Differential Expression of Livin, Caspase-3, and Second Mitochondria-Derived Activator of Caspases in Chronic Rhinosinusitis with Nasal Polyps

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Sponsorships or competing interests that may be relevant to content are disclosed at the end of this article.

Abstract
Background and Objectives. The pathogenesis of human chronic rhinosinusitis with nasal polyps (CRSwNP) remains undetermined. Livin is a member of the inhibitor of the apoptosis protein family proteins. Caspase-3 and second mitochondria-derived activator of caspases (Smac) are critical in the induction of apoptosis. However, little is known about their roles in CRSwNP. We aimed to investigate the expression and role of Livin, caspase-3, and Smac in CRSwNP.

Study Design. Basic research and descriptive study.

Setting. Fuzhou General Hospital, Fuzhou, Fujian, China.

Methods. The immunohistochemistry method was employed for detecting Livin, caspase-3, and Smac protein expression, and real-time polymerase chain reaction was used for assaying mRNA expression of Livin, caspase-3, and Smac in CRSwNP and controls. Moreover, the effects of various stimulators on Livin were evaluated on human nasal epithelial cells (HNECs) culture. Then, the effects of Livin on caspase-3 and Smac were observed on the culture of HNECs.

Results. Stronger protein and mRNA expression of Livin was observed in CRSwNP, especially eosinophilic CRSwNP, weaker protein and mRNA expression of caspase-3 and Smac was observed in CRSwNP, and Livin expression was negatively related to caspase-3 or Smac expression, respectively. Livin mRNA was augmented by interleukin (IL)–4, IL-17A, and IL-1β but suppressed by interferon-γ. Caspase-3 and Smac mRNA expression were inhibited by Livin.

Conclusions. Upregulation of Livin and downregulation of caspase-3 and Smac were observed in CRSwNP, especially in eosinophilic CRSwNP. Livin may exert its anti-apoptosis effect by suppressing caspase-3 and Smac in CRSwNP. IL-4, IL-17A, and IL-1β may be critical for Livin expression.

Keywords
Livin, caspase-3, Smac, chronic rhinosinusitis, nasal polyps

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Table 1. Characteristics of Included Subjects.\(^a\)

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>ECRSwNP</th>
<th>nECRSwNP</th>
<th>Controls</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of subjects</td>
<td>30</td>
<td>28</td>
<td>23</td>
<td></td>
</tr>
<tr>
<td>Age, y</td>
<td>39.0 ± 15.6</td>
<td>40.5 ± 16.0</td>
<td>37.8 ± 15.0</td>
<td>.825</td>
</tr>
<tr>
<td>Gender (male/female)</td>
<td>17/13</td>
<td>15/13</td>
<td>12/11</td>
<td>.944</td>
</tr>
<tr>
<td>Total VAS score</td>
<td>15.7 ± 3.3</td>
<td>13.1 ± 3.0</td>
<td>N/A</td>
<td>.003</td>
</tr>
<tr>
<td>Endoscopy score</td>
<td>9.6 ± 2.8</td>
<td>7.2 ± 2.9</td>
<td>N/A</td>
<td>.002</td>
</tr>
<tr>
<td>CT score</td>
<td>17.1 ± 3.5</td>
<td>15.1 ± 2.6</td>
<td>N/A</td>
<td>.017</td>
</tr>
<tr>
<td>Positive SPT, n (%)</td>
<td>17 (56.7)</td>
<td>7 (25)</td>
<td>0</td>
<td>.029</td>
</tr>
</tbody>
</table>

Abbreviations: CT, computed tomography; ECRSwNP, eosinophilic chronic rhinosinusitis with nasal polyps; n/NA, not applicable; nECRSwNP, noneosinophilic chronic rhinosinusitis with nasal polyps; SPT, skin-prick test; VAS, visual analog scale.

\(^a\)Kruskal-Wallis H test was employed for age comparison between ECRSwNP, nECRSwNP, and the control group. A \(\chi^2\) test was used for gender and SPT comparison between groups. Mann-Whitney U test was employed for VAS, endoscopy, and CT score comparison between the ECRSwNP and nECRSwNP groups.

