Mucin 5AC is significantly upregulated in exosomes from the nasal lavage fluid and may promote the expression of COX-2, VEGF and MMP-9: an implication in nasal polyp pathogenesis*

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Abstract

Background: Exosomes are critical mediators of intercellular communication and could be involved in many human diseases; however, little is known about the role of exosomes in nasal polyps (NP).

Methods: Exosomes in nasal lavage fluids (NLF) were isolated by ultracentrifugation. Exosome identity was validated by nanoparticle tracking analysis (NTA), transmission electron microscopy (TEM) and specific exosomal markers. The exosome proteome was revealed by LC-MS/MS, and the expression of the candidate exosomal protein, mucin 5AC, was confirmed by Western blot analysis and immunohistochemistry (IHC). Cellular uptake of the exosomes was monitored by fluorescence confocal microscopy and the ensuing effects on COX-2, VEGF and MMP-2/MMP-9 were determined by Western blotting, ELISA and gelatin zymography, respectively.

Results: Mass spectrometry analysis and subsequent verification by Western blotting identified that mucin 5AC was significantly upregulated in exosomes from NLFs of NP patients. Moreover, the expression of mucin 5AC was increased in the tissue specimens of the NP patients. Functional assays suggest that the mucin 5 AC-enriched exosomes could be effectively taken up by chronic rhinosinusitis without NP (CRSsNP)-derived fibroblasts, the control cells, resulting in a significant increase in the expression of COX-2, VEGF and MMP-9.

Conclusions: Mucin 5AC, the major airway mucin, cannot only be carried and transferred by nasal exosomes, but may also promote tissue remodeling and angiogenesis and thus could be a potential therapeutic target of NP.

Key words: exosome, mucin 5AC, nasal lavage fluid, nasal polyp

Introduction

Nasal polyps (NP) are noncancerous outgrowths originating from the mucosa of the nose and paranasal sinuses, and are commonly accompanied by chronic rhinosinusitis (CRS). The exact etiology and pathophysiology of NP are still controversial and likely multifactorial. However, mounting evidence indicates

that NP is typically associated with T helper 2 (Th2)-skewed eosinophilic infiltration and high IL-5 levels [1, 2]. In addition, other proinflammatory cytokines and chemokines, such as tumor necrosis factor α (TNF-α) and regulated upon activation normal T cell expressed and presumably secreted (RANTES), are also upregulated in NP tissues [3, 4]. Accordingly, it is generally speculative that chronic and deregulated inflammation arising as a response to various exogenous stimuli, including those triggered by microorganisms, contributes primarily to the disease. As such, many anti-inflammatory agents, in addition to glucocorticoids, have been intensively evaluated for NP treatment [5, 6].

Extracellular vesicles (EVs) are membrane-bound, nano-to-micrometer vesicles secreted from most mammalian cells. Depending primarily upon their intracellular origin, EVs are further classified into three subtypes, including exosomes, microvesicles and apoptotic bodies. Exosomes are spherical to cup-shaped membranous vesicles with a size ranging from 50 to 200 nm in diameter, and can be released by almost all cell types into the extracellular space and body fluids; for instance, saliva, blood, and urine [7]. A wide variety of protein compositions have been found in exosomes [8], including proteins associated with transmembrane transport and fusion, such as Rab GTPases and annexins, as well as those involved in exosome biogenesis, such as Alix and TSG101. Moreover, exosomes are enriched with heat shock proteins (HSP70 and HSP90) and tetraspanins (CD9, CD63, CD81, and CD82) that are commonly used as the molecular markers of exosomes. Furthermore, depending on the cellular origin from which they are derived, exosomes may contain various cell- or state-specific protein cargos [9]. It is generally believed that exosomes i) act as cellular garbage bags that discard excess and/or unwanted cellular constituents, and ii) most importantly, play a major role in intercellular communication, modulating various cellular processes [10].

The role of exosomes in human diseases has been widely documented [8]. Most notably is the association between exosomes and numerous hallmark features of cancer; exosomes have emerged as a promising biomarker for cancer diagnosis and therapy [11]. Compared to cancers, less is known about the relationship between exosomes and inflammatory diseases. It has been reported that exosomes could participate in endothelial cell communication, transferring proinflammatory factors and promoting endothelial dysfunction and inflammation [12]. Besides, plasma-derived exosomes may induce inflammation and contribute to chronic heart failure [13]. Furthermore, epithelial cell-derived exosomes may play a proinflammatory role in allergic airway inflammation [14]. There are several reports that suggest an important role of exosomes in NP. P-glycoprotein (P-gp) was found to be enriched in mucus-derived exosomes from NP patients, and A disintegrin and metalloprotease 10 (ADAM10), a ubiquitously expressed metalloprotease, in NP-derived exosomes has been shown to promote angiogenesis and vascular permeability in vitro [15, 16]. However, a recent study shows that exosomes released by nasal epithelial cells derived from NP patients significantly reduce the proliferation of the control cells [17]. Therefore, the pathophysiological role of exosomes in NP is not fully understood.

In this study, we investigated whether exosomes are involved in the pathogenesis of NP. To potentiate future clinical application, we chose to study the protein composition of exosomes derived from the nasal lavage fluid (NLF) of NP patients. We found that mucin 5AC, the primary gel-forming mucin in human airways, was significantly upregulated in the exosomes. Additionally, our results suggest that the exosomal mucin 5AC in the nasal microenvironment could impact the pathophysiology of NP.

