**Different Patterns of Lung Sirtuin Expression in Smokers With and Without Chronic Obstructive Pulmonary Disease**

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**Key Words:** chronic obstructive pulmonary disease; sirtuin; airflow limitation.

**Summary.** Background and Objective. Chronic obstructive pulmonary disease is characterized by persistent and modified inflammatory responses in lung. Human sirtuin, an antiaging and anti-inflammatory protein, is a metabolic NAD(+)-dependent protein/histone deacetylase that regulates proinflammatory mediators by deacetylating histone and nonhistone proteins. The aim of our study was to compare the expression of sirtuin in large and small airways in non-smokers, asymptomatic smokers, and smokers with chronic obstructive pulmonary disease.

Material and Methods. A total of 12 nonsmokers, 14 asymptomatic smokers, and 12 smokers with moderate chronic obstructive pulmonary disease were enrolled into the study. Immunohistochemical and Western blot methods were used to analyze sirtuin expression in the airways.

Results. The obtained results showed the nonuniform sirtuin expression throughout the bronchial tree. Smokers both with and without chronic obstructive pulmonary disease had decreased sirtuin expression in large airways. However, in small airways, sirtuin expression was decreased only in patients with chronic obstructive pulmonary disease. In addition, a correlation between airflow limitation, smoked pack-years and the number of sirtuin-positive cells in airways was found.

Conclusions. Smoking is characterized by suppressed sirtuin expression in large airways, whereas chronic obstructive pulmonary disease is characterized by more severe suppression of sirtuin expression both in large and small airways.

**Introduction**

Chronic obstructive pulmonary disease (COPD) is characterized by a slowly progressive and irreversible obstruction of the airways. Cigarette smoking is implicated as a major risk factor for the development of COPD because approximately 90% of patients with COPD are smokers (1). However, only a minority of smokers develops COPD. At the same time, practically all the smokers develop chronic bronchitis characterized by inflammation in the large airways.

Sirtuins (SIRTs) belong to class III histone/protein deacetylases (HDACs) and are members of the silent information regulator 2 (Sir2) family.

Members of this evolutionary family of deacetylases include 5 homologues in yeast (ySir2 and Hst1-4) and 7 in humans (SIRT1-7). Unlike class I and II HDACs, which consume a water molecule for direct hydrolysis of the acetyl group, sirtuins require NAD as a cosubstrate for the deacetylation reaction (2).

Gene silencing by this family of enzymes has been correlated directly with a longer lifespan (2). Sirt1 can deacetylate numerous proteins, such as tumor suppressor protein, p53, which modulates various genes that control damaged DNA. The deacetylase activity of SIRT proteins is dependent on the intracellular NAD<sup>+</sup> content (6).

COPD is characterized by oxidative stress and persistent inflammation in the lung tissues (1). Recently, it has been demonstrated that sirtuin regulates cigarette smoke-induced proinflammatory mediator release via RelA/p65 NF-κB expression in macrophages in vitro and in rat lungs in vivo (7). Rajendrasozhan et al. showed that sirtuin levels were reduced in macrophages and lungs of smokers and patients with COPD due to its posttranslational modifications by cigarette smoke-derived reactive components, leading to increased acetylation of RelA/p65 (8).

Resveratrol, probably by activating SIRT1 signaling pathway, inhibits the oxidative stress-dependent phenotypic shift of endothelial cells induced by high shear stress and proinflammatory factors in vitro (9). In addition, it was demonstrated that resveratrol intake was associated with better lung function (10). Recently, significant associations of single-nucleotide polymorphisms of the SIRT-2 gene and COPD...
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have been reported (11).

Cigarette smoke exposure also caused a reduction of SIRT1 expression in the lung tissues of A/J mice, with a concomitant elevation of MMP9. Intranasal treatment with a selective and novel SIRT1 small molecule activator, SRT2172, blocked the increase of MMP9 expression in the lungs (12).

