Role of p63/p73 in epithelial remodeling and their response to steroid treatment in nasal polyposis

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Background: Nasal polyposis (NP) is recognized as aberrant epithelial remodeling, but the molecular mechanism underlying this process is poorly understood. Two important p53 homologues (p63 and p73) play a key role in orchestrating the epithelial development.

Objective: We intended to study whether p63 and p73 are involved in the epithelial remodeling seen in patients with NP and their response to oral glucocorticosteroid (GC) treatment.

Methods: Nasal polyp tissues were obtained from 65 patients, and inferior turbinates were obtained from 19 control subjects without NP. Among patients with NP, 20 were treated with oral prednisone, so that 2 sets of polyp biopsy specimens were taken before (GC naive) and after (GC treated) treatment. Immunohistochemistry and quantitative PCR were performed to determine the expression levels of p63 and p73.

Results: The increase in p63-positive cell numbers was significant in GC-naive NP epithelium (46%) compared with that seen in control epithelium (5%), and it was positively related to the epithelial hyperplasia in patients with NP. The increase in N-terminal transactivation domain p73–positive cell numbers was found in 27% of GC-naive patients with NP and 16% of control subjects, with no statistical difference. The mRNA expression of both p63 and p73 was significantly upregulated in GC-naive patients with NP versus control subjects, and a positive correlation between the p63 and p73 mRNAs was found in all nasal tissues. Furthermore, the improvement of epithelial structure and reduction of p63 mRNA/protein levels were found in patients with NP after GC treatment.

Conclusions: Our results suggest that the ectopic expression of p63 in multiple cell layers is an important pathologic phenomenon in the epithelial remodeling seen in chronically inflamed airway epithelium (eg, in patients with NP), and its aberrant expression can be suppressed with GC treatment. (J Allergy Clin Immunol 2011;127:765-72.)

Key words: *Nasal polyposis, epithelial remodeling, p63, p73, glucocorticosteroids*

The human nasal epithelium not only represents the first line of mucosal defense against various pathogens and allergens but also actively participates in the immunoregulatory response in nasal mucosa. Because the nasal epithelium is susceptible to attack by noxious agents and airborne particles, maintaining its homeostasis and self-renewal properties is critical for the physiological function of the nasal mucosa. Epithelial repair processes in the nasal mucosa are highly organized and well coordinated and involve migration, proliferation, and differentiation of epithelial cells, as well as the interactions between epithelial cells and stromal cells.[1]

Nasal polyposis (NP) is associated with chronic mucosal inflammation and is accompanied by infiltration of inflammatory cells and abnormal tissue remodeling. In patients with NP, the epithelium responds inappropriately to acute or chronic injury and displays signs of dysregulated restitution, which might lead to epithelial damage after aberrant remodeling (hyperplasia or squamous metaplasia). Although a group of transcription factors (eg, activator protein 1),[2] cytokines (eg, IL-6),[3] and growth factors (eg, epidermal growth factor)[1] might play some role in the repair process in airway tissues, the mechanism of epithelial remodeling in patients with NP remains obscure.

p63 is a member of the p53 gene family.[4] Two main isoforms of p63 were identified: one with an N-terminal transactivation domain (TA isoform) and another without it (N-terminal truncated domain [ΔN] isoform). Unlike the tumor suppressor gene p53, overexpression of p63 (especially ΔNp63) has been associated with tumorigenesis in various epithelial cancers.[5-7] p73 is another p53 homologue gene that appears to express at a high level in many human malignancies and is considered to have an oncogenic potential.[8] The function of p73 TA and ΔN isotypes is distinct because TAp73 is generally considered to have similar properties as wild-type p53, whereas ΔNp73 antagonizes the induction of TAp73 and p53-induced genes.[8] In addition, the balance of TAp73/ΔNp73 has been found to be a valuable prognostic factor correlated with the response of chemotherapy and survival of tumors.[9]

In airway epithelium basal cells are considered to have stem/progenitor cell activity, which can differentiate into other epithelial cell types, such as goblet cells and columnar ciliated and nonciliated cells.[10] p63 is constitutively present in the basal cells of many epithelial tissues and is the key gene known to be

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Abbreviations used
ΔN: N-terminal truncated domain
GAPDH: Glyceraldehyde-3-phosphate dehydrogenase
GC: Glucocorticosteroid
H&E: Hematoxylin and eosin
HRP: Horseradish peroxidase
IPA: Ingenuity pathways analysis
NF-κB: Nuclear factor κB
NP: Nasal polyposis
TA: N-terminal transactivation domain

essential for the self-renewal of the stratified epithelial stem cell.[4] p63 knockout mice demonstrated a lack of limbs and a loss of stem cells of all stratified epithelia.[11] Recently, TAp73 has been specifically detected in airway ciliated columnar cells (Mckeon and Xian, unpublished data) and regulates epithelial cell apoptosis.[9,12] Our previous microarray study[2] has also identified a significant upregulation of p63 and p73 in patients with NP compared with that seen in healthy control subjects (unpublished data). In this study we sought to investigate the expression levels of p63 and p73 in patients with NP and explore whether these markers are involved in the NP epithelial hyperplasia and their response to oral glucocorticosteroid (GC) treatment.