Table 1. The demographic characteristics of patients in the immunohistochemical studies.

Demographic data	CRSsNP (n=11)	CRSwNP (n=16)
Gender (M/F)	(5/6)	(12/4)
Age (y), mean	52.73	49.75
Atopy	4	5
Asthma	0	0
Cystic fibrosis	0	0

Materials and methods

Patients and tissues

The study was approved by the Institutional Review Board (IRB) of Kaohsiung Medical University Hospital (KMUHIRB-F(I)-20180020). Based on the definition in European position paper on rhinosinusitis and nasal polyps 2012, patients who were 20 years of age or older with latest diagnosis of bilateral CRS with NPs (CRSwNP) or CRS without NP (CRSsNP) and underwent elective endoscopic sinus surgery at our hospital were recruited. Patients with malignancies, asthma, upper respiratory tract infection within four weeks prior to the surgery, or having any previous nasal surgery were excluded from the study. The presence of atopy was confirmed by patient history and positive Phadiotop test. All the enrolled subjects were instructed to stop any topical or systemic corticosteroid or antibiotics two weeks before the surgery. Nasal polyp tissues (CRSwNP) (n = 20) and control concha bullosa mucosa from CRSsNP (n = 22) were procured during routine endonasal surgeries for subsequent Western blot and/or immunohistochemical analyses. The demographic data of patients studied by the immunohistochemical analysis were summarized in Table 1.

Nasal lavage fluid collection and exosome isolation

Nasal lavage fluids (NLFs) from CRSsNP and CRSwNP patients were collected immediately prior to operation when the patients were under general anesthesia with oral intubation [18]. Briefly, 20

ml of warm saline solution was instilled and irrigated into each nostril with a syringe. NLFs were then collected by aspirating the lavage fluids into another empty syringe. Bleeding of nasal mucosa was avoided during the aspirating procedure. The NLF exosome isolation procedure was adapted from the ultracentrifugation method previously described by Lasser et al. with minor modifications [18]. The samples were centrifuged at 300 × g for 10 min at 4˚C to remove the unwanted cell content. To isolate the NLF's exosomes, the supernatants were first centrifuged at 16,500 × g for 20 min at 4˚C to further remove residual unwanted content, filtered through a 0.2 μm filter and then ultracentrifuged at 85,000 × g for 70 min at 4˚C to spin down the exosomes.

Nanoparticle tracking analysis (NTA)

NTA was performed using a Malvern NanoSight LM10-HS instrument (NTA Nanosight software version 3.0). Briefly, an aliquot of the isolated exosomes were diluted in PBS (1:500-1:1000) to achieve optimal particle density, and 3 sequential measurements (~ 1 min per measurement) at 21°C (viscosity 1.0 cp) were conducted following the manufacturer's instructions.

Transmission electron microscopy (TEM)

The isolated exosomes (10 μg) in PBS were placed on a 200 mesh Formvar/Carbon-coated TEM grid (Ted Pella, Inc, Redding, CA, USA) for 30 min. The grid with the absorbed exosomes was fixed with 2.5% glutaraldehyde in 4% paraformaldehyde for 10 min. After washing with PBS, the grid was incubated with 1% osmium tetroxide (Sigma-Aldrich, St. Louis, MO, USA) for 1 h at 4˚C and then dehydrated with 99% ethanol for 20 min. The grid was air-dried before being examined under the electron microscope (Hitachi HT7700).

Exosome labeling using a fluorescent dye PKH67

The NLF's exosomes were labeled using the PKH67 kit (Sigma-Aldrich) based on the manufacturer's instructions. The exosomes (10 μg) in PBS were resuspended in diluent C and then mixed with PKH67 for 5 min to achieve a final dye concentration of 5 μM in a SW41 centrifuge tube (Beckman Coulter Life Sciences, Indianapolis, IN, USA). To stop the staining, an equal volume of exosome-free fetal bovine serum (FBS) (Gibco, Grand Island, NY, USA) was added and incubated for 1 min, followed by the addition of culture media with 5% exosome-free FBS to fill up the centrifuge tube and then ultracentrifugation at 85,000 × g for 90 min. After washing and pelleting by ultracentrifugation, the exosomes were resuspended in culture media with 5% exosome-free FBS and stored at 4˚C.

Uptake of the fluorescence-labeled NLF's exosomes by CRSsNP-derived fibroblasts

Primary culture of CRSsNP-derived fibroblasts was established and maintained as previously described [19]. The fibroblasts were seeded on a four-well chamber slide (4.5×10^4 cells/well) (SPL Life Sciences, Pocheon-si, Korea) and incubated for 24 h in culture media with 5% exosome-free FBS. PKH67-labeled NLF's exosomes from the CRSwNP patients were then added to the cells and co-incubated for 24 h. The cells were washed three times with PBS and fixed with 4% paraformaldehyde overnight at 4˚C. After staining with DAPI nuclear stain (300 nM) for 3 min, the cells were observed under a confocal microscope.

