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Research article

NOX2 mediates NLRP3/ROS facilitating nasal mucosal epithelial inflammation in chronic rhinosinusitis with nasal polyps

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ABSTRACT

Background: Previous investigations have provided limited insight into the role of oxidative stress in nasal mucosa inflammation. The aim of this study was to investigate the mechanism of oxidative stress in the epithelial cells of chronic rhinosinusitis with nasal polyps CRSwNP utilizing single-cell RNA sequencing data.

Methods: Single-cell RNA sequencing data from HRA000772 were used to assess oxidative stress, inflammasome activation, and nicotinamide adenine dinucleotide phosphate oxidases (NOXs) expression in epithelial cells via integrative rank-based gene set enrichment analysis. The localization of reactive oxygen species (ROS) and NOX2 in nasal mucosa and cell models was visualized using fluorescent probes and immunohistochemistry, respectively. Functional studies on NOX2 involved siRNA and plasmid transfections *in vitro*, while Nucleotide-binding oligomerization domain-like receptor family pyrin domain containing 3 (NLRP3) inflammasome activity was examined using the inducer TMAO and the inhibitor MCC950.

Results: Single-cell RNA sequencing data suggested an increase of oxidative stress score and NLRP3 inflammasome score in CRSwNP epithelial cells. Vitro experiments demonstrated that lipopolysaccharide could induce ROS accumulation, NLRP3 inflammasome activation and epithelial alarmin expression. MCC950 inhibited the expression of epithelia alarmin *in vitro*. Elevated NOX2 in CRSwNP epithelial cells was associated with increased ROS, NLRP3 inflammasome activation, and epithelial alarmin expression. NOX2-targeted siRNA inhibited these effects *in vitro*. Moreover, TMAO reversed the downregulation of epithelial alarmins without impacting ROS levels.

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Conclusion: This study highlights the crucial role of NOX2 as a key regulator of ROS accumulation and NLRP3 inflammasome activation in CRSwNP, underscoring its potential as a valuable therapeutic target for CRSwNP.

1. Introduction

Chronic rhinosinusitis (CRS) presents as persistent inflammatory infiltration of the nasal mucosa, affecting approximately 10 % of the global population and imposing a substantial economic burden on society [1,2]. CRS can be categorized into two phenotypes based on the presence of nasal polyps: CRS with nasal polyps (CRSwNP) and CRS without nasal polyps (CRSsNP) [3,4]. Clinically, CRSwNP is distinguished by more severe symptoms, limited treatment efficacy, and a heightened risk of postoperative recurrence compared to CRSwNP [5–7]. Despite advancements, the etiology and pathogenesis of CRSwNP remain inadequately elucidated, with current perspectives proposing a dysregulated interplay between external environmental triggers and host genetic predispositions leading to epithelial immune dysregulation and chronic inflammation in the upper respiratory tract [8,9].

The pathological changes in CRSwNP primarily involve persistent structural alterations in cells and tissues, which drive sinonasal mucosal remodeling and nasal polyp formation [10–12]. As the primary physiological barrier against external environmental stimuli, the nasal mucosal epithelium responds to oxidative stress when exposed to stimuli, leading to the generation of reactive oxygen species (ROS). These ROS play a crucial role in promoting the pathogenesis of airway inflammatory diseases and are currently a focal point of scientific research [13]. Numerous studies have demonstrated that ROS accumulation within epithelial cells can disrupt tight junctions, resulting in structural and functional abnormalities of the epithelium and increasing epithelial tissue permeability to intercellular fluid and inflammatory cells [14,15]. Studies have demonstrated that mite allergens can induce ROS accumulation in respiratory epithelial cells in asthma, leading to the release of alarmins, including interleukin 33(IL-33) andthymic stromal lymphopoietin (TSLP) [16]. Anti-ROS therapy markedly reduced inflammatory cell infiltration in airway tissue and decreased the frequency of asthma attacks, highlighting the significance of ROS generation and accumulation in epithelial cells in airway immune response [17,18]. Nicotinamide adenine dinucleotide phosphate oxidases (NOXs) catalyze the reduction of oxygen to superoxide anions (O_2^-), a predominant component of ROS and a crucial regulatory factor. Extensive research has demonstrated that NOXs can initiate immune responses through ROS generation and were currently under investigation in numerous inflammatory conditions [19,20]. Deficiency of NOX2 can lower ROS levels in the lungs, consequently reducing IL-13 and Eotaxin expression, and mitigating airway inflammation [21]. However, the involvement of NOXs/ROS in the development and underlying mechanisms of CRSwNP requires further investigation.

The Nucleotide-binding oligomerization domain-like receptor family pyrin domain containing 3 (NLRP3) inflammasome, a cytoplasmic protein complex that includes Caspase-1, recognizes diverse stressors, exogenous microorganisms, and endogenous signals [22]. It is widely distributed in various immune cells and can also be expressed in airway epithelial cells, making it one of the most extensively studied inflammasome subtypes [23–25]. Research indicates that the NLRP3 inflammasome is pivotal in the pathogenesis of airway inflammatory conditions. Once activated, Caspase-1 processes pro-inflammatory cytokines like IL-1β and IL-18 into their active forms [26]. In a murine asthma model, lack of IL-1β markedly diminished the levels of type 2 cytokines, consequently mitigating airway inflammation [27]. Moreover, earlier investigations have revealed a heightened expression of the NLRP3 inflammasome in individuals with CRSwNP, particularly in those with eosinophilic phenotypes, suggesting its implication in the pathogenesis of CRSwNP [28]. However, its role remains elusive. It is noteworthy that ROS have been established as crucial mediators in the activation of the NLRP3 inflammasome within airway epithelial cells, primarily participating in the initial phase of inflammasome activation [29]. Additionally, the aryl hydrocarbon receptor can inhibit ROS accumulation within epithelial cells, thereby suppressing NLRP3 inflammasome production, leading to a reduction in airway mucus secretion and alleviation of asthma-related airway inflammation [30]. Consequently, there is a need to investigate the regulatory mechanisms of ROS and the NLRP3 inflammasome in CRSwNP.

