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Original Article

14-3-3 gene expression exerts isoform-dependent functions in sinonasal pathophysiology

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ABSTRACT

The expression profiles of 14-3-3β and θ isoforms, known to exert both oncogenic and antiapoptotic effects, were assessed in different entities of nasal pathophysiology.

Flow cytometry and immunohistochemistry were used on paraffin-embedded sections of 51 inverted papillomas (IP), 26 nasal polyps (NP), 9 polyps with IP (NPIP) and 10 specimens of normal epithelium (NE).

14-3-3β expression was significantly upregulated in IP as compared with both NP ($p = 0.015$) and NE ($p = 0.002$). 14-3-3β was also increased in NPIP as compared with NE ($p = 0.008$). 14-3-3β cytoplasmic staining was more pronounced in basal cells of the respiratory epithelium although serous glands and the vascular system were often positive as well. High 14-3-3β immunopositivity in IP patients concurred with increased proliferative activity shown by PCNA immunostaining ($p = 0.04$). Expression of 14-3-3θ was also found increased in IP and NPIP patients, compared to NP ($p = 0.005$, $p = 0.002$ respectively) and NE ($p = 0.004$ and $p = 0.001$ respectively). 14-3-3θ cytoplasmic immunopositivity was detected in columnar epithelium, particularly in basal and subluminal cells, whereas no immunoreactivity was observed in NP and NE.

Our results demonstrate differential expression of 14-3-3β and θ isoforms in sinonasal pathophysiology, supporting their implication, respectively, in the proliferative and inflammatory process engaged in the formation of IP.

Introduction

Inverted papillomas (IP) are relatively uncommon benign tumors of the nasal and paranasal sinus epithelium that occur more often in men than in women [1–4]. IP originate from the Schneiderian membrane and are characterized by extensive invaginations of the hyperplastic epithelium into the underlying stroma [5]. IP show an increased tendency to recurrence and malignant transformation, and are thus associated with sinus cancer [6,7]. Bacterial and viral infections, chronic inflammatory conditions, allergies, tobacco and occupational exposures have been implicated in their etiology though their mechanisms of growth and malignant transformation are not as yet fully understood [8].

Sinonasal polyps with epithelial hyperplasia are the most common precursors of Schneiderian papillomas. NPs are abnormal lesions associated with chronic inflammation of nasal and sinus mucous membranes showing no predisposition to age or sex. Eosinophils and basement membrane thickening characterize NPs, while there is no expansive inversion of the often hyperplastic epithelium into the underlying stroma. Unlike polyps in other parts of the body, they are always benign. Sinusitis, allergy, vasomotor rhinitis, infectious rhinosinusitis and asthma are factors contributing to NP growth [9].

Cell proliferation and apoptosis are known as the major causative mechanisms in abnormal lesions. Several studies have investigated epithelial proliferation and apoptotic indicators in both NP and IP. Increased epithelial cell proliferation has been indicated as the main factor involved in the development of NP and IP [10–13].

14-3-3 proteins comprise a large family of small, acidic and highly conserved polypeptides found in all eukaryotic species

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Table 1
Clinicopathological characteristics of patients with inverted papilloma (IP) or with nasal polyps in association with inverted papilloma (NPNP).

Diagnosis	IP	NPIP
Number	51	9
Gender		
Male	41	8
Female	10	1
Age		
Mean ± SE	55.43 ± 1.9	54.66 ± 2.95
Median	56.5	57
Range	31–82	37–64
Location		
Nasal Cavity	37	6
Maxillary	6	3
Ethmoids	6	0
Sphenoids	2	0
Recurrence		
First	4	2
Second	2	0

[14,15]. They are mainly detected in the cytoplasmic compartment and have been shown to play an essential role in regulating cell differentiation, proliferation and transformation [16]. 14-3-3 proteins primarily promote cell survival through sequestration of pro-apoptotic client proteins, including Bax, BAD, FKHRL-1, ASK1 and A20 [17]. 14-3-3θ isoform interacts with, and negatively regulates, pro-apoptotic Bax by inducing its dissociation through caspase-dependent or -independent mechanisms [18]. In contrast, 14-3-3β isoform has been found to produce oncogenic effects, mainly through activation of Raf-1 and augmentation of signaling in the MAPK cascade [19].

Therefore, in order to further investigate the mechanisms governing the formation of different nasal lesions, 14-3-3θ and 14-3-3β expression profiles were studied by flow cytometry and immunohistochemistry in paraffin-embedded tissues of several entities of nasal pathophysiology, including IP, NP, nasal polyps with inverted papilloma (NPIP) and normal nasal epithelium (NE).