Mass spectrometry and protein identification

The exosome samples were analysed by liquid chromatography-tandem mass spectrometry (LC-MS/MS) using the Waters nanoACQUITY Ultra Performance Liquid Chromatograph (UPLC) system (Waters MS Technologies, Manchester, UK) coupled to an electrospray ionization (ESI)- quadrupole-quadrupole-time-of-flight (QqTOF) instrument (Impact HD, Bruker Daltonics, Billerica, MA, USA). For protein identification, tandem mass spectrometry peak lists were extracted and then compared with the Uniprot protein database using the Mascot software Version 2.5 (Matrix Science, London, UK). The detailed protocol for LC-MS/MS and criteria for protein identification were elsewhere described [20].

Western blot analysis and antibodies

Five micrograms of each exosome sample was mixed with SDS sample buffer and run on 12% SDS-PAGE gels for detecting the exosome markers, with the remaining procedures being carried out according to our previous protocol [21]. To detect mucin 5AC with high molecular weight (> 500 kDa), a 5% SDS-PAGE without the upper stacking gel was applied. Antibodies used in Western blot analysis were anti-CD9, -COX-2 and -annexin V (Cell Signaling, Danvers, MA, USA), anti-mucin 5AC and -CD63 (arigo Biolaboratories, Taiwan) and anti-TSG101 (GeneTex, Irvine, CA, USA). For Western blot analysis on tissue samples, tissue lysates were prepared as previously described [19] and 10 μg of each sample was analysed by SDS-PAGE as above.

Immunohistochemistry

A non-biotin, two-step polymeric detection system was used to detect mucin 5AC protein expression based on our previous protocol [19]. The intensity of immunostaining was scored based on the percentage of positively stained cells in the mucosal epithelia as follows: negative, <5%; weak, 5-30%; moderate, 30-60%; and strong, >60%.

NLF's exosome treatment and VEGF detection by ELISA

CRSsNP-derived fibroblasts were seeded on 12-well culture dishes (1×10^5 cells/well). The cells were then treated with NLF's exosomes (1:1000 or 2 ng/ml exosomal protein) from CRSwNP patients for 24 h. The culture media were replaced with fresh ones containing 5% exosome-free FBS, and the cells were incu-