In this study, we initially observed a notable accumulation of ROS in the epithelial cells of CRSwNP. Subsequently, we identified a significant upregulation of NADPH oxidase 2(NOX2) in CRSwNP, which was significantly associated with eosinophilic infiltration and epithelial inflammation. The upregulation of NOX2 led to the generation of ROS and the formation of the NLRP3 inflammasome in

Table 1
Demographic characteristics of enrolled subjects.

Variables	Control	CRSsNP	CRSwNP	*P*-value
Gender, male/female	18/12	13/7	31/9	0.268
Age, years	39.7 ± 14.7	43.6 ± 6.1	40.0 ± 17.3	0.602
BMI, kg/m^2	23.7 ± 2.5	23.7 ± 2.8	23.2 ± 2.8	0.664
AR	0	1	16	< 0.001
Asthma	0	0	6	0.018
Peripheral blood eosinophil count (10^9/L)	0.1 ± 0.1	0.2 ± 0.1	0.3 ± 0.2	0.007
Peripheral blood eosinophil percentage (%)	2.4 ± 1.3	2.6 ± 2.3	4.9 ± 3.9	0.001
Tissue eosinophil count (10^9/L)		7.1 ± 5.5	24.5 ± 20.4	< 0.001
Tissue eosinophil percentage (%)		0.1 ± 0.1	13.5 ± 10.7	< 0.001

Abbreviations: BMI, Body mass index; AR, allergic rhinitis.

nasal mucosal epithelial cells, thereby promoting the expression of damage-associated molecular patterns (DAMPs). Overall, our findings demonstrate that NOX2 plays a key role in inducing the accumulation of ROS and activating the NLRP3 inflammasome in CRSwNP. This is correlated with the severity of both intrinsic and clinical symptoms, and it provides potential therapeutic targets.

2. Methods

2.1. Subjects

The disease group comprised 40 patients with CRSwNP and 20 patients with CRSsNP. All CRS patients were diagnosed according to the diagnostic criteria outlined in the EPOS 2020 [2]. In addition, the control group consisted of 30 patients who underwent nasal septoplasty surgery and without inflammatory airway diseases. Details of the participant characteristics are summarized in Table 1. All participants included in the study were approved by the Ethics Committee of Xiangya Hospital, Central South University (Approval number: 202103112) with fully informed consent. Patients were excluded from the study if they met any of the following criteria: under 18 years of age; had taken oral corticosteroids, antibiotics, or other immune-regulating drugs in the past month; were experiencing acute inflammation or uncontrolled asthma; or had been diagnosed with fungal rhinosinusitis, allergic fungal rhinosinusitis, or sinonasal tumors. Middle turbinate mucosa tissues were collected from controls and CRSsNP patients, and nasal polyps were collected from CRSwNP patients under endoscopy after general anesthesia. The collected specimens were divided into two parts, with one part fixed in formaldehyde solution for dehydration and paraffin embedding, and the other part stored in liquid nitrogen for subsequent experiments.

2.2. Single-cell RNA sequencing data processing

The single-cell RNA sequencing data was sourced from the HRA000772 dataset. Following the Cell Ranger analysis, the downstream data were processed using steps such as quality control, integration, and dimensionality reduction, employing the Seurat package (version 4.4.0) [31]. Quality control involved the removal of doublets, filtering for a red blood cell percentage <3 %, a mitochondrial percentage less than 20 %, and a gene count ranging from 200 to 7000 per cell. The data was normalized with NormalizeData. High variable genes were identified with FindVariableFeatures. All genes were then normalized using ScaleData, followed by dimensionality reduction through RunPCA. Sample integration was performed using Harmony. Cells were categorized into Lymphocytes, Epithelial cells, Dendritic cells, Endothelial cells, Mesenchymal cells, and Myeloid cells based on markers (Supplementary Table 1). Subsequently, irGSEA (version 2.1.5), based on the Molecular Signatures Database [32], was utilized to score oxidative stress and inflammasome activity in epithelial cells. All analysis were performed with R software (version 4.3.0, Vienna, Austria.).

2.3. Cytokine detection

In the cellular models, we detected various cytokines and enzymes including epithelial-derived cytokines like IL-33, IL-25, and TSLP; NOX enzymes such as NOX1, NOX2, NOX3, NOX4, NOX5, dual oxidase 1 (DUOX1), and DUOX2; along with components of the NLRP3 inflammasome including NLRP3, Caspase-1, IL-1β, and IL-18. RNA was extracted using Trizol (NCM Biotech, Suzhou, China), with subsequent reverse transcription and real-time quantitative PCR to assess mRNA relative expression levels employing the $\Delta\Delta$CT method, as detailed previously. The primer sequences utilized are listed in Supplementary Table 2. For protein analysis, extraction was performed using RIPA buffer (NCM Biotech, Suzhou, China), proteins were then denatured and their bands visualized via Western blot. Information on the primary and secondary antibodies used is available in Supplementary Table 3. Grayscale values of the protein bands were quantified using Image J (NIH, Bethesda, USA) to determine the relative expression levels of the target proteins [33].

2.4. Immunohistochemistry

The tissues were harvested during surgery and fixed in 4 % paraformaldehyde for 48 h and embedded in a wax block. After sectioning, deparaffinization, dehydration, and antigen retrieval, the sections were incubated with H_2O_2 for 10 min, then blocked with 10 % goat serum for 30 min at room temperature. The samples were incubated with primary and secondary antibodies specific to the targets. Information on the primary and secondary antibodies used is available in Supplementary Table 3. The antibodies were bound to the specific antigens, forming antigen-antibody complexes that underwent a color reaction. The count of positive cells was conducted using Fiji.