However, to our knowledge, there are no data in the literature where sirtuin expression is compared both in large and small airways of smoking humans. Therefore, we attempted to compare the expression of sirtuins in large and small airways of nonsmokers, smokers with normal lung function, and smokers with COPD.

Material and Methods

Patients. A total of 38 subjects undergoing lung resection for a solitary peripheral non–small cell carcinoma were enrolled into the study. They were subdivided into 3 groups: 12 subjects were nonsmokers with normal lung ventilation function, 14 subjects were current smokers with normal lung function, and 12 current smoking subjects had moderate COPD.

The diagnosis of COPD was established according to the definition of GOLD guidelines (1). The patients with COPD had FEV1/FVC<70% and 50%<FEV1<80% of predicted. The study was approved by the local Ethical Committee, and it conforms to the declaration of Helsinki; informed consent was obtained from all the subjects. The clinical characteristics of the groups are presented in Table.

Study Design. The lung tissue specimens for the evaluation of small airways were taken from the subpleural parenchyma, but for the evaluation of large airways, the bronchial rings were taken from the segmental bronchus of the lobe obtained at surgery as far away as possible from the tumor site. The samples were fixed without inflation in 10% neutral buffered formalin, processed, and embedded routinely.

### Table. Characteristics of the Study Population

| Characteristic       | Nonsmokers (n=12) | Current Smokers (n=14) | Current Smokers With COPD (n=12) |
|----------------------|-------------------|------------------------|----------------------------------|
| Age, years           | 62 (15)           | 68 (7)                 | 66 (8)                           |
| Smoking history, pack-years | NA              | 29 (13)                | 32 (6)                           |
| FEV1, %              | 97 (10)           | 94 (12)                | 59 (11)*                         |
| FEV1/FVC, %          | 79 (7)            | 74 (5)                 | 60 (7)*                          |
| Male/female ratio    | 11:1              | 12:2                   | 10:2                             |
| Height, cm           | 170 (9)           | 173 (6)                | 176 (5)                          |
| Weight, kg           | 81 (14)           | 74 (12)                | 78 (15)                          |

Data are presented as mean (SD) unless otherwise stated. FEV1, forced expiratory volume in one second; FVC, forced vital capacity; NA, not applicable. *P<0.05 compared with smokers and nonsmokers with normal lung function.

Pulmonary Function Tests. Pulmonary function testing was performed on a Jaeger MasterScreen spirograph (Jaeger Gmbh, Germany) according to the British Thoracic Society Guidelines within one week before the surgery (13).

Immunohistochemistry. For immunohistochemistry, a formalin-fixed paraffin-embedded tissue was cut into 4-μm thick sections. Antigen retrieval was achieved by treatment in a domestic microwave for 30 minutes in citrate buffer (pH 6.0). The sections were incubated in 3.0% H2O2/PBS to quench endogenous peroxidase activity and then blocked with a protein block (Dako). The slides were then incubated for 1 hour with a primary monoclonal rabbit anti-human antibody against sirtuin, SIRT1 (Abcam, code ab 32441, clone E104).

An EnVision kit (Dako, Denmark) was used for the visualization of binding with the primary antibody. 3′,3′-Diaminobenzidine-tetrahydrochloride (DAB) was applied as a chromogen (7 minutes). For a positive control, human colon carcinoma (SIRT1 control) tissue was used. Negative controls were performed by omitting the primary antibody.

Analysis of airways and cell counting were performed using a light microscope connected to a video recorder linked to a computerized image system (Motic Image Advanced 3.2 software, Motic Ltd, China, Xyamen).

Cell Counting. Large airways were defined as cartilaginous bronchi with submucosal glands. The number of sirtuin-positive cells in large airways was counted randomly by a blinded investigator in the epithelium and subepithelium of each section, excluding the areas of smooth muscle and mucous secreting glands where these were identified.