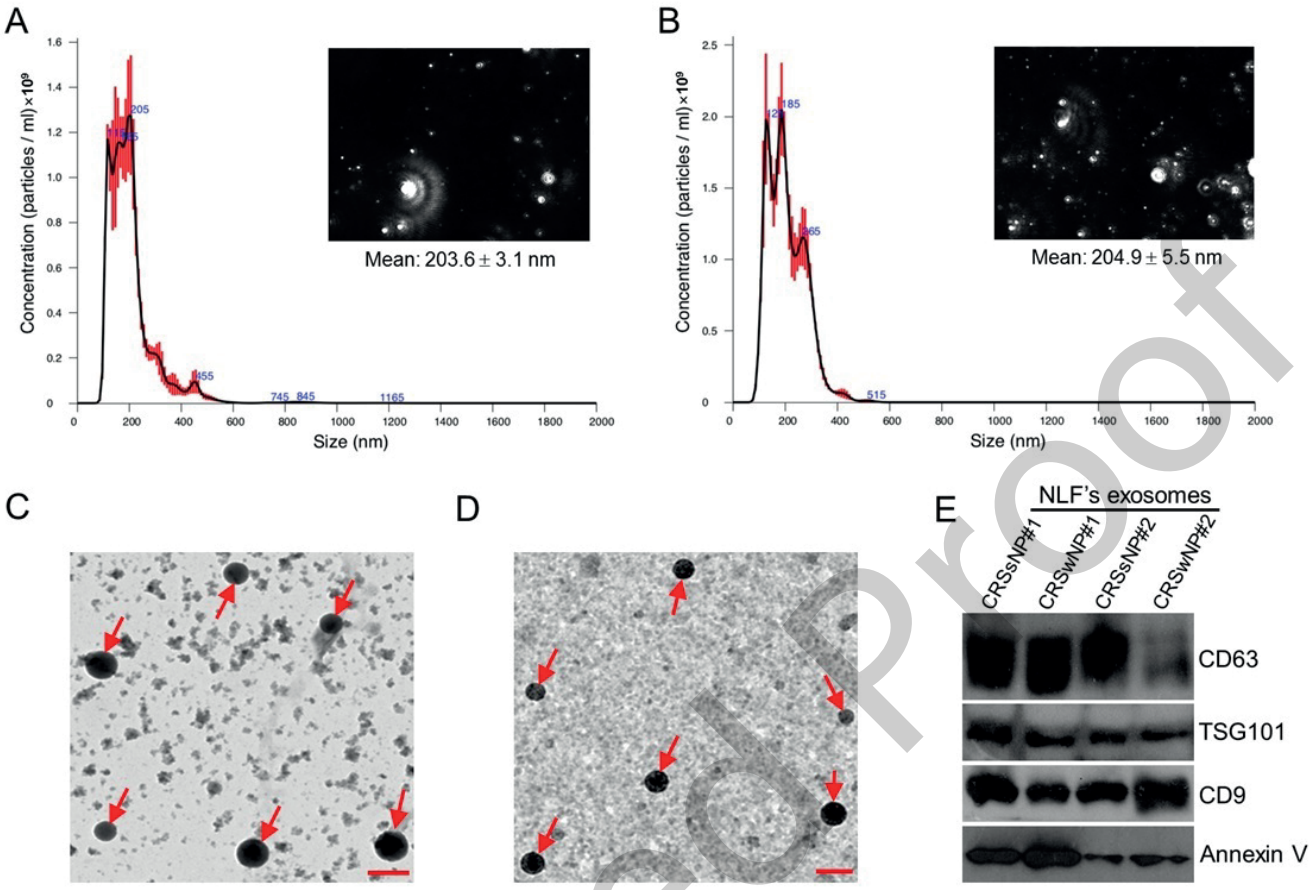

Figure 1. Verification of the identity and purity of the nasal lavage fluid (NLF) exosomes. The mean diameter size, size distribution and concentration of exosomes from the NLF of a CRSsNP patient (A) and a CRSwNP patient (B) were analysed by nanoparticle tracking analysis (NTA). The upper right panels show the exosome particle images. Transmission electron microscopy (TEM) images of the exosomes (indicated by the arrows) from the CRSsNP patient (C) and the CRSwNP patient (D). Scale bars: 100 nm. (E) The exosome characterization was further confirmed by the expression of four known exosomal protein markers.

bated for another 24 h. The conditioned media were collected for ELISA assay, and the cell lysates (10 μg/each) were used for the detection of mucin 5AC and COX-2 expression by Western blot analysis. The ELISA assay was performed using the Human VEGF Quantikine ELISA Kit (R&D Systems, Minneapolis, MN, USA) based on the manufacturer's instructions.

Gelatin zymography

The production of MMP-2 and MMP-9 was measured by gelatin zymography. The conditioned culture media of CRSsNP-derived fibroblasts were first concentrated by a speed vacuum and then separated on a 7.5% SDS-PAGE gel containing 2 mg/ml gelatin (Sigma-Aldrich, St. Louis, MO, USA) under non-reducing conditions. The gels were washed in wash buffer containing 2.5% Triton X-100 in 50 mM Tris-HCl, pH 7.4, followed by incubation in reaction buffer (50 mM NaCl, 15 mM $CaCl_2$, 0.05% Brij-35 in 50 mM Tris-HCl, pH 7.4) at 37˚C for 24 h. To visualize the gelatinolytic bands, the gels were stained with Coomassie Blue and then washed in destaining buffer until clear bands are visible.

Statistical analysis

The immunohistochemical results were further evaluated by Fisher's exact (chi-square) test to reveal the correlation between mucin 5AC expression and CRSwNP. The ELISA results of VEGF expression were analysed by Student's t-test. Probability value below 0.05 ($p < 0.05$) was considered statistically significant.

Results

Identification and characterization of exosomes from the nasal lavage fluid (NLF)

After collection and purification of extracellular vesicles from the NLF of CRSsNP and CRSwNP patients, to confirm the exosome identity, we first used nanoparticle tracking analysis (NTA) to reveal the concentration, mean size and size distribution of the vesicles. The majority of the vesicles in the NLF of a NP patient (CRSwNP#1) and a control subject (CRSsNP#1) had particle sizes ranging from 50-250 nm (Figure 1A and B). Consistently, transmission electron microscopy (TEM) images showed that most of the vesicles had a diameter of around 100-150 nm (Figure 1C

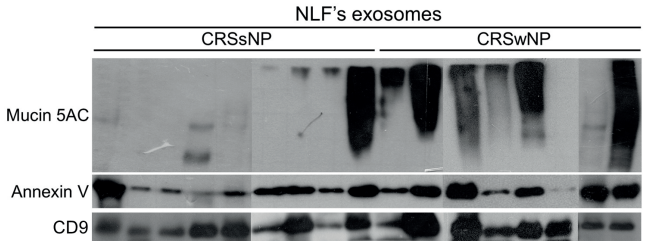

Figure 2. An increased level of mucin 5AC in the NLF's exosomes of CRSwNP patients. Following the LC-MS/MS analysis, mucin 5AC was chosen for further verification by Western blotting in 8 and 9 exosome samples from CRSwNP and CRSsNP patients, respectively. Note that mucin 5AC has a molecular weight of 586 kDa, and is expected to be a smear on the blot due possibly to deglycosylation and degradation. Annexin V is a known exosomal marker.

and D). Furthermore, this and an additional pair of vesicle samples from the NLF of CRSwNP#2 and CRSsNP#2 were analysed by immunoblotting using antibodies against four widely accepted exosome markers, including CD63, Annexin V, TSG101 and CD9. We found that the expression of all the four markers could be detected in the vesicle samples, indicating successful isolation of exosomes (Figure 1E). Together, these data have confirmed the identity of the isolated vesicles as exosomes.

Increased expression of mucin 5AC in exosomes from the NLF of NP patients

The isolated exosomes of CRSwNP#1 and CRSsNP#1 were analysed for protein composition by LC-MS/MS. Five candidates with consistently higher scores and a larger number of matched non-repeated peptides were selected for further verification (supplementary Table 1). While the expression of all the candidates in the NLF's exosomes was verified, mucin 5AC appeared to be more significantly upregulated in the NP patient (supplementary Figure 1). Furthermore, to confirm this result in additional samples, we found that mucin 5AC was highly expressed in 6 out of 8 NLF's exosomes of NP patients, as opposed to only 1 out of 9 NLF's exosomes of the control subjects (Figure 2). These results suggest that exosomal mucin 5AC is selectively upregulated in the NLF of NP patients.