2.5. Tissue ROS detection

ROS detection was conducted following the manufacturer's instructions (S0033S, Beyotime, Shanghai, China). The DCFH-DA (S0033S-1, Beyotime, Shanghai, China) was added to the tissue paraffin slides and incubated for 20 min at 37 °C in darkness. Then thoroughly wash the slides three times. The nuclei were counterstained with DAPI. Finally, perform anti-fade treatment, mount the slides, and capture images under a fluorescence microscope.

2.6. Cell culture and transfection

After thawing the previously stored human nasal epithelial cells (HNEpC), they were cultured in Roswell Park Memorial Institute 1640 medium (RPMI 1640, Solarbio, Beijing, China), supplemented with 10 % fetal bovine serum (Basalmedia, Shanghai, China), at 37 °C in an atmosphere containing 5 % CO_2. Chronic inflammatory cell model was established with lipopolysaccharide (LPS, 2 μg/mL, Solarbio, Beijing, China) for 48 h, while control group was cultured with comparable quantity of phosphate buffered saline (PBS, Servicebio, Wuhan, China). Following the manufacturer's instructions, Lipofectamine 3000 (Thermofisher, Waltham, USA) was used to transfect cells with NOX2 siRNA or NOX2 plasmids for 24 h. Then complete medium and subsequent interventions were delivered according to the experimental design. The siRNA and plasmids were purchased from Sangon Biotech (Wuhan, China).

2.7. Cell intervention

In the experimental design, the cell cultures were divided into treatment and control groups. The treatment group received either an MCC950 (T3701, TopScience, Shanghai, China) solution (10 μM) to inhibit NLRP3 activity or a Trimethylamine N-oxide (TMAO, TMPH-00683, TopScience, Shanghai, China) solution (150 μM) to act as an inducer, with exposures lasting for 1 h. In contrast, the control group was administered an equivalent volume of PBS to serve as a baseline comparison.

2.8. Cell ROS detection

ROS detection was conducted following the manufacturer's instructions (S0033S, Beyotime, Shanghai, China). The diluted DCFH-DA (10 μM, S0033S-1, Beyotime, Shanghai, China) was added to the cell wells after experimental intervention and incubate for 20 min.

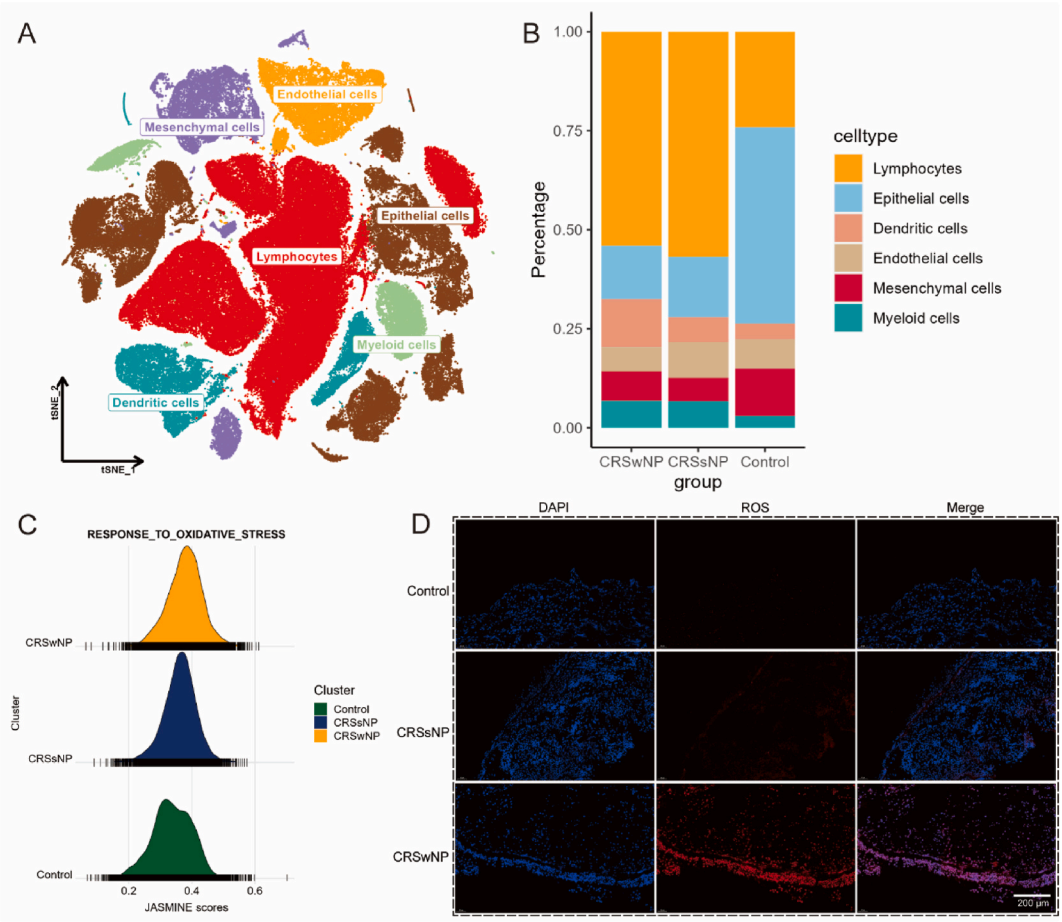

Fig. 1. Single-cell RNA sequencing reveals enhanced oxidative stress in the nasal epithelium of CRSwNP patients. A, tSNE plots display 149,029 cells from 21 individuals (5 controls, 5 CRSsNP patients, 11 CRSwNP patients) separated into 6 main clusters. B, Stacked bar chart shows significant differences in cellular composition between the control, CRSsNP, and CRSwNP groups, with lymphocytes, epithelial cells, and dendritic cells being the major cellular components of CRSwNP. C, Ridgeline plots by irGSEA suggest oxidative stress is present in the epithelial cells of the CRSwNP group. D, The ROS fluorescence intensity is significantly enhanced in the CRSwNP group, especially in the epithelial cells.

Then thoroughly wash the cells three times with RPMI 1640 medium, and subsequently observe the expression of ROS under a fluorescence microscope.

