Increased Activation and Differentiated Localization of Native and Phosphorylated STAT3 in Nasal Polyps

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Key Words
Chronic rhinosinusitis · JAK-STAT pathway · Nasal polyp · Phosphokinase · STAT3

Abstract
Background: Chronic rhinosinusitis with nasal polyps (CRSwNP) is a multifactorial disease; the underlying mechanisms of cell signalling are not fully understood. STAT3 (signal transducer and activator of transcription 3) is a phosphokinase and a key signalling molecule implicated in cell cycle regulation. We studied the distribution and expression of STAT3 to examine the role of STAT3 in the pathogenesis of CRSwNP. Methods: We investigated tissue samples of the nasal polyps and inferior turbinates of patients with CRSwNP as well as samples of the inferior turbinate of subjects without chronic sinusitis. The expression levels of STAT3 and its activated form pSTAT3 were analysed using Western blotting, protein array, DNA microarray and immunohistochemistry. Results: No significant differences were found in STAT3-mRNA levels between the samples of nasal polyps and inferior turbinates of the same patient. However, the amount of pSTAT3 was increased in the polyp tissue compared to the inferior turbinates from both CRSwNP patients and control subjects (p < 0.01), indicating an activation of STAT3 in polyps. We identified a varying distribution pattern of pSTAT3; pSTAT3 was primarily found in superficial epithelial cells but not in the basal layer of the epithelium of the turbinate, whereas pSTAT3 was located in all layers of the epithelium of the polyp and mostly noted in the basal layer. Conclusions: Our results of the activation and varying localisation of STAT3 and its phosphorylated form in nasal polyps suggest that pSTAT3 plays a crucial role in the proliferative development of nasal polyps.

Introduction

Chronic rhinosinusitis (CRS) is one of the most common diseases in Western Europe [1] and the United States [2]. Generally, it can be distinguished between chronic rhinosinusitis with nasal polyps (CRSwNP) and chronic rhinosinusitis without nasal polyps (CRSsNP) [3, 4], but it is difficult to differentiate these two entities based on clinical impression alone [5]. CRSwNP and CRSsNP show different inflammatory patterns [6]. CRSwNP is characterised by higher eosinophilia, immunoglobulin E and interleukin 5 (IL-5) compared with CRSsNP [3, 4]. However, it is difficult to differentiate these two entities based on clinical impression alone [5]. CRSwNP and CRSsNP show different inflammatory patterns [6]. CRSwNP is characterised by higher eosinophilia, immunoglobulin E and interleukin 5 (IL-5) compared with CRSsNP [3, 4]. However, it is difficult to differentiate these two entities based on clinical impression alone [5].
The pathogenesis of nasal polyps is caused by a deregulated regulation of cell growth, whereas the underlying causal molecular mechanisms and signalling pathways are not fully understood [9]. The regulation of these pathways is complex, and various phosphokinases are involved in intracellular signalling.

STAT (signal transducer and activator of transcription) proteins were discovered 20 years ago as mediators of interferon-induced gene expression. STAT proteins are involved in growth control, differentiation, apoptosis and transformation of cells, and can be activated by virtually every cytokine and growth factor [10].

STAT3, an 89-kDa protein, is one of the 7 members of the STAT family of proteins that have been identified so far. STAT3 is a key signalling molecule: normal STAT3 signalling is tightly controlled with finite kinetics, which mediates standard cellular responses [11]. STAT3 deficiency leads to early embryonic lethality [12].

STAT3 is expressed in most tissues and is activated by a large number of different ligands [13]. The linking of these molecules to the Janus kinase (JAK) receptor leads to the phosphorylation of STAT3 at tyrosine 705 (Y705; thereafter, pSTAT3). pSTAT3 dimerises in homo- and heterodimers and migrates to the nucleus. Following phosphorylation at serine 727 (S727), pSTAT3 activates the transcription of various target genes [14].

The aberrant activation of STAT3 is observed in numerous human cancers and is widely recognised as a critical molecular abnormality and a master regulator of tumour processes [15]. Constitutively active STAT3 promotes uncontrolled growth and survival through the dysregulation of gene expression. Persistently active STAT3 induces tumour angiogenesis and modulates immune functions in favour of tumour immune evasion [11, 16].

STAT3 is a well-known phosphokinase involved in oncogenesis and the regulation of inflammation, and may have a function in CRSwNP. The objective of this study was to investigate the expression, activity and distribution of STAT3 in nasal polyp tissue and examine the roles of STAT3 in the pathogenesis of nasal polyps.

