Impairment of cilia architecture and ciliogenesis in hyperplastic nasal epithelium from nasal polyps

Ying Ying Li, BM, MS,[a]* Chun Wei Li, BM, PhD,[a]* Siew Shuen Chao, MD,[a] Feng Gang Yu, PhD,[a] Xue Min Yu, MD, PhD,[b] Jing Liu, BM, MS,[a] Yan Yan, PhD,[a] Liang Shen, PhD,[c] William Gordon, PhD,[d] Li Shi, MD, PhD,[b,e]* and De Yun Wang, MD, PhD[a] *Singapore, Shandong, China, and Irvine, Calif*

Background: Aberrant airway epithelial remodeling is one of the cardinal histopathologic features of inflammatory airway diseases, but whether it alters the mucociliary apparatus remains unknown.

Objective: We sought to investigate the morphologic pattern of motile cilia and ciliogenesis-associated makers in hyperplastic nasal epithelium from nasal polyps (NPs) both *in vivo* and *in vitro*.

Methods: Biopsy specimens obtained from patients with NPs (n = 44) and inferior turbinate from healthy control subjects (n = 38) were analyzed by using scanning electron microscopy, immunofluorescence staining, single-cell (cytospin) staining, quantitative real-time PCR, and human nasal epithelial stem/progenitor cell culture and differentiation.

Results: Abnormal cilia architecture (untidy, overly dense, and lengthened) was more commonly observed in patients with NPs by using scanning electron microscopy. Ectopic lengthened cilia were visualized by means of immunofluorescence (patients with NPs: 6.33 μm [5.51-7.43 μm] vs control subjects: 3.73 μm [3.50-4.27 μm], $P < .0001$), at the site of epithelial hyperplasia in isolated single cells (patients with NPs: 6.55 ± 0.23 μm vs control subjects 4.89 ± 0.24 μm, $P < .0001$), and in differentiated ciliated cells derived from human nasal epithelial stem/progenitor cells (patients with NPs: 9.20 ± 0.56 μm vs control subjects: 5.21 ± 0.37 μm, $P < .0001$). Ciliary beat frequency was found to be significantly slower in patients with NPs than control subjects *in vitro*. Both protein and mRNA levels of ciliogenesis-associated markers (centrosomal protein 110 [CP110], forkhead box J1 [Foxj1], and P73 isoform with an N-terminal transactivation domain [TAp73]) were significantly increased in patients with NPs versus those seen in control subjects and were positively correlated with cilia length.

Conclusion: For the first time, this study demonstrates for that motile cilia impairment is a co-condition of epithelial hyperplasia in patients with NPs, and this impairment of function is a likely cause of chronic mucosal inflammation or infection (eg, biofilm) observed in patients with chronic rhinosinusitis. (J Allergy Clin Immunol 2014;134:1282-92.)

Key words: *Impairments of cilia architecture, nasal polyps, hyperplasia*

The nasal epithelium not only serves as a mechanical barrier to protect against environmental factors, microorganisms, and toxins but also participates in both innate and adaptive immune responses.[1-3] In the normal airway epithelium, a delicate and tight balance of self-renewal and physiologic differentiation is regulated by key gene expression networks and molecular pathways.[4,5] Aberrant signaling under various disease conditions can easily disrupt this homeostatic balance, leading to pathologic airway remodeling. During inflammatory stress, the nasal epithelium frequently undergoes injury, followed by a rapid remodeling phase. This response can range from epithelial hyperplasia, to goblet cell metaplasia, to denudation, to loss of cilia, fibrosis, or even basement membrane thickening.

Nasal polyps (NPs) are associated with chronic mucosal inflammation and are accompanied by abnormal epithelial remodeling. In patients with NPs, the epithelium appears to respond inappropriately to injury, and this can lead to aberrant epithelial damage, including hyperplasia or squamous metaplasia.[2,6] Multiple reports have also demonstrated that an excess of ciliated cells showing abnormal cilia or loss of cilia can be found on squamous metaplastic epithelium.[7-9] However, the underlying mechanisms leading to epithelial damage and formation of abnormal cilia remain unknown.