Mucin 5AC expression is significantly upregulated in NP tissues

Since the NLF's exosomes are conceivably secreted from the nasal mucosa, we then investigated whether mucin 5AC expression in the mucosal tissues might correlate with that in the NLF's exosomes. Tissue lysates from 12 CRSsNP and 15 CRSwNP patients were analysed by Western blotting for the detection of mucin 5AC expression. Compared to the CRSsNP samples, mucin 5AC seemed to be more significantly expressed in the CRSwNP samples (Figure 3A). Immunohistochemical (IHC) analysis on paraffin-embedded tissue sections revealed that mucin 5AC expression was localized primarily to the upper epithelial layers of the CRSwNP tissue specimens (Figure 3B). Furthermore, consistent with the results in Figure 3A, the vast majority of the CRSsNP tissues (9/11) had negative to weak mucin 5AC staining

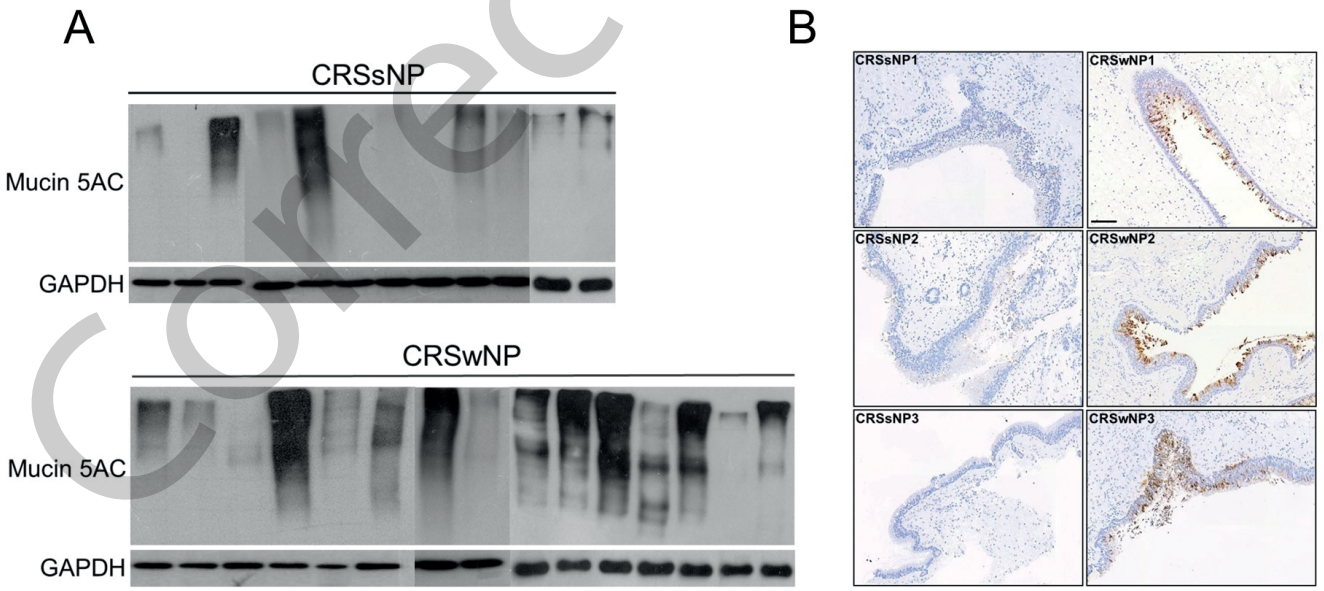

Figure 3. The expression of mucin 5AC is significantly upregulated in CRSwNP tissue specimens. (A) Mucin 5 AC expression in 12 CRSsNP and 15 CRSwNP fresh tissues was examined by Western blot analysis. GAPDH was the loading control. (B) Paraffin-embedded tissue sections of 15 CRSsNP and 18 CRSwNP were analysed by IHC using anti-mucin 5AC antibody. Shown here are the three representative results of positively stained CRSwNP and negatively stained CRSsNP, respectively. Mucin 5AC was mostly expressed at the upper epithelial layers. Scale bar: 100 μm for all the results. Magnification: x 100.