**Materials and Methods**

**Patients, Inclusion and Exclusion Criteria**

We examined tissue samples of nasal polyps and the inferior turbinate, as an internal control, which were harvested from 13 patients with CRSwNP during sinus surgery. Special emphasis was placed on the gentle removal of polyps without bruising or tearing. Only patients with eosinophilic CRSwNP, which was determined by histopathological examination, were included in the study to examine a subgroup of polyps that was as homogenous as possible. No patient was treated with systemic or topical corticosteroids within 4 weeks prior to surgery. Patients with asthma and aspirin hypersensitivity were excluded to minimise overlapping effects on the phosphokinase profile of the patients.

Additionally, we examined 8 samples of the inferior turbinate of patients without any history of sinusitis or allergy who underwent septal surgery as an external control.

The study was approved by the Human Ethics Committee of the University of Luebeck (AZ_10-201) and conducted in accordance with the ethical principles for medical research formulated in the WMA Declaration of Helsinki. All patients provided signed informed consent.

**DNA Microarray**

Fresh tissue samples of nasal polyps and the inferior turbinate of 6 patients with CRSwNP were snap-frozen in liquid nitrogen immediately after sampling, stored at −80°C and shipped on dry ice to Miltenyi Biotec (Bergisch Gladbach, Germany) for microarray analysis. The RNA was isolated and quality checked via an Agilent 2100 Bioanalyser platform (Agilent Technologies, Santa Clara, Calif., USA), and the RNA Integrity Number was calculated. Agilent whole human genome microarrays (4 × 44K) were performed following the manufacturer’s protocols. The Agilent human genome CGH microarray kit 44K is a high-resolution tool for genome-wide DNA copy number variation profiling without amplification or complexity reduction. Over 43,000 coding and non-coding human sequences are represented. One glass slide is formatted with 4 high-definition 44K arrays. Gene expression was calculated using the Rosetta Resolver® gene expression data analysis system (Rosetta Biosoftware, Seattle, Wash., USA).

**Protein Array**

The Proteome Profiler™ human phosphokinase array kit (R&D Systems Inc., Minneapolis, Minn., USA) allows the simultaneous detection of the relative phosphorylation levels of 46 kinase phosphorylation sites. The protein array contains mitogen-activated protein kinases (MAPKs), MAPK kinases, MAPK-activated protein kinases, STATs, other transcription factors and signal transduction adaptor proteins. Capture and control antibodies are spotted in duplicate on nitrocellulose membranes.

Tissue homogenates of samples of polyps and inferior turbinates from 5 patients with CRSwNP as an internal control (250 μg of total protein/array) were applied to the phosphoprotein array following the manufacturer’s instructions.

The tissue extracts were diluted and incubated overnight with the array. The array was washed to remove unbound proteins, followed by incubation with a cocktail of biotinylated detection antibodies. Streptavidin-horseradish peroxidase (HRP) and chemiluminescent detection reagents were applied, and the signal produced at each capture spot corresponded to the amount of phosphorylated protein bound.

Images were acquired using Fusion FX 7®. Pixel density was analysed using the Bio-1D software® (both Vilber Lourmat, Marne-la-Vallée, France).

**Western Blot Analysis**

Tissue lysates of nasal polyps (n = 7) and inferior turbinates of patients with CRSwNP (n = 7), and inferior turbinates of patients with healthy mucosa (n = 8) were denatured by boiling for 5 min in 1× SDS sample buffer. The protein concentrations were deter-
Studies of STAT3-mRNA in Nasal Polyps

Western blot analysis showed no differences in the expression of unphosphorylated STAT3 in the polyps (0.87; SD 0.23; n = 7) and turbinate samples of patients with CRSwNP (0.89; SD 0.24; n = 7) compared with healthy mucosa (1.00; SD 0.33; n = 8). We detected a 1.72-fold increase in pSTAT3 in the nasal polyps (n = 7) compared with the inferior turbinate from the same patient (n = 6); the mean fold change was 0.99 (SD 0.23; fig. 1).

Increased Activity of STAT3 in Nasal Polyps

The evaluation of the DNA microarray (NM_213662) showed no quantitative difference in the amount of STAT3-mRNA between the samples of nasal polyps and the inferior turbinate from the same patient (n = 6); the mean fold change was 0.99 (SD 0.23; fig. 1). Western blot analysis showed no differences in the expression of unphosphorylated STAT3 in the polyps (0.87; SD 0.23; n = 7) and turbinate samples of patients with CRSwNP (0.89; SD 0.24; n = 7) compared with healthy mucosa (1.00; SD 0.33; n = 8). We detected a 1.72-fold increase in pSTAT3 in the nasal polyps (n = 7) compared with the inferior turbinate from the same patient (n = 6); the mean fold change was 0.99 (SD 0.23; fig. 1).