2.9. Statistical analysis

Quantitative clinical and demographic variables were expressed as the mean ± standard deviation (mean ± SD). In contrast, experimental data were shown as mean ± standard error of the mean (mean ± SEM). For the comparison between two groups,

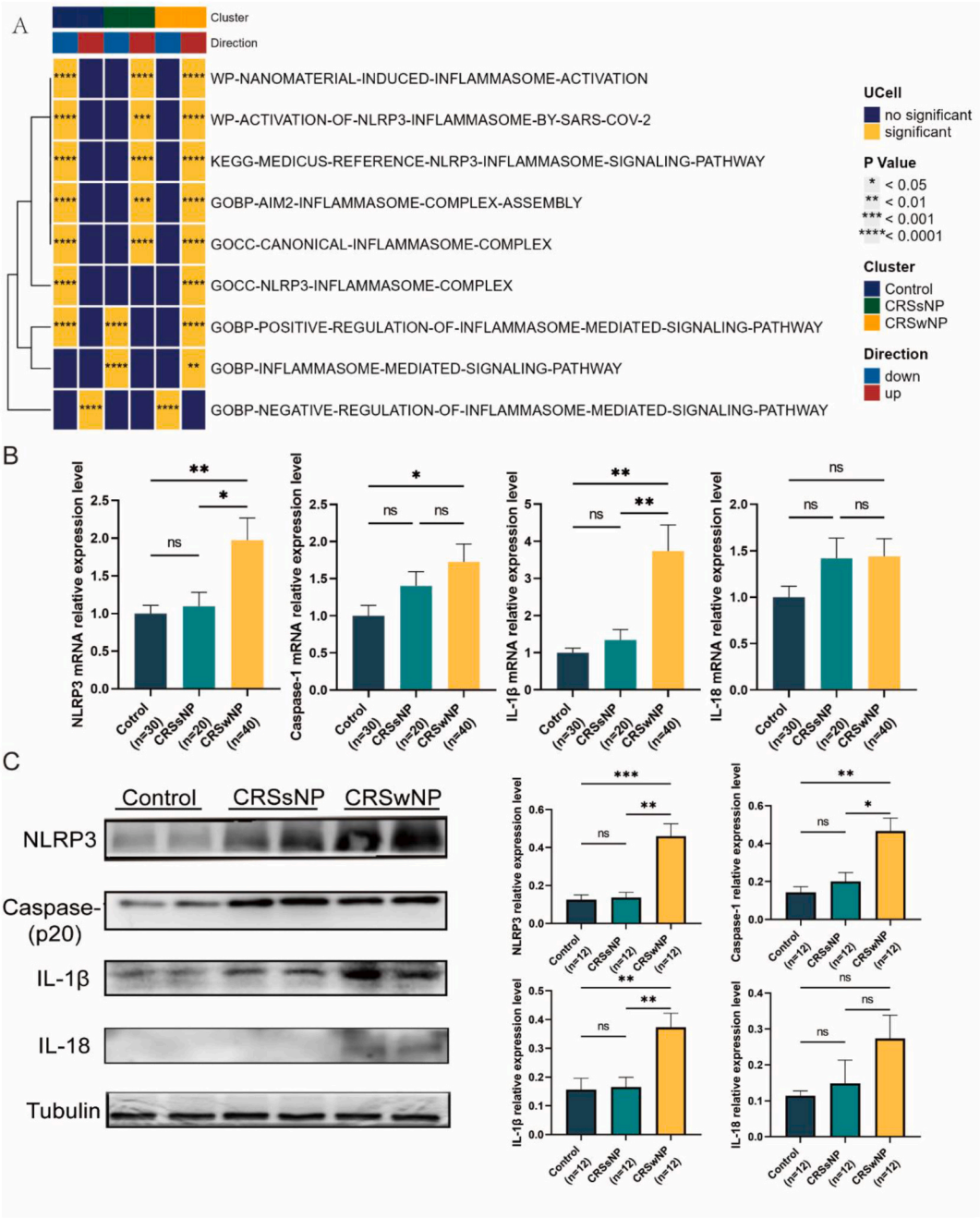

Fig. 2. The NLRP3 inflammasome are enhanced in the nasal polyps and contributes to the pathological mechanism of CRSwNP. A, The heatmap generated by irGsea indicates significant upregulation of inflammasome-related annotations in CRSwNP, particularly the NLRP3 inflammasome. B, qRT-PCR and Western blot analyses show that the tissue RNA and protein levels of NLRP3, Caspase-1, and IL-1β, which are key components of the NLRP3 inflammasome, are significantly increased in the CRSwNP patients. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

independent sample t-tests were used. For comparing more than two groups, one-way analysis of variance (ANOVA) was applied if the data adhered to the assumptions of normality and homogeneity of variances. When these assumptions were not met, non-parametric tests were used instead. Categorical variables were reported as frequencies, and comparisons between groups were made using the chi-square test. Statistical analyses were performed using SPSS version 26 (IBMCorp., Armonk, USA) and GraphPad Prism version 9.5 (GraphPad Software, San Diego, USA). A *p*-value less than 0.05 was considered to indicate statistical significance.

3. Results

3.1. ROS is involved in the pathological mechanism of CRSwNP

The analysis of oxidation stress in CRSwNP, based on the HRA000772 dataset, encompassed 5 control subjects, 5 CRSsNP subjects and 11 CRSwNP subjects. Following quality control, scaling and dimension reduction, 149029 cells were retained for subsequent analysis. Cell clustering identified six primary cell types based on the gene expression of cell markers, including epithelial cells, endothelial cells, myeloid cells, mesenchymal cells, lymphocytes, and dendritic cells (Fig. 1A). The proportions of these primary cell types varied significantly among groups, highlighting the heterogeneity of CRSwNP (Fig. 1B). Overall, lymphocytes, epithelial cells, and dendritic cells were the predominant components of nasal polyps.

