

Conclusion

The results of this study indicate that CLC mRNA levels in nasal brushing was upregulated in CRSwNP patients with polyp recurrence after ESS and was positively correlated with eosinophil percentages in both polyp tissue and peripheral blood. The expression level of CLC mRNA in nasal brushing which could be detected by a non-invasive method was identified as an effective predictive factor for polyp recurrence and could guide medical and surgical therapy, postoperative follow-up, and further help to prevent or delay the recurrence of polyps.

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Authorship contribution

All authors participated in the drafting/revision of the manuscript, final approval of the manuscript, and interpretation of findings. SQ and BY contributed to most of the experimental management, including study design, biochemical techniques, data analysis and manuscript drafting. CL participated in parts of experimental procedures. LZ and CW were involved in the design of the study, enrollment of the patients and revision of the manuscript. All authors agree to be accountable for all aspects of the work.

Conflict of interest

All authors declare no financial or commercial conflicts of interest.

References

1. Fokkens WJ, Lund VJ, Mullol J, et al. European Position Paper on Rhinosinusitis and Nasal Polyps 2012. Rhinol Suppl 2012; 23:3 p preceding table of contents, 1-298.
2. Stevens WW, Schleimer RP, Kern RC. Chronic Rhinosinusitis with Nasal Polyps. J Allergy Clin Immunol Pract 2016; 4:565-72.
3. Wynn R, Har-El G. Recurrence rates after endoscopic sinus surgery for massive sinus polyposis. Laryngoscope 2004; 114:811-3.
4. Jankowski R, Pigret D, Decroocq F, Blum A, Gillet P. Comparison of radical (nasalisation) and functional ethmoidectomy in patients with severe sinonasal polyposis. A retrospective study. Rev Laryngol Otol Rhinol (Bord) 2006; 127:131-40.
5. Punekar YS, Ahmad A, Saleh HA. Estimating the effect of nasal steroid treatment on repeat polypectomies: survival time analysis using the General Practice Research Database. Rhinology 2011; 49:190-4.
6. Zhang Z, Linkin DR, Finkelman BS, et al. Asthma and biofilm-forming bacteria are independently associated with revision sinus surgeries for chronic rhinosinusitis. J Allergy Clin Immunol 2011; 128:221-3 e1.
7. Sil A, Mackay I, Rowe-Jones J. Assessment of predictive prognostic factors for functional endoscopic sinus surgery in a 5-year prospective outcome study. Am J Rhinol 2007; 21:289-96.
8. Matsuwaki Y, Ookushi T, Asaka D, et al. Chronic rhinosinusitis: risk factors for the recurrence of chronic rhinosinusitis based on 5-year follow-up after endoscopic sinus surgery. Int Arch Allergy Immunol 2008; 146 Suppl 1:77-81.
9. Nakayama T, Yoshikawa M, Asaka D, et al. Mucosal eosinophilia and recurrence of nasal polyps - new classification of chronic rhinosinusitis. Rhinology 2011; 49:392-6.
10. Nakayama T, Asaka D, Kanaya H, Kuboki A, Haruna S. Prognostic factors for recurrence after endoscopic sinus surgery for chronic rhinosinusitis with nasal polyps. Auris Nasus Larynx 2016; 43:641-7.
11. Lou H, Meng Y, Piao Y, Wang C, Zhang L, Bachert C. Predictive significance of tissue eosinophilia for nasal polyp recurrence in the Chinese population. Am J Rhinol Allergy 2015; 29:350-6.
12. Batzakakis D, Stathas T, Mastronikolis N, Kourousis C, Aletras A, Naxakis S. Adhesion molecules as predictors of nasal polyposis recurrence. Am J Rhinol Allergy 2014; 28:20-2.
13. Akhtar S, Ikram M, Azam I, Dahri T. Factors associated with recurrent nasal polyps: a tertiary care experience. J Pak Med Assoc 2010; 60:102-4.