Quartitative analysis of the protein array showed a significantly increased level of pSTAT3 in the polyp tissue in each pair of the polyp and turbinate samples from the same patient with CRSwNP. The mean concentration of pSTAT3 in the polyps was 2.08-fold higher than that observed in the inferior turbinate (n = 5, p < 0.01; fig. 3).

Altered Localisation of STAT3 in Nasal Polyps

Haematoxylin and eosin staining of the nasal turbinates of patients with both healthy mucosa and CRSwNP showed the typical multi-row ciliated epithelium, with goblet cells included. Variable degrees of hyperplasia and focal squamous metaplasia were found in the epithelium of the nasal polyps. Data from the histopathological examination of the polyps are listed in table 2.
Immunohistochemically, STAT3 staining was particularly present in the outer cell layer of the epithelium of both the nasal turbinate of patients with CRSwNP and the healthy mucosa (fig. 4a, b). STAT3 was uniformly expressed in the cytoplasm of epithelial cells in all layers of nasal polyp tissue (fig. 4c). The percentage of stained cells in the basal layer of the epithelium was significantly different between the polyps (n = 6) and the turbinates of patients with CRSwNP (n = 6; p < 0.05) and healthy mucosa (n = 6; p < 0.05).

The different distribution of pSTAT3 in polyp tissue was even more striking. pSTAT3 was evident in only a few nuclei in the epithelium of the turbinates of patients with healthy mucosa (fig. 4d). In the turbinates of patients with CRSwNP, pSTAT3 was found in the nuclei of almost all cells in the superficial cell layer and sporadically in the basal layer (fig. 4e).

However, pSTAT3 was observed in both the superficial and basal layer of the epithelium of the polyps. The difference between the percentage of stained cells in the basal layer of the epithelium between the polyps (n = 6) and both the turbinates of patients with CRSwNP (n = 6; p < 0.001) and the healthy mucosa (n = 6; p < 0.01) is highly significant (fig. 4f).

A semiquantitative analysis of the distribution pattern of immunohistochemical staining is listed in table 3.

**Discussion**

The etiopathogenesis of CRS and especially of nasal polyps still needs to be clarified. Epithelial damage, aberrant tissue remodelling and hyperplasia are typical features of CRSwNP. Cellular growth seems to be dysregulated in polyps. A greater proportion of proliferating cells and increased apoptosis have been identified in the epithelial cells of polyps compared with normal mucosa [22].

### Table 1. Clinical and demographic characteristics of the patients

<table>
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<tr>
<th>No.</th>
<th>Gender</th>
<th>Age (years)</th>
<th>Lund-Mackay score</th>
<th>Lund-Kennedy score</th>
<th>Duration of complaints, months</th>
<th>Smoker, pack-years</th>
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<td>9</td>
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Mean ± SD 49.3±12.6 17.3±4.2 10.1±1.7 15.6±9.6 9±12.2

M = DNA microarray; P = protein array; WB = western blot; IHC-F = immunohistochemistry (frozen).
The contribution of signalling pathways that regulate the growth of nasal polyps remains poorly defined. The activation or inactivation of the enzymes involved in these pathways is often carried out by various phosphokinases, such as STAT3.

So far, only two studies exist on the role of STAT3 and pSTAT3 in the development of nasal polyps and they have contradictory results. Peters et al. [23] measured the concentration of STAT3 and pSTAT3 by Western blot in tissue samples of nasal polyps and inferior turbinates of patients with CRSwNP compared with healthy mucosa. **p < 0.01.

Fig. 2. Representative example of Western blotting. a STAT3 and pSTAT3 in polyps (p), turbinates (t) and healthy mucosa (h). Re-probing with an antibody against β-actin demonstrates equal protein loading in each lane. b Mean levels (±SD) of STAT3 and pSTAT3 in polyps and inferior turbinates of patients with CRSwNP compared with healthy mucosa. **p < 0.01.