Key markers of airway epithelium and the ciliogenesis process include centrosomal protein 110 (CP110), forkhead box J1 (Foxj1), and P73 isoform with an N-terminal transactivation domain (TAp73). It was demonstrated by a recent study that increased expression of CP110 in mucosa from patients with chronic

From [a]the Department of Otolaryngology, Yong Loo Lin School of Medicine, National University of Singapore; [b]the Department of Otolaryngology, Qilu Hospital, Shandong University; [c]the Biostatistics Unit, National University of Singapore, National University Health System, Singapore; [d]the Department of Medicine and Biological Chemistry, University of California, Irvine; and [e]the Key Laboratory of Otorhinolaryngology, Ministry of Health, Shandong University.

*These authors contributed equally to this work.

Supported by grants from the National Medical Research Council (NMRC; CIRG12Nov033) and from the Singapore Immunology Network (SIgN; SIgN 10-028) of Singapore and the National Nature Science Foundation of China (grant awarded number 81170897).

Disclosure of potential conflict of interest: Y. Y. Li, C. W. Li, S. S. Chao, J. Liu, Y. Yan, and D. Y. Wang have received research support from the National Medical Research Council (Singapore) and the Singapore Immunology Network and are employed by the National University of Singapore. F. G. Yu has received research support from the Singapore Immunology Network and is employed by the National University of Singapore. L. Shi and X. M. Yu have received research support from the National Nature Science Foundation of China and are employed by Shandong University (China). L. Shen is employed by the National University of Singapore. W. Gordon is employed by the University of California, Irvine.

Received for publication February 17, 2014; revised June 19, 2014; accepted for publication July 15, 2014.

Available online September 4, 2014.

Corresponding author: De Yun Wang, MD, PhD, Department of Otolaryngology, Yong Loo Lin School of Medicine, National University of Singapore, 1E Kent Ridge Rd, Singapore 119228. E-mail: entwdy@nus.edu.sg. Or: Li Shi, MD, PhD, Department of Otolaryngology, Qilu Hospital, Shandong University, 107 Wenhua West Rd, Jinan, Shandong, P. R. China 250012. E-mail: shili126@sina.com.

0091-6749/$36.00

http://dx.doi.org/10.1016/j.jaci.2014.07.038

Abbreviations used
ALI: Air-liquid interface
CBF: Ciliary beat frequency
hNESPC: Human nasal epithelial stem/progenitor cell
IF: Immunofluorescence
IHC: Immunohistochemistry
NP: Nasal polyp
SEM: Scanning electron microscopy
TEER: Transepithelial electrical resistance
TFI: Total fluorescence intensity

rhinosinusitis might contribute to the poor ciliation observed in these patients[10]; however, the pathway involved remains unclear. Foxj1 is a commonly reported ciliogenesis-associated maker that participates in multiple steps of this process, including centrosome multiplication, docking, and cilia elongation, and is often used as a specific marker in monitoring motile cilia formation.[11] TAp73 is a specific marker of airway ciliated columnar cells, and in our previous study its mRNA levels were found to be upregulated in patients with NPs.[12,13] A combined study of these markers might provide greater insights into the pathogenesis of chronic rhinosinusitis with NPs and the pathways involved in ciliated cell development and function during the disease process.

Recently, we have been successful in isolating adult human nasal epithelial stem/progenitor cells (hNESPCs) from nasal biopsy specimens of healthy subjects and patients with NPs.[14-16] Single cell–derived colonies stain uniformly for basal cell markers, such as p63 and keratin 5, and about 80% of colonies show long-term self-renewal potential because they can be propagated successively for at least an estimated 20 to 50 additional doublings while maintaining an immature phenotype.[14] The lineage potential of these cells has been assessed through multiple differentiation assays. Pedigree lines developed from single cells can differentiate into properly stratified mucociliary airway epithelium composed of both ciliated columnar cells and goblet cells. The aim of this study was to investigate the morphologic pattern of motile cilia and ciliogenesis-associated makers (eg, Foxj1, CP110, and TAp73) in primary nasal mucosal tissue from healthy subjects and patients with NP, with further confirmation performed *in vitro* with hNESPCs derived from the same donors.