14. Chen J, Zhou Y, Zhang L, et al. Individualized Treatment of Allergic Rhinitis According to Nasal Cytology. Allergy Asthma Immunol Res 2017; 9:403-9.
15. She W, Yang J, Wang C, Zhang L. Diagnostic Value of Nasal Cytology in Chronic Rhinosinusitis Assessed by a Liquid-based Cytological Technique. Am J Rhinol Allergy 2018; 32:181-7.
16. Groger M, Bernt A, Wolf M, et al. Eosinophils and mast cells: a comparison of nasal mucosa histology and cytology to markers in nasal discharge in patients with chronic sino-nasal diseases. Eur Arch Otorhinolaryngol 2013; 270:2667-76.
17. Acharya KR, Ackerman SJ. Eosinophil granule proteins: form and function. J Biol Chem 2014; 289:17406-15.
18. Lavin J, Min JY, Lidder AK, et al. Superior turbinate eosinophilia correlates with olfactory deficit in chronic rhinosinusitis patients. Laryngoscope 2017; 127:2210-8.
19. Tan BK, Klingler AI, Poposki JA, et al. Heterogeneous inflammatory patterns in chronic rhinosinusitis without nasal polyps in Chicago, Illinois. J Allergy Clin Immunol 2017; 139:699-703 e7.
20. Nakayama T, Sugimoto N, Okada N, et al. JESREC score and mucosal eosinophilia can predict endotypes of chronic rhinosinusitis with nasal polyps. Auris Nasus Larynx 2019; 46:374-83.
21. Persson EK, Verstraete K, Heyndrickx I, et al. Protein crystallization promotes type 2 immunity and is reversible by antibody treatment. Science 2019; 364:eaaw4295.
22. Global Initiative For Asthma. Global strategy for asthma management and prevention. 2014. Available at: www.ginasthma.org
23. Brozek JL, Bousquet J, Baena-Cagnani CE, et al. Allergic Rhinitis and its Impact on Asthma (ARIA) guidelines: 2010 revision. J Allergy Clin Immunol 2010; 126:466-76.
24. Ownby DR, Johnson CC, Peterson EL. Exposure to dogs and cats in the first year of life and risk of allergic sensitization at 6 to 7 years of age. Jama 2002; 288:963-72.
25. Gevaert P, Calus L, Van Zele T, et al. Omalizumab is effective in allergic and nonallergic patients with nasal polyps and asthma. J Allergy Clin Immunol 2013; 131:110-6 e1.
26. Lund VJ, Kennedy DW. Staging for rhinosinusitis. Otolaryngol Head Neck Surg 1997; 117:S35-40.
27. Shen PH, Weitzel EK, Lai JT, Wormald PJ, Lin CH. Retrospective study of full-house functional endoscopic sinus surgery for revision endoscopic sinus surgery. Int Forum Allergy Rhinol 2011; 1:498-503.
28. Varvyanskaya A, Lopatin A. Efficacy of long-term low-dose macrolide therapy in preventing early recurrence of nasal polyps after endoscopic sinus surgery. Int Forum Allergy Rhinol 2014; 4:533-41.
29. Luo Q, Chen F, Liu W, et al. Evaluation of long-term clarithromycin treatment in adult Chinese Patients with chronic rhinosinusitis without nasal polyps. ORL J Otorhinolaryngol Relat Spec 2011; 73:206-11.
30. Sambrook J RD. Molecular Cloning: A Laboratory Manual. 3rd edition. In: Sambrook J, Russell DW, eds. Extraction, purification and analysis of mRNA from eukaryotic cells. New York: Cold Spring Harbor Laboratory Press, 2001; pp. 7.7–7.10.
31. Yan B, Wang Y, Li Y, Wang C, Zhang L. Inhibition of arachidonate 15-lipoxygenase reduces the epithelial-mesenchymal transition in eosinophilic chronic rhinosinusitis with nasal polyps. Int Forum Allergy Rhinol 2019;9(3):270–80.
32. DeLong ER, DeLong DM, Clarke-Pearson DL. Comparing the areas under two or more correlated receiver operating characteristic curves: a nonparametric approach. Biometrics 1988; 44:837-45.
33. Hopkins C, Gillett S, Slack R, Lund VJ, Browne JP. Psychometric validity of the 22-item Sinonasal Outcome Test. Clin Otolaryngol 2009; 34:447-54.
34. Smith TL, Litvack JR, Hwang PH, et al. Determinants of outcomes of sinus surgery: a multi-institutional prospective cohort