Fig. 3. Protein array results. a Example of a representative protein array of a polyp and an inferior turbinate from the same patient. The spot for pSTAT3 on the membrane is marked. b Mean levels (±SD) of pSTAT3. **p < 0.01.
patients with CRSwNP and performed immunohistochemistry in tissue samples of the nasal polyps and uncinate of patients with CRSwNP. They found lower levels of pSTAT3 in the tissue samples from polyps compared to controls. The group of Cao and Zhang performed immunohistochemistry in tissue samples of the nasal polyps and inferior turbinates of patients who underwent septal surgery as controls. The positive rate of STAT3 and pSTAT3 was significantly higher in nasal polyps than in controls [24, 25].

We examined tissue samples of polyps from a well-defined subpopulation of patients with eosinophilic bilateral CRSwNP and two different types of controls with a battery of four tests. We did not find a difference in the amount of mRNA between polyps and inferior turbinates of patients with CRSwNP or healthy mucosa. Furthermore, no difference could be detected in the concentra-

Table 2. Histopathological examination of the polyps

<table>
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<tr>
<th>Histologic measures</th>
<th>Mean ± SD</th>
<th>%</th>
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<td><strong>Cellular markers, absolute n/HPF</strong></td>
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<tr>
<td>Eosinophils</td>
<td>50.1±26.3</td>
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<tr>
<td>Neutrophils</td>
<td>3.5±1.7</td>
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<tr>
<td>Lymphocytes</td>
<td>27.2±8.2</td>
<td></td>
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<tr>
<td>Plasma cells</td>
<td>27.0±6.9</td>
<td></td>
</tr>
<tr>
<td>Macrophages</td>
<td>15.0±6.6</td>
<td></td>
</tr>
<tr>
<td>Mast cells</td>
<td>6.3±2.9</td>
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</tr>
<tr>
<td><strong>Epithelial markers</strong></td>
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<tr>
<td>Goblet cells, %</td>
<td>7.0±1.8</td>
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<tr>
<td>Thickness of the basal membrane, μm</td>
<td>7.5±3.1</td>
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<tr>
<td>Epithelial hyperplasia</td>
<td>83</td>
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<tr>
<td>Squamous metaplasia</td>
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<tr>
<td><strong>Stromal markers</strong></td>
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<td></td>
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<tr>
<td>Subepithelial oedema</td>
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<td></td>
</tr>
<tr>
<td>Mucosal fibrosis</td>
<td>17</td>
<td></td>
</tr>
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</table>

Table 3. Semiquantitative analysis of immunohistological staining of STAT3 and pSTAT3 in the epithelium (n = 6)

<table>
<thead>
<tr>
<th>Localization</th>
<th>STAT3</th>
<th>pSTAT3</th>
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<tbody>
<tr>
<td>Superficial layer</td>
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<td></td>
</tr>
<tr>
<td>Healthy mucosa (control)</td>
<td>16.7 (10.4)</td>
<td>10.0 (6.1)</td>
</tr>
<tr>
<td>Turbinate (CRSwNP)</td>
<td>50.7 (15.9)</td>
<td>58.7 (20.0)</td>
</tr>
<tr>
<td>Polyp</td>
<td>47.5 (23.1)</td>
<td>58.3 (21.4)</td>
</tr>
<tr>
<td>Basal layer</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Healthy mucosa (control)</td>
<td>2.8 (1.3)</td>
<td>17.5 (4.8)</td>
</tr>
<tr>
<td>Turbinate (CRSwNP)</td>
<td>4.3 (1.6)</td>
<td>6.7 (2.6)</td>
</tr>
<tr>
<td>Polyp</td>
<td>43.8 (22.1)</td>
<td>54.3 (16.5)</td>
</tr>
</tbody>
</table>

Mean percentages of stained cells (SD) are shown.

Fig. 4. Representative examples of immunostaining with haematoxylin counterstaining. STAT3 is located mainly in the superficial layer (arrow) of the epithelium of both the inferior turbinate of healthy mucosa (a) and the inferior turbinate of a patient with CRSwNP (b). In polyps, a uniform cytoplasmic expression of STAT3 was found in all layers of the epithelium (c). The cells of the epithelium in healthy mucosa hardly express pSTAT3 (d). pSTAT3 is expressed in almost all nuclei of the superficial epithelial layer (arrow) of the inferior turbinate of a patient with CRSwNP (e). pSTAT3 is expressed in the superficial layer and in the proliferating basal epithelial layer (arrows) of a nasal polyp (f).
fection of non-phosphorylated STAT3 protein between polyps and inferior nasal turbinate of patients with CRSwNP or healthy mucosa. However, pSTAT3 concentration was increased in nasal polyps compared with inferior turbinates of patients with CRSwNP and healthy mucosa, suggesting that this critical point of the STAT3 cascade is altered. We conclude that in CRSwNP the regulation of STAT3 is disrupted exclusively at the phosphorylation level.