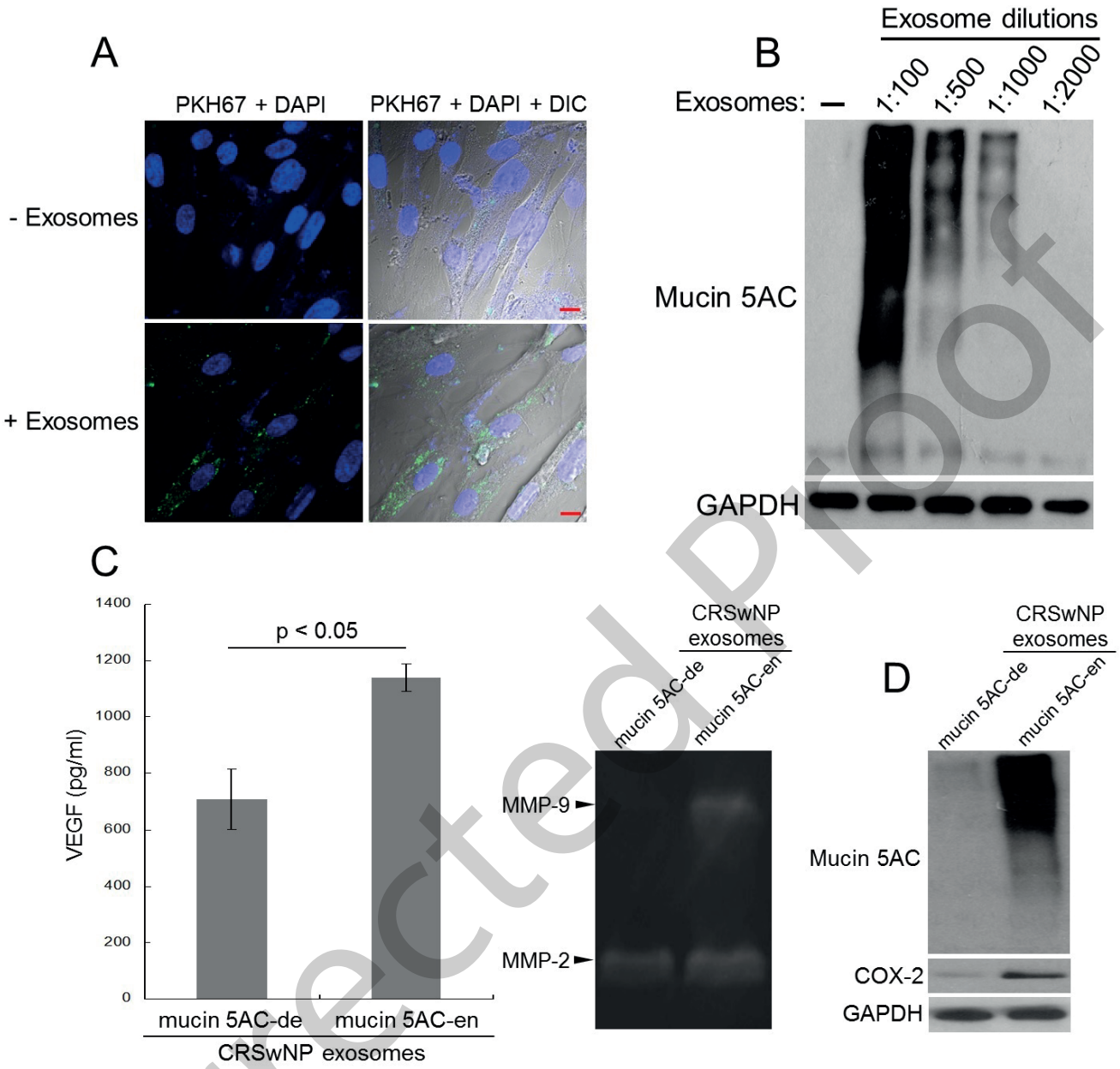

Figure 4. Exosome uptake and the effect of exosomal mucin 5AC in CRSsNP-derived fibroblasts. (A) CRSsNP-derived fibroblasts were co-cultured with PKH67-labeled exosomes isolated from the NLF of CRSwNP patients for 24 h (1:1000 dilution). The CRSsNP cells cultured in exosome-free but PKH67-containing media were served as the negative control. Nuclei were stained with DAPI (blue) and exosomes were the green fluorescent puncta in the cytoplasm. Images were captured under a confocal microscope. DIC: differential interference contrast. Scale bar: 10 mm. (B) CRSsNP-derived fibroblasts were treated with the indicated increasing dilutions (initial exosomal protein concentration = 2 mg/ml) of exosomes from the NLF of the CRSwNP patients for 24 h. The cell lysates were analysed by Western blotting for the detection of mucin 5AC expression. The fibroblasts were then treated with the 1:1000 dilution of NLF's exosomes from the CRSwNP patients for 24 h. After another 24 h, the conditioned media were subjected to ELISA assay for VEGF and gelatin zymography for MMP-2/MMP-9 (MMP-2: 72 kDa; MMP-9: 92 kDa) (C), while the corresponding cell lysates were analysed by Western blotting for mucin 5AC and COX-2 expression (D). Mucin 5AC-de: mucin 5AC-deficient; mucin 5AC-en: mucin 5AC-enriched.

intensity, whereas nearly 70% of the CRSwNP tissues (11/16) had moderate to strong mucin 5AC staining intensity ($p < 0.01$) (Table 2).

Uptake of the mucin 5AC-enriched exosomes of NP patients increases the expression of COX-2, VEGF and MMP-9 in CRSsNP-derived fibroblasts

To evaluate the role of exosomal mucin 5AC in NP pathophysiology, we first tested whether the NLF's exosomes could mediate

Table 2. Immunohistochemical and statistical studies on the expression of mucin 5AC in nasal tissues.

Tissue Types	Staining intensity		p value[a]
	Negative + Weak	Moderate + Strong	
CRSsNP	9	2	0.0097**
CRSwNP	5	11	

[a] Data were analysed by Fisher's exact test.

**$p < 0.01$ indicates significant difference in the expression level of mucin 5AC between CRSsNP and CRSwNP.

the transfer of mucin 5AC to the recipient cells. The purified NLF's exosomes from the NP patients were labeled with a green fluorescent PKH67 dye and then added to the culture media of CRSsNP-derived fibroblasts. Following a 24 h co-incubation period, the cells were examined by a confocal microscope. The microscopic results demonstrated that the labeled exosomes could be effectively taken up by the fibroblasts, as evidenced by the punctate fluorescence in the cytoplasm (Figure 4A). More importantly, mucin 5AC protein carried by the exosomes could be readily detected by Western blotting after the co-incubation of CRSsNP-derived fibroblasts with various dilutions of the NLF's exosomes (Figure 4B). These data have validated protein cargo transfer (i.e. mucin 5AC) by the NLF's exosomes to the recipient cells.