study. Otolaryngol Head Neck Surg 2010; 142:55-63.
35. Tosun F, Arslan HH, Karslioglu Y, Deveci MS, Durmaz A. Relationship between postoperative recurrence rate and eosinophil density of nasal polyps. Ann. Otol. Rhinol. Laryngol. 2010; 119:455-9.
36. DeConde AS, Mace JC, Levy JM, Rudmik L, Alt JA, Smith TL. Prevalence of polyp recurrence after endoscopic sinus surgery for chronic rhinosinusitis with nasal polyposis. Laryngoscope 2017; 127:550-5.
37. Bousquet J, Chanez P, Lacoste JY, et al. Eosinophilic inflammation in asthma. N Engl J Med 1990; 323:1033-9.
38. Braunstahl GJ, Fokkens W. Nasal involvement in allergic asthma. Allergy 2003; 58:1235-43.
39. Pantanowitz L, Balogh K. Charcot-Leyden crystals: pathology and diagnostic utility. Ear Nose Throat J 2004; 83:489-90.
40. Stevens WW, Peters AT, Tan BK, et al. Associations between inflammatory endotypes and clinical presentations in chronic rhinosinusitis. J Allergy Clin Immunol Pract 2019;7(8):2812–20.e3.
41. Furuta GT, Kagalwalla AF, Lee JJ, et al. The oesophageal string test: a novel, minimally invasive method measures mucosal inflammation in eosinophilic oesophagitis. Gut 2013; 62:1395-405.
42. Devouassoux G, Pachot A, Laforest L, et al. Galectin-10 mRNA is overexpressed in peripheral blood of aspirin-induced asthma. Allergy 2008; 63:125-31.
43. Chua JC, Douglass JA, Gillman A, O'Hehir RE, Meeusen EN. Galectin-10, a potential biomarker of eosinophilic airway inflammation. PLoS One 2012; 7:e42549.
44. Noh S, Jin S, Park CO, et al. Elevated Galectin-10 Expression of IL-22-Producing T Cells in Patients with Atopic Dermatitis. J Invest Dermatol 2016; 136:328-31.
45. Almkvist J, Karlsson A. Galectins as inflammatory mediators. Glycoconj J 2002; 19:575-81.
46. Negrete-Garcia MC, Jimenez-Torres CY, Alvarado-Vasquez N, et al. Galectin-10 is released in the nasal lavage fluid of patients with aspirin-sensitive respiratory disease. Scientific World Journal 2012; 2012:474020.
47. Liu C, Yan B, Qi S, Zhang Y, Zhang L, Wang C. Predictive Significance of Charcot-Leyden Crystals for Eosinophilic Chronic Rhinosinusitis With Nasal Polyps. Am J Rhinol Allergy 2019;33: 671-80.
48. Weller PF, Goetzl EJ, Austen KF. Identification of human eosinophil lysophospholipase as the constituent of Charcot-Leyden crystals. Proc. Natl. Acad. Sci. U.S.A. 1980; 77:7440-3.
49. Huang F, Subbaiah PV, Holian O, et al. Lysophosphatidylcholine increases endothelial permeability: role of PKCalpha and RhoA cross talk. Am J Physiol Lung Cell Mol Physiol 2005; 289:L176-85.
50. Humbles AA, Lloyd CM, McMillan SJ, et al. A critical role for eosinophils in allergic airways remodeling. Science 2004; 305:1776-9.
51. Ueki S, Tokunaga T, Melo RCN, et al. Charcot-Leyden crystal formation is closely associated with eosinophil extracellular trap cell death. Blood 2018; 132:2183-7.
52. Ueki S, Miyabe Y, Yamamoto Y, et al. Charcot-Leyden Crystals in Eosinophilic Inflammation: Active Cytolysis Leads to Crystal Formation. Current Allergy and Asthma Reports 2019;19(8):35.
53. Rodríguez-Alcázar JF, Ataide MA, Engels G, et al. Charcot–Leyden Crystals Activate the NLRP3 Inflammasome and Cause IL-1β Inflammation in Human Macrophages. J Immunol 2019; 202:550-8.
54. Kubach J, Lutter P, Bopp T, et al. Human CD4+CD25+ regulatory T cells: proteome analysis identifies galectin-10 as a novel marker essential for their anergy and suppressive function. Blood 2007; 110:1550-8.
55. Allen JE, Sutherland TE. Crystal-clear treatment for allergic disease. Science 2019; 364:738-9.