In agreement with the group of Cao and Zhang, we observed a higher amount of pSTAT3 in nasal polyps compared to controls [24, 25], but we did not observe a higher expression of STAT3. In contrast to our study, Peters et al. [23] included patients who were treated with corticosteroids. Because corticosteroids are effective in the conservative treatment of CRSwNP [4], they most likely change the phosphokinase profile of the tissue. Furthermore, Peters et al. [23] studied mucosa of the uncinate of patients with CRSwNP (tissue of the middle meatus) as a control in their immunohistochemistry analysis. We used the mucosa of the inferior meatus, where polyps never occur. The differing results of the three studies could be explained by the different patient selection criteria and different types of controls.

Although no major histological difference was found in the nasal mucosa and polyps obtained from African, Chinese and Caucasian patients [26], different inflammatory patterns have been discussed in patients of different races [4, 27–30]. The different origin of the patients is another possible explanation of the diverse results. Other possible confounders, including biofilms [31] and infection/colonisation with fungi [32] or *Staphylococcus aureus* [33], were not examined in our study or in the studies of the other research groups because these aspects were beyond the scope of the studies.

This work differs from many purely molecular biological studies in that we have measured not only the amount of protein but also its spatial distribution. The real surprise in our results was the different distribution of pSTAT3 in the cells of the epithelium of polyps and inferior turbinates, which had not been reported previously. In the 1990s, a rupture of the basal membrane, which was followed by interstitial oedema, was suspected as the cause of polyp formation based on light-microscopic findings in a rat model [34, 35]. Our studies provide clear indications, obtained using an immunohistochemical method, that the STAT3 pathway is turned on, which is known in oncology, and that the phosphorylation rate of a key signalling molecule is up-regulated in the basal cell layer of the pathologically altered epithelium of nasal polyps.

The distribution of STAT3 and pSTAT3 in the turbinates of patients with CRSwNP and healthy mucosa is very similar. Our findings provide a possible explanation as to why nasal polyps rarely occur in the region of the inferior turbinate.

Distinct and partially contradictory roles of STAT3 in the immune system have been described. Although anti-inflammatory effects have been reported [36], STAT3 has been considered an activator of the expression of pro-inflammatory genes [37, 38] and may play a critical role in the control of mucosal immune tolerance [39].

STAT3 is a key element of the downstream signalling of IL-6 [40]. Increased amounts of IL-6 were reported in patients with CRSwNP [23, 41]. IL-17 has been found in the upper respiratory tract, and it stimulates the survival and degranulation of eosinophils [42]. An enhanced Th17 response in nasal polyps has also been demonstrated [43, 44, 45], regardless of eosinophilic or non-eosinophilic inflammation [43]. An impaired balance of Th17 and regulatory T cells was reported in patients with CRSwNP [46], as Th17 airway inflammation is enhanced by an IL-6- and IL-17-positive feedback loop [47, 48]. Our findings provide evidence that STAT3, an element of the downstream signalling of both IL-6 and IL-17, is activated in nasal polyps.

As early as 1863, Rudolf Virchow hypothesised that micro-inflammation resulting from irritation leads to enhanced cell proliferation [49, 50]. STAT3 regulates the expression of target genes involved in cell-cycle progression and apoptosis and promotes cellular transformation as well as abnormal cell proliferation. STAT3 is considered a suppressor of apoptosis [51–53]. It is commonly discussed as a link between inflammation and cancer [54].

CRSwNP is a recurrent, benign and extremely proliferative disease that is triggered by inflammation. STAT3, which is activated in nasal polyps and localised in epithelial cells facing the basal membrane, may drive the development of nasal polyps by inhibiting apoptosis in the epithelium, which is otherwise induced by a chronic inflammatory condition.

Curcumin (diferuloylmethane) is a naturally occurring compound found in the plant *Curcuma longa* that has numerous medicinal properties, including anti-inflammatory and anti-tumor effects [55]. Several small molecule STAT3 inhibitors have been developed by modifying curcumin, and some have shown promising activity both in vitro and in mouse xenograft models [56–58]. Agents with biologic activity inhibiting STAT3-related cellular functions may have therapeutic potential in the treatment of malignant tumours [59] and possibly...
References