The oxidative stress scores were evaluated in epithelial cells using functional terms associated with "oxidative stress". The result indicated that the scores significantly raised in CRSwNP group (Fig. 1C). ROS comprises oxidative stress products. To validate the level of oxidative stress, we assessed the ROS accumulation in nasal tissues. The results demonstrated a significant enhancement of ROS fluorescence signal in the CRSwNP group compared to the control and CRSsNP groups, particularly concentrated in the epithelial area of mucosa (Fig. 1D). This suggested that the accumulation of ROS in the nasal epithelium may be involved in the pathological process of CRSwNP.

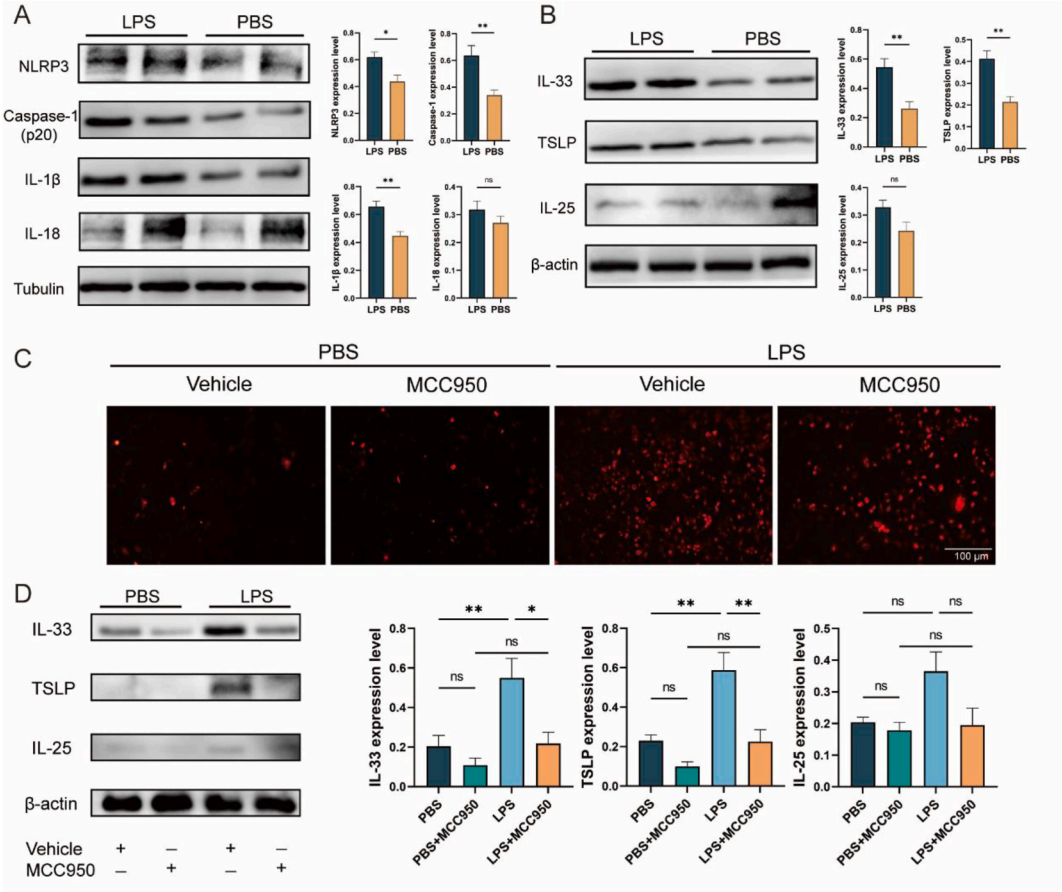

Fig. 3. LPS induces activation of NLRP3 inflammasome and promotes epithelial alarmin release in HNEpC. A, LPS stimulation significantly increases NLRP3 inflammasome synthesis in HNEpC. B, Epithelial alarmin IL-33 and TSLP are significantly upregulated in HNEpC after LPR stimulation. C, After treatment with the NLRP3 inhibitor MCC950, no significant difference was observed in ROS expression between the PBS and LPS groups. The red fluorescence represents ROS. D, MCC90 significantly attenuates LPS-induced increase of IL-33 and TSLP in HNEpC. $*P < 0.05$, $**P < 0.01$.