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SUPPLEMENTARY DATA

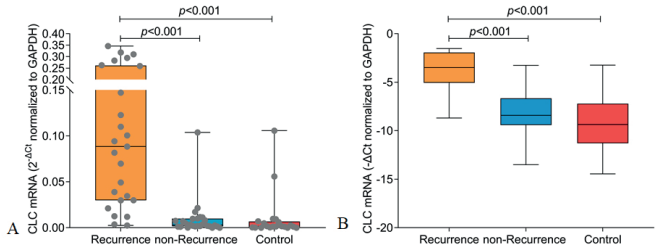

Figure S1. The relative Charcot-Leyden Crystal (CLC) mRNA levels in CRSwNP patients with recurrence/non-recurrence of polyps and control subjects. (A) The relative CLC mRNA levels (2-ΔCt) normalized to GAPDH. (B) The linearized value by log2 for CLC mRNA levels normalized to GAPDH (-ΔCt). Recurrence of CRSwNP was based on 12-18-month follow-up, and significance of differences in the CLC mRNA levels between the groups was analyzed using Kruskal-Wallis test with Dunn's corrections.

Table S1. Comparison of CLC level between recurrence and non-recurrence groups for patient with and without asthma.

	Asthma			non-Asthma		
	Recurrence (n=15)	non-Recurrence (n=7)	p-value	Recurrence (n=10)	non-Recurrence (n=19)	p-value
CLC(2-ΔCt), median (IQR)	0.11 (0.03-0.26)	0.00 (0.00-0.01)	<0.001	0.08 (0.04-0.12)	0.00 (0.00-0.01)	<0.001

IQR: interquartile range

Table S2. Comparison of CLC level between recurrence and non-recurrent groups for patient with different degrees of olfaction.

	Olfaction score (>7)			Olfaction score (≤ 7)		
	Recurrence (n=18)	non-Recurrence (n=7)	p-value	Recurrence (n=7)	non-Recurrence (n=19)	p-value
CLC($2^{-\Delta Ct}$), median (IQR)	0.12 (0.03-0.28)	0.00 (0.00-0.01)	<0.001	0.05 (0.03-0.09)	0.00 (0.00-0.01)	0.034

IQR: interquartile range. Olfaction score (VAS≤ 7) was categorized as 'mild- moderate', and olfaction score (VAS > 7) was categorized as 'severe', according to EPOS[E1].

Table S3. Comparison of CLC level between recurrence and non-recurrence groups for patient with different degrees of rhinorrhea.

	Rhinorrhea score (>7)			Rhinorrhea score (≤ 7)		
	Recurrence (n=5)	non-Recurrence (n=3)	p-value	Recurrence (n=20)	non-Recurrence (n=23)	p-value
CLC($2^{-\Delta Ct}$), median (IQR)	0.12 (0.04-0.26)	0.01 (0.00-0.01)	<0.001	0.09 (0.03-0.18)	0.00 (0.00-0.01)	<0.001

IQR: interquartile range. Rhinorrhea score (VAS≤ 7) was categorized as 'mild- moderate', and Rhinorrhea score (VAS > 7) was categorized as 'severe', according to EPOS[E1].

Table S4. Comparison of CLC level between recurrence and non-recurrence groups for patient with different degrees of Lund Mackay scores.

	Lund Mackay score (≥19)			Lund Mackay score (<19)		
	Recurrence (n=16)	non-Recurrence (n=9)	p-value	Recurrence (n=9)	non-Recurrence (n=17)	p-value
CLC($2^{-\Delta Ct}$), median (IQR)	0.10 (0.03-0.27)	0.00 (0.00-0.01)	<0.001	0.09 (0.03-0.10)	0.01 (0.00-0.01)	<0.001

IQR: interquartile range. The Lund Mackay score≥19 was categorized as higher Lund Mackay score, and Lund Mackay score <19 was categorized as lower Lund Mackay score, according to the average value (18.8) of the LM score in this research.