We then investigated how the exosomal mucin 5AC could impact the cellular and physiological function of the recipient cells. After treating CRSsNP-derived fibroblasts with the NLF's exosomes of the NP patients, the production of VEGF and MMP-2/MMP-9 in the conditioned media was measured by ELISA and gelatin zymography, respectively. The results showed that cells treated with mucin 5AC-enriched exosomes had a significantly increased level of VEGF ($p < 0.05$) and MMP-9, but not MMP-2, in comparison to those treated with mucin 5AC-deificent exosomes (Figure 4C). Furthermore, the expression of COX-2 was higher in the cells treated with mucin 5AC-enriched exosomes than those treated with mucin 5AC-deficient exosomes (Figure 4D). These results indicate that exosomal mucin 5AC may enhance the expression of COX-2, VEGF and MMP-9 in CRSsNP-derived fibroblasts.

Discussion

Due possibly to the complex yet controversial etiology and pathophysiology [22], NP remains one of the most difficult challenges in clinical rhinology. To date, the exact pathophysiological and etiological mechanisms leading to the development of nasal polyps are poorly understood. However, it is generally agreed that the polyp tissue is infiltrated with different types of immune cells, many of which may produce a wide variety of cytokines, chemokines and growth factors, thus helping to sustain inflammation and growth of NP presumably through autocrine- or paracrine-based regulation. Thus, it is conceivable that there is intense demand for cell-cell communication in NP microenvironment. Exosomes, being important cell-cell communicators and transporters, therefore warrant in-depth research in NP pathogenesis.

To potentiate future clinical application, instead of studying exosomes from cultured cells, we chose to investigate exosomes from the nasal lavage fluid (NLF) of NP patients. After successful isolation and verification of exosomes from the NLF of CRSsNP and CRSwNP patients, the exosomal protein composition was revealed by LC-MS/MS. Among the candidates whose presence in the exosomes was identified by the LC-MS/MS analysis, mucin 5AC, the major airway glycoprotein, was found to be more significantly expressed in the NLF's exosomes of a cohort of NP patients. Consistently, several mucins, including mucin 5AC, were previously identified to be enriched in exosomes from primary airway epithelial cells and non-small cell lung cancer (NSCLC) cell lines and patients [23, 24]. Although several proinflammatory cytokines can induce the overexpression of mucin 5AC in NP [25-28], how these biological effects might impact the downstream cellular and physiological responses remains virtually unknown. Thus, together with its known functions relevant to the disease (supplementary Table 1), we decided to further investigate the role of mucin 5AC in NP pathophysiology.

Because exosomes are extracellular vesicles originated from the parent cells, we speculated that the high mucin 5AC expression in the NLF's exosomes might therefore suggest increased cellular mucin 5AC expression. Indeed, mucin 5AC was highly expressed in more than 50% (8/15) of the fresh tissues of CRSwNP, in sharp contrast to those of CRSsNP in which only about 17% (2/12) of the samples had high mucin 5AC expression. Furthermore, IHC studies on tissue sections showed that most of the CRSwNP specimens (11/16 or 69%) were stained moderately or strongly for mucin 5AC ($p < 0.01$). These results are consistent with previous reports that mucin 5AC is upregulated in CRSwNP [25-29]. On the other hand, mucin 5AC is known to be secreted from the goblet cells, the highly polarized epithelial cells with mucin-containing granules at the apical side [30]. This may thus help to confirm our observation that mucin 5AC expression was mostly localized toward the apical surface of the nasal epithelium.

Aside from its widely acknowledged role as a defensive and protective barrier in airway mucosa, whether or not mucin 5AC might have additional biological functions in NP microenvironment remains obscure. Given that exosomes are important mediators of intercellular communication, we therefore reasonably predicted that exosomal mucin 5AC could play a regulatory role, exerting a significant impact on neighboring cells. To understand the biological effect of exosomal mucin 5AC, we first

tested if cultured cells could take up the mucin 5AC-enriched exosomes from the NLF. After the co-incubation of CRSsNP-derived fibroblasts with green fluorescent dye (PKH67)-labeled exosomes from the NLF of the CRSwNP patients, there were plentiful fluorescent puncta in the cytosol, indicating effective exosome uptake. Furthermore, in agreement with this result, mucin 5AC could be delivered into the cells via the exosomes in a dose-dependent manner.