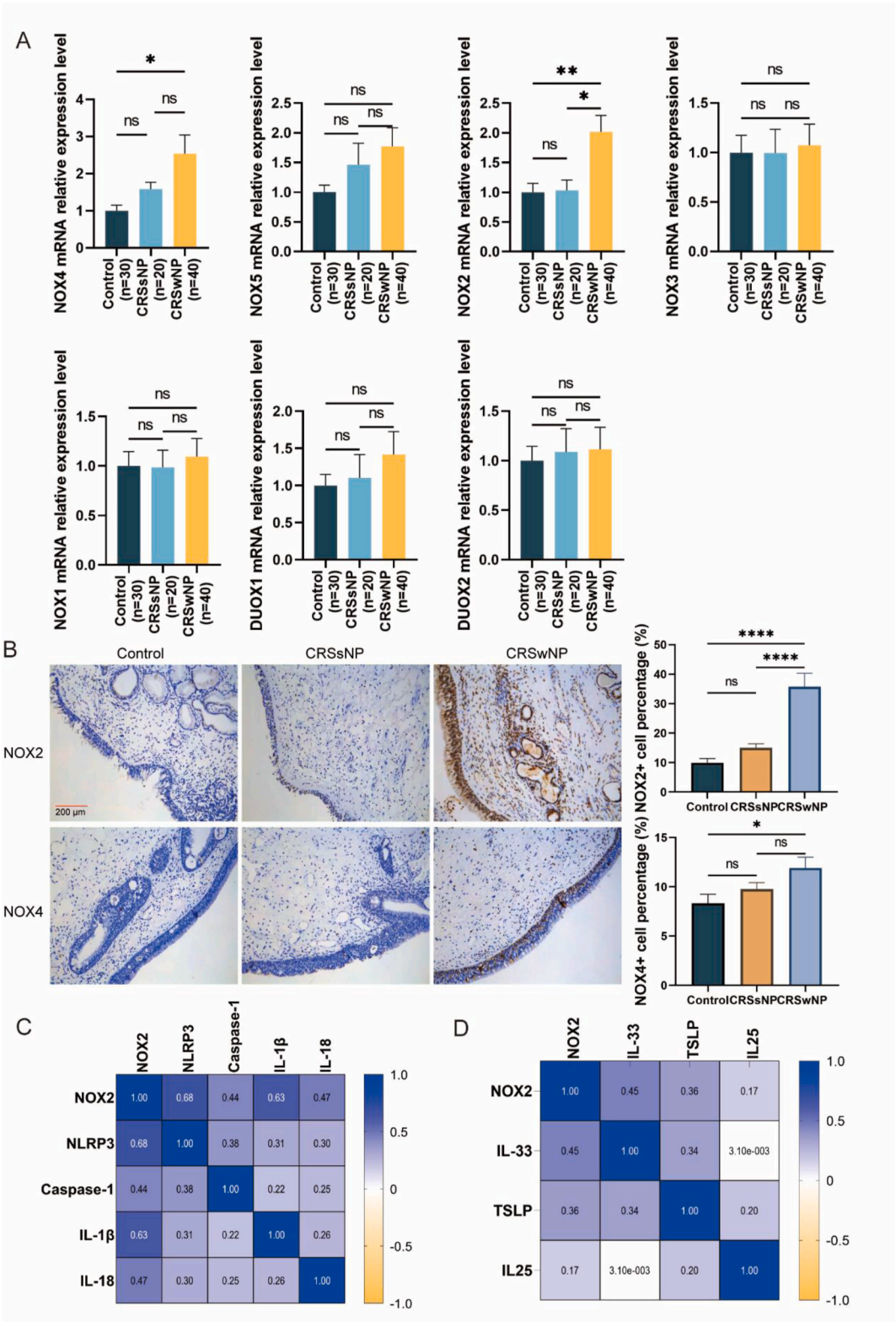

Fig. 4. NOX2 expression is significantly elevated in CRSwNP patients and linked to NLRP3 inflammasome and epithelial alarmin. A, Tissue NOX2 and NOX4 mRNAs are significantly upregulated in the CRSwNP group. B, Immunohistochemistry reveals higher NOX2 expression in the CRSwNP group, predominantly localized in the epithelial layer of the nasal polyps. C. The heatmap indicates a positive correlation between NOX2 and both the NLRP3 inflammasome and epithelial alarmin. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$.

3.2. Enhanced NLRP3 inflammasomes facilitate the release of epithelial alarmins contributing to the pathogenic mechanisms of CRSwNP

Considering the accumulation of ROS in the nasal epithelium in CRSwNP, we subsequently investigated the role of ROS in the generation of epithelial alarmins in nasal epithelial cells. Prior studies have suggested that ROS could activate inflammasomes, leading to their assembly and activation, thereby triggering inflammatory responses. Upon activation, inflammasomes process pro-inflammatory cytokines like IL-1β and IL-18 into their active forms, subsequently initiating inflammatory and immune responses