METHODS

Study population

Approval to conduct this study was obtained from the National Healthcare Group Domain-Specific Review Board of Singapore (Singapore) and from the Institutional Review Boards of Qilu Hospital, Shandong University (China). Adult patients with NP and healthy subjects (control subjects) were recruited from the Department of Otolaryngology, National University Health System (Singapore), and the Department of Otolaryngology, Qilu Hospital (China). The clinical characteristics of the study subjects are shown in Table I. Tissues from patients with NPs were obtained from 44 patients with bilateral NPs (grade 2 or 3, which have partially or totally blocked nasal pathway)[1] who underwent functional endoscopic sinus surgery. Nine patients received a short course (3 days) of oral glucocorticoid treatment before surgery. Physician-diagnosed asthma was reported in an additional 9 patients, and some of them were being treated with either a β_2-agonist (n = 3) or β_2-agonist plus glucocorticoid inhaler (n = 2). Control biopsy specimens were obtained from the inferior turbinate of 38 patients with septal deviation who were scheduled for septal plastic surgery. None of the control subjects had upper respiratory tract infections or any type of rhinosinusitis; 10 control subjects had allergic rhinitis, but they were not taking any forms of glucocorticoids, antiallergic agents (eg, antihistamines and antileukotrienes), or antibiotics within 3 months before the study.

Fresh tissue specimens were prepared for different analyses: scanning electron microscopy (SEM), immunofluorescence (IF) staining, single-cell (cytospin) staining, quantitative real-time PCR, and hNESPC culture and differentiation. Because of the limited size of some tissue biopsy specimens in both patients with NPs and control subjects, not all specimens could be used for every analysis (see details of sample use in Table II).

hNESPC culture and differentiation at the air-liquid interface

Primary epithelial cells were isolated from fresh nasal specimens, and hNESPCs were cultured in our progenitor cell culture system.[14] The hNESPCs were then transferred to an air-liquid interface (ALI) system to become fully differentiated ciliated cells within 4 weeks.[17] During the differentiation process, transepithelial electrical resistance (TEER) measurements were performed on a weekly basis. Details of the ALI culture and TEER procedure are provided in the Methods section in this article's Online Repository at www.jacionline.org.

SEM

Tissue specimens and differentiated cells in Transwell inserts (Corning, Corning, NY; 35 ± 2 days in ALI culture) were prepared according to a standard protocol for SEM (the full method is described in the Methods section in this article's Online Repository). The surfaces of both tissue epithelium and Transwell inserts were examined with a JEM-1010 scanning electron microscope (JEOL, Tokyo, Japan).

Immunohistochemistry and IF

Cytospin samples (primary cells from nasal biopsy specimens and epithelial cells from ALI cultures), nasal tissue sections, and Transwell membranes were used to perform immunohistochemistry (IHC) and IF staining. Details of antibodies and staining procedures are provided in the Methods section in this article's Online Repository at www.jacionline.org.

Evaluation of staining results

The researchers independently assessed all cases in a blind fashion to have a standardized histologic evaluation of the staining (including both IHC and IF).

Evaluation of histologic staining. For evaluation of the epithelial structure, an arbitrary scoring system was developed (for full details, see the Methods section in this article's Online Repository), and epithelial tissue with more than 4 layers was considered hyperplastic.[12] Evaluation of epithelial cell markers (p63 and TAp73) and leukocyte counts (eosinophil and neutrophil) were evaluated histologically. Details of the evaluation methods are described in the Methods section in this article's Online Repository.

Measurement of cilia length, cilia area, and ciliated cell percentage by using IF staining. Cilia length was evaluated in nasal tissues, primary cells isolated from nasal biopsy specimens, and ciliated cells from ALI cultures by assessing βIV-tubulin staining at ×400 magnification. Five areas of cilia staining from the tissue sections or cytospin cells were randomly selected. Cilia length was measured with ImageJ software.[18] The mean values of cilia length were calculated from 20 measurements for each paraffin-embedded section, 10 to 30 measurements for each primary cell sample, and 30 to 70 measurements for cells from each ALI culture.