We next investigated whether the exosomal mucin 5AC might play a causative role in the development of NP. Intriguingly, mucin 5AC was shown to enhance the expression of integrins, matrix metalloproteinases (MMPs) and vascular endothelial growth factor (VEGF) in pancreatic cancer cells, suggesting its role in regulating cancer cell adhesion and motility [31]. Significantly higher levels of MMPs and VEGF in patients with CRSwNP are also well documented [32-37]. Furthermore, both MMPs and VEGF have been shown to play pivotal roles in tissue remodeling and angiogenesis [38, 39], the distinctive characteristics and critical processes during NP formation. Since fibroblasts are one of the major stromal cells that produce MMPs and VEGF, accordingly, we tested the possibility that the exosomal mucin 5AC might promote the production of MMP-2/MMP-9 and VEGF in CRSsNP-derived fibroblasts. To more specifically evaluate the effect of exosomal mucin 5AC on the recipient cells, we compared the production of MMP-2/MMP-9 and VEGF between cells treated with mucin 5AC-deficient and mucin 5AC-enriched exosomes from the NLF of the CRSwNP patients. We found that the expression level of VEGF and MMP-9, but not MMP-2, was significantly increased in CRSsNP-derived fibroblasts treated with the mucin 5AC-enriched exosomes. Given that COX-2, the enzyme catalyzing the conversion of arachidonic acid into prostaglandin E2 (PGE2), was reported to act upstream and enhance the expression of MMP-9 and VEGF [40-42], we verified whether COX-2 might be upregulated by mucin 5AC in the cells. Indeed, the expression level of COX-2 was higher in the cells treated with mucin 5AC-enriched exosomes. These results suggest that mucin 5AC might promote the production of MMP-9 and VEGF via the COX-2/PGE2 pathway; whether or not mucin 5AC can enhance the activity of the pathways in vivo might be demonstrated in the NP mouse model. Besides, the exact molecular mechanism underlying the induction of COX-2, MMP-9 and VEGF by mucin 5AC requires further investigation.

Conclusion

Exosomes have emerged as critical mediators of intercellular communication, impacting several human diseases; however, previous implications in the role of exosomes in NP are rather limited. Although a number of recent studies suggest that NP-derived exosomes may affect the biological functions, including cell proliferation and angiogenesis, in NP pathophysiology [15, 17], which specific component in the exosomes accounts for such effects awaits further verification. Our results suggest that mucin 5AC, the major mucin in nasal mucosa, can not only be carried and transferred by nasal exosomes, but might also impact the cellular and physiological functions in nasal microenvironment via such exosome-mediated intercellular communication. We showed that mucin 5AC was significantly upregulated in the NLF's exosomes and tissue specimens of the NP patients. Most importantly, the exosomal mucin 5AC of the NP patients could be effectively delivered into the control cells, resulting in a significant increase in the expression of COX-2, VEGF and MMP-9. Together, these findings suggest that exosomal mucin 5AC could modulate tissue remodeling and angiogenesis and thus could be a potential therapeutic target for NP.

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Authorship contribution

Study concept and design: JYFC, LFW; sample collection and processing: LFW, CCH, YRW; data acquisition, analysis and interpretation: LFW, CHL, SSL, CCH; writing and drafting of the manuscript: JYFC; critical review of the manuscript: JYFC, CYC, CHL; obtaining funding: LFW, JYFC; study supervision: JYFC; final approval: all authors.

Conflict of interest

None to declare.

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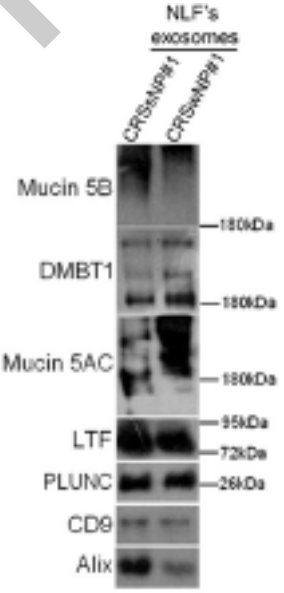
SUPPLEMENTARY DATA

Table 1 Five exosomal protein candidates identified by mass spectrometry and their known biological functions

Protein Name	Background information	Mass data (score/matched non-repeated peptides)		
		CRSsNP	CRSwNP #1	CRSwNP #2
Mucin 5AC	1. high molecular weight, heavily glycosylated proteins produced by epithelial tissues in most animals. 2. key components of the gel-like airway mucus, functioning from lubrication to cell signaling to forming biochemical and physical barriers as the first line of defense. 3. mucin 5AC has been linked to functions other than innate immunity, such as cell proliferation, adhesion, invasion and inflammation.	1633/22	828/19	2797/47
Mucin 5B		1615/19	841/19	2640/44
BPI Fold-Containing Family A Member 1 (PLUNC)	1. a secretory protein specifically expressing in the airways and nasopharyngeal regions. 2. inhibits the epithelial sodium channel, and also has anti-microbial functions. 3. plays a role in innate immunity in the airways.	4604/22	434/6	376/6
Deleted In Malignant Brain Tumors 1(DMBT1)	1. a tumor suppressor gene that plays a role in the interaction of tumor cells and the immune system. 2. In addition to the role in mucosal innate immunity that defends against invading pathogens, DMBT1 has been shown to affect cell proliferation, differentiation, migration, inflammation and angiogenesis.	2333/14	1354/15	1989/18
Lactotransferrin (LTF)	1. an iron-binding glycoprotein of the transferrin family. 2. widely distributed in various exocrine secretions, such as milk, saliva, tears, and nasal mucus. 3. has antimicrobial activity and is an important part of innate immune system.	820/5	1655/19	1388/25



Sup Figure 1. Identification and verification of mucin 5AC upregulation in the NLF's exosomes of the CRSwNP. The five candidates identified from the mass spectrometry results (Table1) were verified by Western blot analysis. CD9 and Alix are known exosomal markers. Molecular weights: mucin 5B: 590 kDa; DMBT1: 260 kDa; mucin 5AC: 580 kDa; LTF: 78 kDa; PLUNC: 27 kDa.