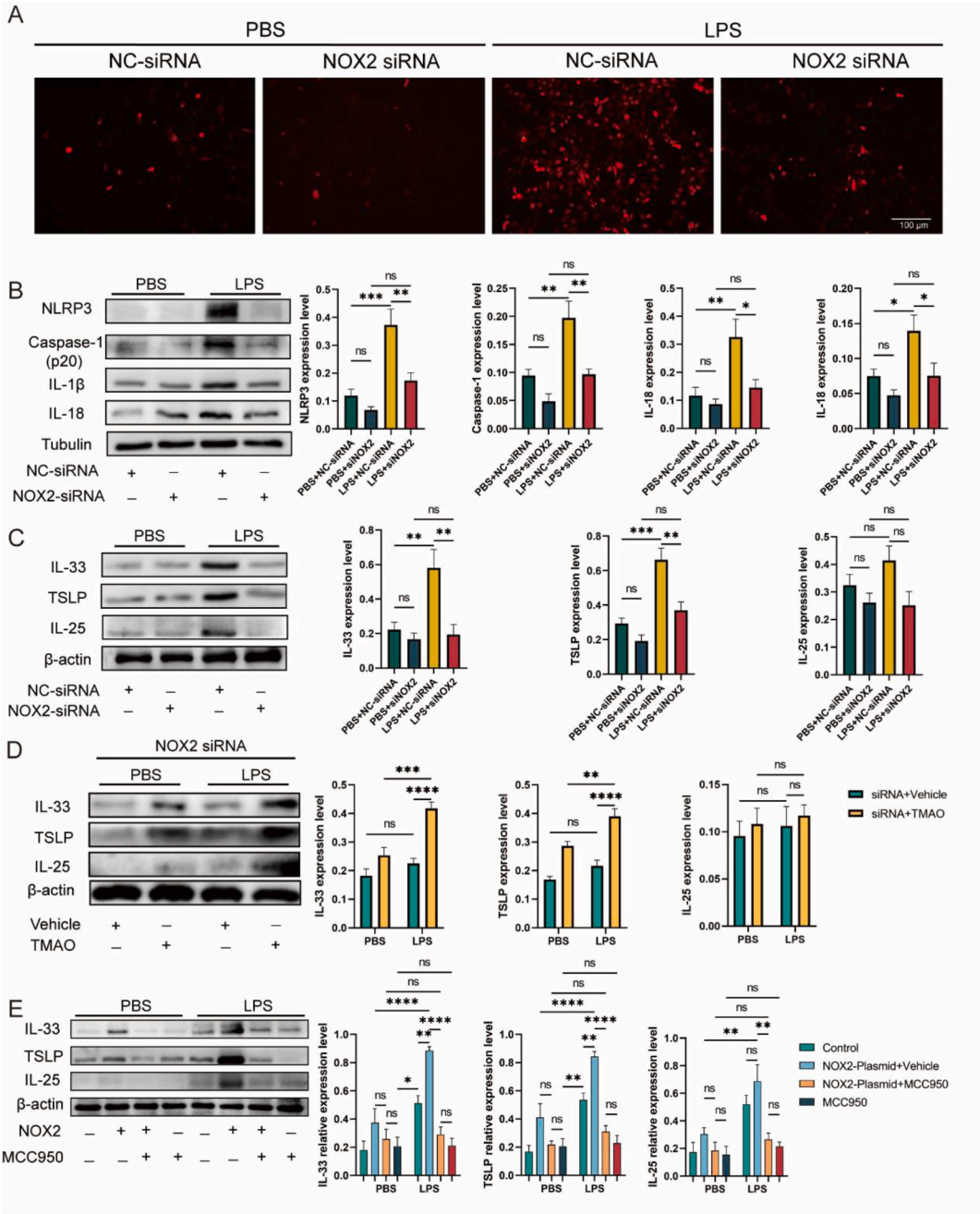

Fig. 5. NOX2 induces the release of epithelial alarmin in HNEpC via ROS/NLRPR inflammasome axis. A, Knockdown of NOX2 markedly reduces the LPS-induced increase in ROS levels in HNEpC, as indicated by the red fluorescence representing ROS. B, Knockdown of NOX2 significantly reduces the LPS-induced synthesis of the NLRP3 inflammasome in HNEpC. C-D, Knockdown of NOX2 reduces the LPS-induced synthesis of IL-33 and TSLP in HNEpC, but this effect is reversed by the NLRP3 inflammasome inducer TMAO. E, Transfection with the NOX2 overexpression plasmid enhances the LPS-induced synthesis of IL-33 and TSLP in HNEpC, and this effect is diminished by the NLRP3 inflammasome inhibitor MCC950. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$.

[34]. Consequently, we assessed the inflammasomes in epithelial cells, and the results revealed differential expression of multiple inflammasome-related annotations in CRSwNP (Fig. 2A). Notably, NLRP3 inflammasome annotation was significantly enriched in CRSwNP, suggesting its potential involvement in the pathogenesis of CRSwNP (Fig. 2A). We evaluated the mRNA and protein levels of NLRP3, Caspase-1, IL-18, and IL-1β in nasal mucosal tissues. The findings indicated significant increases in NLRP3, Caspase-1, and IL-1β in the CRSwNP group, which supports their potential role in the development of CRSwNP (Fig. 2B and C).

To investigate whether NLRP3 inflammasomes triggered the release of epithelial alarmins, we stimulated nasal epithelial cells with LPS and evaluated the NLRP3 inflammasomes and alarmins *in vitro*. The results demonstrated a significant upregulation of NLRP3, Caspase-1, IL-1β, IL-33, and TSLP expression in HNEpC following LPS stimulation (Fig. 3A and B). Subsequently, we targeted the NLRP3 inflammasome using MCC950 in HNEpC. Interestingly, MCC950 did not inhibit the expression of LPS-induced ROS (Fig. 3C). Moreover, upon NLRP3 inhibition, the expression of Caspase-1, IL-1β, IL-33, and TSLP markedly decreased compared to the PBS group (Fig. 3D and Supplementary Fig. 1A). These findings suggest that the activation of NLRP3 inflammasomes may influence the regulation of epithelial alarmins in HNEpC cells after LPS stimulation.

3.3. NOX2/ROS mediates activation of NLRP3 inflammasome and promotes release of epithelial alarmins

NOX enzymes have been identified as the primary source of ROS production and are known to play a crucial role in airway inflammatory diseases [17,35]. In our investigation of the role of NOXs in CRSwNP, we compared the expression of NOX1, NOX2, NOX3, NOX4, NOX5, DUOX1, and DUOX2 among groups. The results indicated significant upregulation of NOX2 and NOX4 in polyp tissues (Fig. 4A). Immunohistochemistry analysis further demonstrated elevated levels of NOX2 and NOX4 in the CRSwNP group, with NOX2 displaying more pronounced expression primarily in the mucosal epithelium (Fig. 4B). Hence, we propose that NOX2 and NOX4 may play a role in the pathogenesis and progression of CRSwNP. Notably, NOX2 exhibited a positive correlation with eosinophil counts and proportions in both peripheral blood and CRSwNP tissue samples (Supplementary Fig. 1B). Furthermore, our correlation analysis between NOX2 and NLRP3 inflammasomes and alarmins in nasal tissues indicated a significant correlation between NOX2 and NLRP3, Caspase-1, IL-1β, IL-33, and TSLP (Fig. 4C and D). In conclusion, with NOX2 specifically linked to nasal mucosal epithelial inflammation, it represents a promising target for future investigation.

To investigate whether NOX2 promotes the expression of epithelial alarmins through ROS/NLRP3, we further conducted functional experience *in vitro*. Following the silencing of NOX2 in HNEpC, there was a notable decrease in intracellular ROS levels (Fig. 5A), and the protein levels of NLRP3, Caspase-1, IL-1β, IL-18, IL-33, and TSLP were significantly diminished (Fig. 5B and C). This suggested that NOX2 may be an essential regulator of ROS accumulation, NLRP3 inflammasome activation, and epithelial alarmin expression in HNEpC. Furthermore, upon administering TMAO, a NLRP3 inducer, the expression of IL-33 and TSLP increased again (Fig. 5D). In another perspective, HNEpC was treated with the NOX2 overexpression plasmid together with MCC950. The results demonstrated that NOX2 overexpression increased the intracellular levels of IL-33 and TSLP, and inhibiting NLRP3 inflammasome activation reduced the upregulation of IL-33, TSLP, and IL-25 induced by NOX2 (Fig. 5E). In conclusion, NOX2 appears to play a role in the progression of chronic inflammation by triggering ROS accumulation, activating NLRP3 inflammasomes, and fostering oxidative stress in nasal epithelium.