Materials and Methods

Subjects

We recruited 58 patients who suffered from CRSwNP, comprising 30 patients with eosinophilic chronic rhinosinusitis with nasal polyps (ECRSwNP) and 28 patients with noneosinophilic chronic rhinosinusitis with nasal polyps (nECRSwNP), and 23 control subjects for immunohistochemistry and real-time polymerase chain reaction (PCR) and human nasal epithelial cells (HNECs) culture in this study. CRSwNP was diagnosed on the basis of previously published criteria.\(^9\) A 10\% cutoff of percentage eosinophils in total inflammatory cells was chosen as the demarcation standard between ECRSwNP and nECRSwNP, as described previously.\(^4\) Human normal nasal mucosas were collected from the inferior turbinates of 23 patients subjected to septorhinoplasty due to anatomic variations. These control subjects had no allergy history, a negative skin-prick test (SPT), and no sinus disease. Subject information was composed of age, gender, total visual analog scale (VAS) scores, endoscopy scores (Lund-Kennedy), computed tomography (CT) scores (Lund-Mackay), and results of an SPT. Detailed characteristics of included subjects are summarized in Table 1. Moreover, subjects receiving any immunosuppressant, antihistamine, antileukotriene, antibiotic, or oral or topical steroid 1 month before the surgery were excluded.

Nasal polyps from CRSwNP patients or inferior turbinate mucosal samples from controls were harvested during surgery. At the time of surgery, the tissue samples were immediately cut into several portions and stored at −80°C. Samples were divided into several sections for hematoxylin-eosin (HE) staining, immunohistochemistry, real-time PCR, or HNECs culture. Livin, caspase-3, and Smac protein expression were investigated by the immunohistochemistry method, and the mRNA expression of Livin, caspase-3, and Smac was assessed using real-time PCR. In addition, the effects of various stimulators with different concentrations and time were evaluated on HNECs culture.

This study was approved by the ethical committee of Fuzhou General Hospital, and every subject provided informed consent.

Eosinophil Counting and Immunohistochemistry

The tissues were fixed by 4% paraformaldehyde and then embedded in paraffin, serially sectioned (4 μm), and stained with HE. The number of eosinophils was counted microscopically in 10 high-power fields (400×), and a 10% cutoff of percentage eosinophils in total inflammatory cells was chosen as the demarcation standard between ECRSwNP and nECRSwNP, as described previously.\(^4\) The immunohistochemistry method was performed using the streptavidin biotin complex kit (Boster Biological Technology, Pleasanton, California) as described by the manufacturer’s protocol. Following deparaffinization and rehydration, the sections were rinsed using phosphate buffered solution (PBS) 3 times and then microwaved for 10 minutes in citrate buffer. Then, 3% hydrogen peroxide was added to block endogenous peroxidase for 20 minutes. After the incubation of primary antibody (rabbit anti-human Livin and Smac, 1:100 dilution, and rabbit anti-human caspase-3, 1:50 dilution; Abcam, Cambridge, United Kingdom) at 37°C for 60 minutes, the sections were rinsed with PBS and then incubated with secondary anti-rabbit antibody for 20 minutes. For visualization, 3,3-diaminobenzidine was applied. A section incubated with PBS instead of the primary antibody was chosen as a negative control. Ten fields of each section were randomly selected and microscopically examined, and Livin immunoreactivity was analyzed by measuring the integrated optical density (IOD) by Image Pro Plus 6.0 (Media Cybernetics, Rockville, Maryland) as described previously.\(^10\) Following the calibration of optical density, we used hue saturation intensity mode (hue, 0-30; saturation, 0-255; intensity, 0-230) to select the area of interest, and the IOD that indicated immunoreactivity of Livin, caspase-3, or Smac was counted.

RNA Extraction and Reverse Transcription

After homogenization of the tissues or cells, TRIzol reagent was added to extract total RNA. The purity and integrity were assessed by measuring absorbance ratios at 260/280 nm (1.8-2.0 was considered eligible) and agarose gel electrophoresis, respectively. For reverse transcription,
Superscript First-Strand Synthesis System (Invitrogen, Carlsbad, California) was applied to reversely transcribe 2 μg total RNA into cDNA as a template for real-time PCR, and cDNA was then stored at −80°C for further experiment.

**Real-Time PCR**

Platinum SYBR Green qPCR SuperMix (Invitrogen) was employed to detect the mRNA expression of Livin, caspase-3, and Smac. Primers were designed and synthesized (Sangon Biotech, Shanghai, China). Each sample was analyzed in triplicate. Livin (170 bp) primers were forward primer, 5-AGTCTGAAAGTGCCACGGAG-3 and reverse primer, 5-CACAAAGACGAATGGACACGG-3. Caspase-3 (153 bp) primers were forward primer, 5-TGAGCCATGGTGAAGAAGGA-3 and reverse primer, 5-AGTCCAGTTCTGTAACACCGG-3. Smac (146 bp) primers were forward primer, 5-TCGCTGGAGAAGGACTCCTGACC-3 and reverse primer, 5-ATGATGTTCTGGAAGAGCC-3. Relative mRNA level was expressed as the relative fold change and calculated using the formula 2−ΔΔCT = 2(ΔCT(Sample)−ΔCT(calibrator)), where each ΔCT = ΔCTTarget−ΔCGAPDH. One sample without any treatment from the control subject was designated as a calibrator.