METHODS

Study subjects and sample collection

Adult patients with NP and control subjects without NP were recruited from the Department of Otolaryngology of the First Affiliated Hospital, Sun Yat-Sen University, and the Department of Otolaryngology in the Qilu Hospital, Shandong University, China. The clinical characters of the studied subjects are shown in Table I. NP tissues were obtained from 65 patients with bilateral NP (grade 2 or 3, which includes a partially or totally blocked nasal pathway[13]) who underwent functional endoscopic sinus surgery. Among the patients with NP, 20 subjects were treated with oral prednisone (10 mg thrice per day for 10 days) immediately after hospitalization, so that 2 sets of polyp biopsy specimens were taken from the same patient before the initiation of treatment (GC naive) and after treatment (GC treated). Biopsy specimens of inferior turbinate mucosa were obtained from 19 subjects without NP with septal deviation who were scheduled for septal plastic surgery as healthy control subjects. None of the patients with NP and control subjects had upper respiratory tract infections or undertook any forms of GC and antibiotics within 3 month before the study. All tissues (both patients with NP and control subjects) were fixed in formalin for histologic evaluation. Tissue samples from control subjects (n = 19), GC-naive patients with NP (n = 51), and GC-treated patients with NP (n = 20) were preserved with RNAlater (Ambion, Austin, Tex) for detecting gene expression. Approval to conduct this study was obtained from the Institutional Review Boards of Sun Yet-Sen University (China), Shandong University (China), and the National University of Singapore (Singapore).

Immunohistochemistry

Retrieved nasal biopsy specimens (from patients with NP and control subjects) were embedded in paraffin and sectioned at 4 μm with a Leica microtome (Leica, Wetzlar, Germany). Paraffin sections of nasal samples were stained with hematoxylin and eosin (H&E) and immunohistochemical staining. Eosinophils were observed by using H&E staining, whereas neutrophils were stained with murine anti-human neutrophil elastase mAb (clone NP57; Dako A/S, Glostrup, Denmark).

Protein expression of neutrophil elastase, p63, and TAp73 was examined by means of immunohistochemical staining with a modified horseradish peroxidase (HRP) technique with the DakoCytomation EnVision+System-HRP (Dako A/S). Slides were processed with Target Retrieval Buffer (Dako A/S). Endogenous peroxidase activity was blocked with 3% H_2O_2. They were stained with murine anti-human neutrophil elastase mAb (Clone NP57, Dako A/S), murine anti-human p63 mAb (clone 4A4),[14] and murine anti-human TAp73 mAb (Clone 3A6; Mckeon and Xian, unpublished data)[12] at dilutions of 1:2,000, 1:300, and 1:10, respectively. TAp73 is the isoform of p73 that contains the TA domain. Species- and subtype-matched antibodies were used as negative controls (N-Universal Negative Control for murine IgG, Dako A/S). Slides stained with primary antibodies and negative controls were incubated at 4°C overnight. The slides were then incubated with DAKO EnVision+System-HRP (Dako A/S) at room temperature for 30 minutes. Diaminobenzidine was used as a substrate for color development. All slides were counterstained with hematoxylin.

Evaluation of immunohistochemical patterns

Eosinophil and neutrophil counting. Two hundred leukocytes were counted (at ×400 magnification) in 5 individual fields with infiltration of inflammatory cells, and then the percentage of eosinophils or neutrophils was calculated by using the following formula:

$$\left(\frac{\text{Positive - staining cells}}{200\ \text{Leukocytes}} \times 5\right) \times 100\%.$$

Nasal tissues were categorized as eosinophilia or neutrophilia when the percentage of eosinophils or neutrophils exceeded 10%.

Evaluation of epithelial cell markers. For evaluation of the epithelial structure, an arbitrary scoring system was developed: score 1, normal or no obvious hyperplastic epithelial structure (the layer of epithelial cells is ≤4); score 2, epithelial hyperplasia (the layer of epithelial cells is >4).

For evaluation of p63 and TAp73, a modified semiquantitative scoring system considering the extent of immunoreactivity was performed.[2] The extent of immunoreactivity of p63 and TAp73 antibodies within the epithelial region was graded as follows: score 0, negative staining; score 1, 1 to 2 layers of positive cells; and score 2, more than 2 layers of positive cells. Overexpression of p63 and TAp73 is defined when the score is 2.

To have a standardized histologic evaluation of the staining (including both H&E staining and immunostaining), the researcher independently assessed all cases in a blind fashion.

Quantitative PCR

One microgram of the total RNA was reverse transcribed with TaqMan Reverse Transcription Reagents Kit (Applied Biosystems, Foster City, Calif) based on the manufacturer's protocol. Real-time RT-PCR analysis was performed to evaluate the expression levels of p63, p73, and two p73 isoforms (TAp73 and ΔNp73). The TaqMan assays (Applied Biosystems) included the following: p63, Hs00978343_m1; p73, Hs01056230_m1; TAp73, Hs01056228_m1; and ΔNp73, Hs01065727_m1. Glyceraldehyde-3-phosphate dehydrogenase *(GAPDH)* was used as a housekeeping gene and was purchased from TaqMan Endogenous control (Applied Biosystems). Both target and reference *(GAPDH)* genes were amplified in separate wells in triplicate. Relative gene expression was calculated by using the comparative $2^{-\Delta\Delta Ct}$ methods,[15] with *GAPDH* as a reference.

Microarray and Web-based bioinformatics analysis

DNA microarrays containing 38,500 genes on the HG-U133 Plus 2.0 arrays were used to characterize the global gene expression profile in 8 pairs of patients with NP (GC naive and GC treated) and 5 healthy control subjects in a previous study.[2] The p63-related biological functions and diseases and the p63 upstream and downstream genes were explored by using ingenuity pathways analysis (IPA; version 8.7, Ingenuity Systems, www.ingenuity.com). IPA is a Web-delivered application that enables the easy search and summarization of the scientific literature for a single gene or a group of gene molecules (eg, microarray data). Each gene identifier is mapped to its corresponding gene object in the ingenuity pathway knowledge base, which provides up-to-date high-quality knowledge of the gene information. Once keying in "p63"