4. Discussion

This study conducted a preliminary exploration of the interplay among NOXs, ROS, and the NLRP3 inflammasome in nasal mucosal epithelium. The findings revealed that the increased expression of NOX2 can intensify ROS accumulation and the release of epithelial alarmins in the nasal mucosal epithelium. Additionally, the use of NLRP3 inhibitors can attenuate the excessive production of epithelial alarmins induced by NOX2 upregulation, thereby impeding the progression of nasal mucosal inflammation.

The nasal mucosa epithelium, a crucial component of the innate immune system, plays a significant role in the occurrence and development of CRSwNP by mediating epithelial inflammation. Upon exposure to external environmental factors or pathogens, the nasal mucosa undergoes a robust oxidative stress response within epithelial cells, leading to the release of epithelial alarmins, such as IL-33, IL25, and TSLP [36]. These alarmins trigger immune cell infiltration, culminating in an inflammatory response and tissue damage within the nasal mucosa [37]. As a byproduct of oxidative stress, ROS are produced by epithelial cells during this response, with ROS accumulation being a key contributor to tissue damage and disease advancement in various chronic inflammatory conditions [38,39]. Our research has revealed heightened ROS production in mucosal epithelial cells of CRSwNP patients compared to control and CRSsNP groups, indicating a potential role of ROS in CRSwNP development. Furthermore, research has shown that *Staphylococcus aureus* enterotoxin B presence can elevate ROS levels in epithelial cells in polyp tissues of CRSwNP patients [40]. Although the primary focus of this study was on epithelial cells, the significant presence of ROS in the subepithelial region suggests its broader role in driving inflammation. A comprehensive understanding of the origins and effects of ROS production in both epithelial and immune cells will ultimately enhance our knowledge of CRSwNP development and progression. Our results confirmed that LPS can induce ROS synthesis, leading to increased expression of epithelial alarmins, implying a connection between ROS and inflammation in nasal mucosa epithelial cells. Therefore, further exploration into the origins of ROS production will enhance our understanding of CRSwNP pathogenesis.

NOX enzymes, which generate superoxide anion ($O_2^{\bullet-}$) and hydrogen peroxide (H_2O_2), play a significant role in various physiological and pathological processes [41,42]. Previous studies have suggested that NOXs may contribute to the accumulation of ROS, thereby influencing the development of airway inflammation [43,44]. Our findings have revealed a notable overexpression of NOX2 in epithelial layer of nasal polyps. This overexpression correlated positively with the proportion of eosinophils in peripheral blood and

tissues, indicating a potential influence of NOX2 on the progression of CRSwNP. Additionally, our findings proposed that ROS, as a consequence of NOX2 activity, served as a secondary messenger in the inflammatory response of epithelial cells, leading to the release of epithelial alarmins and promoting inflammation in nasal epithelial cells. Hitherto, further elucidation is required to clarify the specific mechanisms underlying the involvement of ROS mediated by NOX2 in the progression of CRSwNP.

As previously mentioned, the nasal mucosa epithelium serves as the first physiological barrier against external environmental stimuli, and acts as the frontline for pattern recognition receptors such as Toll-like receptors, NOD-like receptors, and protease-activated receptors in pathogen detection [17]. Earlier research on asthma has demonstrated that exposure of the airway epithelium to allergens can activate pattern recognition receptors, thereby leading to NLRP3 inflammasome activation and the release of inflammatory factors [45]. The NLRP3 inflammasome composed the NLRP3, Caspase-1, and the adaptor ASC, which leads to the release of active forms of IL-1β and IL-18, thereby inducing an inflammatory response [46]. Based on this, we confirmed upregulating expression of NLRP3, Caspase-1, IL-1β, IL-33, and TSLP in CRSwNP. Their expression levels positively correlate with NOX2, indicating that the NLRP3 inflammasome could be one of the downstream molecules of NOX2. Vitro experiments demonstrated that silencing NOX2 significantly reduced ROS expression and concurrently inhibits the expression of NLRP3, Caspase-1, IL-1β, IL-33, and TSLP. It indicated that NOX2 participated in the occurrence of chronic nasal mucosa inflammation through the NLRP3 inflammasome [47]. Notably, the NLRP3 inflammasome functions as a mediator of ROS, as studies suggested that ROS can trigger its activation, leading to subsequent inflammatory responses [47]. Our results indicated that the production of alarmins in nasal epithelium induced by NOX2 can be inhibited by MCC950, yet without substantial improvement in ROS accumulation. This implied that ROS might be an intermediary molecule in the activation of the NLRP3 inflammasome by NOX2.

5. Conclusions

In summary, our research identifies NOX2 as a novel target for inhibiting chronic inflammation in the nasal mucosa of CRSwNP for the first time. We elucidated the regulatory role of NOX2 on ROS and NLRP3, demonstrating its ability to activate the NLRP3 inflammasome through ROS, thereby triggering the release of alarmins and facilitating disease progression. Our research paves a new path for understanding the inflammatory process in the nasal mucosa through oxidative stress. Given the association between ROS and the NLRP3 inflammasome with various inflammatory diseases, exploring the potential of NOX2 knockdown in regulating oxidative stress may hold promise for treating chronic inflammatory diseases.

Ethics approval and consent to participate

All participants included in the study were approved by the Ethics Committee of Xiangya Hospital, Central South University (Ethics number: 202103112) with fully informed consent.

Availability of data and materials

The datasets generated and/or analysed during the current study are available in the China National center for Bioinformation, https://ngdc.cncb.ac.cn/gsa-human/browse/HRA000772.

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CRediT authorship contribution statement

Sijie Jiang: Writing – original draft, Software, Methodology, Data curation. **Benjian Zhang:** Methodology, Investigation. **Sihui Wen:** Investigation. **Shenghao Cheng:** Writing – original draft. **Yingchun Shen:** Data curation. **Shaobing Xie:** Writing – review & editing. **Zhihai Xie:** Conceptualization. **Weihong Jiang:** Writing – review & editing, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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