**Cell Culture and Stimulation**

Isolation of HNECs from human inferior turbinate mucosa specimens and culture of HNECs were performed as described previously. Briefly, nasal specimens were rinsed in PBS 3 times and then incubated in Dulbecco’s modified Eagle’s medium (DMEM)/F12 (Sigma, St Louis, Missouri) containing 20% FBS and 0.1% protease type XIV for 16 hours at 4°C. Following PBS washing 3 times and then incubated in Dulbecco’s modified Eagle’s medium (DMEM) containing 20% FBS and 0.1% protease type XIV for 16 hours at 4°C. Then, 10% fetal bovine serum (FBS; Sigma) was used to block protease activity, and cell suspensions were filtered and centrifuged at 1200 rpm for 5 minutes. Following PBS washing 3 times, the cell pellets were planted into DMEM containing 20% FBS and 0.1% protease type XIV for 16 hours at 4°C.

**Statistical Analysis**

Statistical analysis software (GraphPad Prism 5.0 and SPSS version 20.0 software) was applied to conduct the analyses, and a P value <.05 was deemed statistically significant. The Kruskal-Wallis H test was employed for comparison among 3 groups and Mann-Whitney U test for comparison between 2 groups. Differences test in proportions between groups was employed by the χ² test. Correlations were assessed by Spearman test. Moreover, 2-way analysis of variance and Bonferroni post hoc test were employed for evaluating the effects of various stimulators on Livin mRNA expression. Values were shown as mean ± standard deviation.

**Results**

**Patient Characteristics**

As indicated in Table 1, significant elevation in total VAS score, endoscopy score, CT score, and rate of positive SPT were found in the ECRSwNP group compared with the nECRSwNP group (P = .003, P = .002, P = .017, and P = .029, respectively), indicating that more severe symptoms and signs, poorer image manifestations, and a higher atopy rate were found in ECRSwNP.

**Livin, Caspase-3, and Smac Immunoreactivity in Nasal Tissues**

As depicted by immunohistochemistry staining (Figure 1), upregulation of Livin protein and downregulation of caspase-3 and Smac protein levels were observed in the CRSwNP groups compared with the control group, and the positive staining was shown mainly in the nasal epithelium, glands, and submucosal inflammatory cells. Quantitative analysis of immunostaining (IOD) demonstrated significant upregulation of Livin protein and downregulation of caspase-3 and Smac protein levels in the ECRSwNP group compared with controls (P < .001, P < .001, and P < .001, respectively) and in the nECRSwNP group compared with controls (P < .001, P < .001, and P < .001, respectively; Figure 2A-C). Furthermore, these alterations of Livin, caspase-3, or Smac were more obvious in the ECRSwNP group than in the nECRSwNP group and in the ECRSwNP group than in the nECRSwNP group (P = .007, P = .001, and P = .002, respectively; Figure 2A-C), and no significant discrepancy in Livin, caspase-3, or Smac protein expression was found between atopic patients and nonatopic patients (P = .28, P = .33, and P = .15, respectively). Moreover, Livin protein was negatively correlated with caspase-3 or Smac protein in CRSwNP patients (R = −0.521, P = .00003; and R = −0.476, P = .00016, respectively).

**Livin, Caspase-3, and Smac mRNA Levels in Nasal Tissues**

As showed in Figure 2D-F, significant upregulation of Livin mRNA and downregulation of caspase-3 and Smac mRNA levels were found in the ECRSwNP group compared with controls (P < .001, P < .001, and P < .001, respectively) and in the nECRSwNP group compared with controls (P < .001, P < .001, and P < .001, respectively). Livin mRNA was significantly more strongly expressed and caspase-3 and Smac mRNA were significantly more weakly
expressed in the ECRSwNP group compared with the nECRSwNP group ($P = .001$, $P = .001$ and $P = .002$, respectively; Figure 2D-F). For these genes, no significant difference was found between atopic patients and nonatopic patients ($P = .23$, $P = .30$, and $P = .10$, respectively).