Materials and methods

Patients

The patient cohort included 51 patients diagnosed with IP and 9 patients with NPIP (Table 1). Twenty-six patients with inflammatory nasal polyps (NP group: 18 men and 8 women) aged 48.38 ± 3.084 (range 16–81, median 49.5) and 10 specimens of normal nasal epithelium (NE group) were also included. Among the IP patients, three presented a local recurrence once, while two patients relapsed twice (recurrence rate 9.8%). The mean recurrence time was 38 months. This study was approved by the ethical and scientific committee of the 'Hippokrateio' General Hospital, and all subjects gave their informed consent.

Methods

Flow cytometric analysis of epithelial cells apoptosis

Apoptosis of epithelial cells was determined with flow cytometry analysis on cellular aliquots retrieved from rehydrated paraffin sections, using an antibody detecting the M30 neo-epitope of caspase-cleaved CK18, namely the M30-FITC CytoDeath monoclonal antibody (1:100) (Alexis Biochemicals 804–590) according to the manufacture protocol.

Flow cytometric analysis of PCNA, 14-3-3β and 14-3-3θ expression

Aliquots of cell suspension retrieved from rehydrated paraffin sections were fixed and permeabilized using 1% paraformaldeyde-1% saponin in 1 × PBS for 20 min at 4 °C. The following antibodies were used: PCNA (dilution 1:400, Monoclonal Mouse Anti-Proliferating Cell Nuclear Antigen PC10, M 0879, DAKO, Denmark) labeled with Polyclonal Goat Anti-Mouse Immunoglobulins/RPE Goat F(ab′)2 (R 0480 DAKO, Denmark), 14-3-3 β-PE (SC-1657PE, Santa Cruz Biotechnology, Inc., USA; 10 μl/100 μl cell suspension) and 14-3-3θ (USA SC-732, Santa Cruz Biotechnology, Inc., USA; 1 μl/sample) labeled with fluorescein (FITC) using anti-rabbit IgG (F0382 Sigma–Aldrich Co., USA). After 20 min of incubation at room temperature, the samples were washed once with 1xPBS solution and analyzed on a COULTER®.

EPICS® XL-MCL™ flow cytometer. Negative controls of unstained cells and cells stained only with secondary antibody were processed in parallel and used as references.

Immunohistochemical analysis of 14-3-3 isoforms

Paraffin sections were stained with anti-14-3-3θ (Santa Cruz Biotechnology; sc-732; dilution 1:80) and anti-14-3-3 β (Santa Cruz Biotechnology; sc-1657; dilution 1:100) using the Dako REAL EnVision Detection System Peroxidase/DAB1 (Dako; K5007) according to the manufacturer's protocol. Sections were subjected to microwave antigen retrieval (10 min in 0.1 M citrate acid buffer solution, pH 6) before incubation overnight at 4 °C with the respective antibody. Appropriate positive and negative controls were performed in parallel.

For double stain tests, EnVision G/2 Doublestain System (Dako, DK) was used, as instructed by the manufacturer. Slides were first incubated with PCNA (dilution 1:1500 o/n, 4 °C) and then with anti-14-3-3 β. PCNA was detected with DAB-chromogen (nuclear staining), and 14-3-3β with Permanent Red (cytoplasmic immunoreactivity). Specimens were counterstained with hematoxylin. Slides were washed between steps with 1xPBS. Negative controls were obtained by omitting the primary antibodies.

Two independent pathologists evaluated the percentage and intensity of 14-3-3 isoforms expression at 5 high power (×40) microscopic fields randomly picked for quantification. The immunohistochemical evaluation was performed according to the method proposed by Liu et al. [20]. The percentage of positive cells in the entire depth of the epithelium was scored between 0 and 4 (0 = no staining, 1 = 1–20%, 2 = 21–50%, 3 = 51–75% and 4 > 75%). Immunostaining intensity was scored from 0 to 3 (0 = no stain, 1 = light staining, 2 = moderate staining, 3 = strong staining). The two scores were then multiplied, producing a final scoring scale of 14-3-3 immunoreactivity ranging between 0 and 9. 14-3-3-immunoreactivity was rated as 0: for no staining, 1–3 points: low; 4–6 points: moderate; 8–12 points: high. The same method was used for evaluating the immunoreactivity in the stromal components.

Statistical analysis

SPSS 16.0 (SPSS Inc., Chicago, IL) was used for analysis. Data are presented as mean ± SEM values. Nonparametric statistics was used for the analysis of quantitative variables. Differences between groups were analyzed by Mann Whitney *U* test or χ^2 test and were considered to be statistically significant when the two-sided probability value was <0.05.