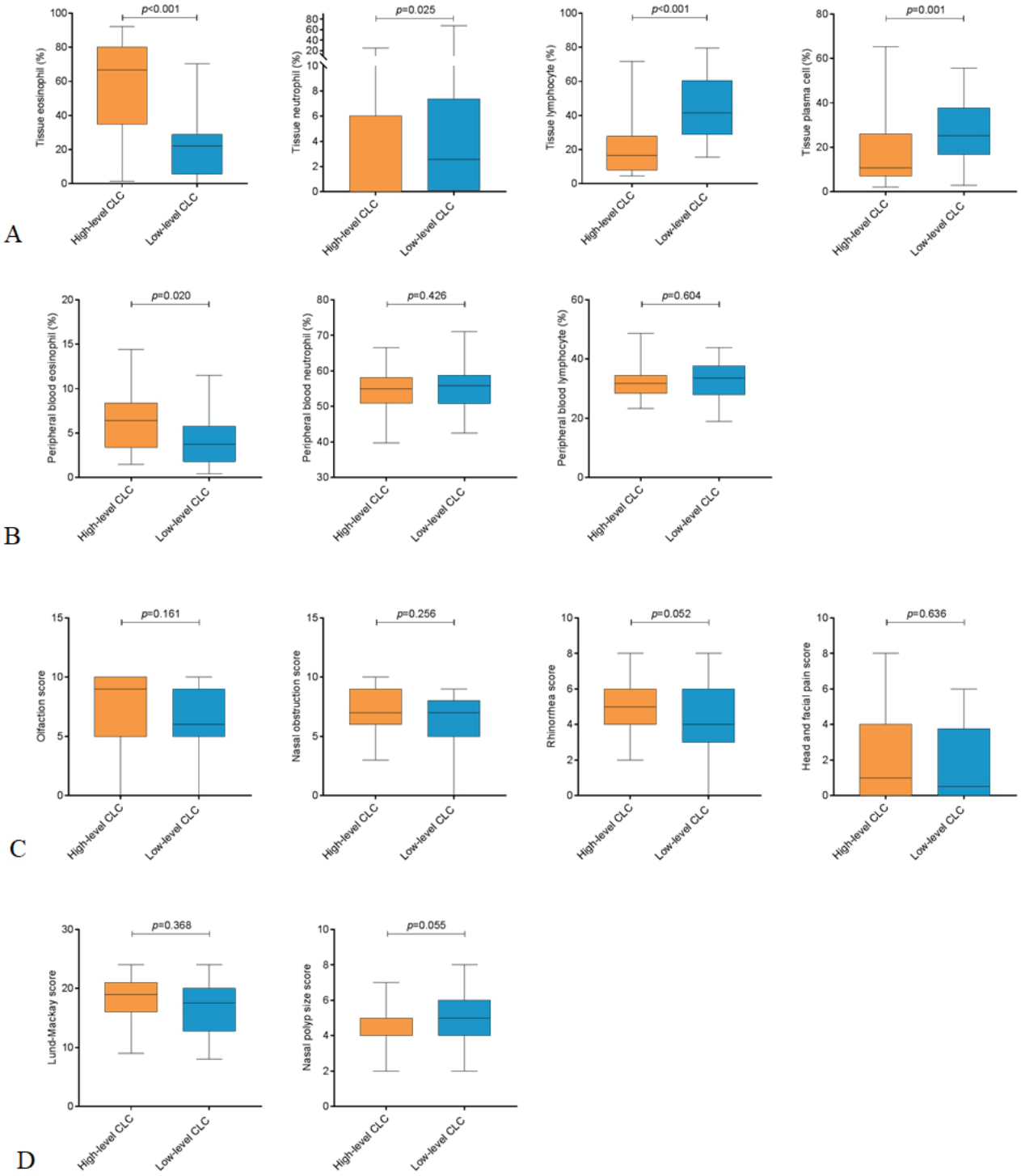

Figure S2. The comparison of clinical characteristics between high- and low- level of CLC divided by the cut-off value in (A) Percentages of immune cells in tissue samples; (B) Percentages of immune cells in peripheral blood; (C) Visual analogue scale (VAS) scores and (D) Lund-Mackay scores and polyp size scores.

References

1. Fokkens WJ, Lund VJ, Mullol J, et al. European Position Paper on Rhinosinusitis and Nasal Polyps 2012. Rhinol Suppl 2012; 23:3 p preceding table of contents, 1-298.