Moreover, Livin mRNA was negatively correlated with caspase-3 or Smac mRNA in CRSwNP patients ($R = –0.497$, $P = .00007$; and $R = –0.481$, $P = .00013$, respectively).

### Effects of Cytokine Stimulation on Livin mRNA Production in HNECs

In cultured HNECs, significantly increased Livin mRNA expression was found after incubation with IL-4, IL-17A, and IL-1β in a concentration- and time-dependent manner ($P < .001$; Figure 3A-C). Conversely, significantly reduced Livin mRNA expression was found after incubation with IFN-γ ($P < .001$; Figure 3D). Moreover, no significant alteration of Livin mRNA expression was observed after incubation with IL-5 and IL-13 (IL-5, 10 ng/mL, $P = .21$ [12 hours], $P = .12$ [24 hours]; IL-5, 50 ng/mL, $P = .10$ [12 hours], $P = .07$ [24 hours]; IL-13, 10 ng/mL, $P = .19$ [12 hours], $P = .10$ [24 hours]; and IL-13, 50 ng/mL, $P = .11$ [12 hours], $P = .08$ [24 hours], respectively).

### Effects of Livin Stimulation on Caspase-3 and Smac mRNA Production in HNECs

To explore the functional mechanism of Livin, the production of caspase-3 and Smac mRNA in HNECs after stimulation with recombinant human Livin was assayed using real-time PCR. Notably, caspase-3 and Smac mRNA were significantly downregulated by Livin in a concentration- and time-dependent manner ($P < .001$; Figure 3E, F).

### Discussion

CRSwNP is a common chronic inflammation characterized by nasal obstruction, purulent discharge, headache, and hyposmia. It has been demonstrated that inhibition of apoptosis in inflammatory cells plays a critical role in the formation of CRSwNP, and classification of CRSwNP by...
eosinophil and non-eosinophil infiltration has aroused great interest among rhinologists. However, the exact pathophysiological mechanism of inflammation in CRSwNP has still not been fully unraveled. It has been shown that Livin participates in the process of cell apoptosis inhibition and promotes cell proliferation. Thus, we aimed to clarify the exact role of Livin in CRSwNP by classification of eosinophil infiltration.

As shown in Table 1, ECRSwNP exhibited different characteristics compared with nECRSwNP, indicating that eosinophils triggered a more severe form of inflammation compared with non-eosinophilic inflammatory cells in CRSwNP, and this was consistent with published results.

As indicated by immunohistochemistry staining, we found that upregulation of Livin and downregulation of caspase-3 and Smac protein expression were observed in CRSwNP patients, and positive staining was mainly in the nasal epithelium, glands, and submucosal inflammatory cells, indicating that Livin may exert an anti-apoptosis effect by inhibiting caspase-3 and Smac on nasal epithelial cell growth, gland and endothelial cells proliferation, and submucosal inflammatory cell infiltration in CRSwNP, and these effects were more obvious in ECRSwNP patients compared with nECRSwNP patients.

Through the analysis of real-time PCR results, we found that mRNA expression of Livin was elevated and mRNA expression of caspase-3 and Smac was reduced in the ECRSwNP groups, especially in the ECRSwNP group. Livin mRNA was negatively correlated with caspase-3 or Smac, respectively. Similarly, in HNECs culture and stimulation, caspase-3 and Smac mRNA were significantly downregulated by Livin. These results indicate that Livin may exert its anti-apoptosis effect by suppressing caspase-3 and Smac in the pathogenesis of CRSwNP.

To explore the regulatory mechanism of Livin production, HNECs were incubated with a variety of stimulators at different concentrations and times. Results indicated that significant upregulation of Livin mRNA was observed after incubation with IL-4, IL-17A, and IL-1β, whereas Livin mRNA expression was significantly reduced by IFN-γ, indicating that the anti-apoptosis effect of Livin may be enhanced in the Th2/Th17 type and proinflammatory microenvironment but attenuated in the Th1-type microenvironment.

**Conclusion**

Upregulation of Livin and downregulation of caspase-3 and Smac were observed in patients with CRSwNP, especially ECRSwNP. Livin may exert its anti-apoptosis effect by suppressing caspase-3 and Smac in the pathogenesis of CRSwNP. IL-4, IL-17A, and IL-1β may be critical for Livin gene expression.

**Author Contributions**

Hai Lin, designed study, collected and analyzed data, wrote article; Dong Lin, designed study, collected and analyzed data, revised article; Xisheng Xiong, collected and analyzed data, revised article.

**Disclosures**

**Competing interests:** None.

**Sponsorships:** None.

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