Small airways were considered as membranous bronchioles without cartilage or glands and with an internal diameter of less than 2 mm, as previously described. At least 4 intact airways with a diameter of less than 2 mm were identified for each patient. In each airway, the number of sirtuin-positive cells in the epithelium, submucosa, and adventitia were quantified. The submucosa was defined as the area that extends from the distal edge of the basement membrane to the internal edge of smooth muscle, whereas the adventitia was defined as the area that extends from the outer edge of smooth muscle to the alveolar attachments.

Analysis of large and small airways and cell counting was performed using a light microscope (Motic BA400) connected to a video recorder linked to a computerized image system (Motic Image Advanced 3.2 software, Motic Ltd, China, Xyamen). An area of at least 10 mm2 was analyzed for each specimen. The cases were coded, and the measurements were made in a blinded fashion without knowledge of clinical data for a given patient’s spec-
imem. All cell counts were expressed as the number of cells per mm$^2$.

**Western Blot Analysis.** For the Western blot analysis, frozen lung tissues from the segmental bronchus (for large airways) or the subpleural parenchyma (for small airways) were homogenized using RIPA lysis buffer and protease inhibitors. The supernatant was decanted and used for PAGE-Western blot analysis to detect sirtuin protein. The proteins were transferred onto a PVDF membrane (Immobilon, Millipore) for 60 minutes at 150 mA. The membranes were blocked with 3% BSA in PBS for 1 hour and then incubated overnight at 4°C with a monoclonal rabbit anti-human antibody against sirtuin, SIRT1 (1:10000; Abcam, code ab 32441, clone E104) and rabbit polyclonal anti β-actin (1:5000). After washing with PBS, the blots were incubated for 30 minutes with peroxidase-coupled goat antirabbit IgG (1:10.000, Sigma-Aldrich) and then washed again in PBS. The blots were developed using chemiluminescence reagents (GE Healthcare). Band densitometry analysis of the membrane was performed using the scanned images of nonsaturated immunoblot films. Pixel intensities of the bands obtained in each experiment were normalized using β-actin signals.

**Statistical Analysis.** Group data were expressed as mean (SD) for functional data or median (range) for morphological data. Differences between groups were analyzed using analysis of variance (ANOVA) for functional data and the Kruskal-Wallis test for morphological data. When the differences were significant, the ANOVA test was followed by the unpaired $t$ test, and the Kruskal-Wallis test was followed by the Mann-Whitney $U$ test for comparison between groups.

Correlation coefficients were calculated using the Spearman rank method. A $P$ value of $<0.05$ was considered statistically significant. Data analysis was performed using the GraphPadPrism 5.0 version software.

**Results**

**Clinical Findings.** The patients’ clinical characteristics and the data of lung function are presented in Table.

As expected from the selection criteria, the values of FEV$_1$% of predicted and FEV$_1$/FVC ratio were significantly different in the patients with COPD, as compared to both nonsmokers and asymptomatic smokers. All patients except the nonsmokers’ group were current smokers.

No significant differences in age, weight, and height were documented among 3 selected groups. The asymptomatic smokers and the patients with COPD did not differ substantially by smoking history (29 [SD, 13] versus 32 [SD, 6] pack-years, $P>0.05$). In all the groups, there were more men than women.

All the study subjects suffered a stage I solitary non–small cell peripheral carcinoma. No significant differences were found regarding the histological types of tumors between nonsmokers, current smokers, and patients with COPD that excludes a possible influence of tumor type on the results obtained.

**Sirtuin Expression in Large Airways.** Fig. 1A–B shows the number of sirtuin-positive cells and the expression of sirtuin protein in large airways. Sirtuin was expressed by epithelial cells, macrophages, and lymphocytes.

The number of sirtuin-positive cells in large airways in the patients with COPD (10 cells/mm$^2$; range, 2–80) and the smokers with normal lung function (14 cells/mm$^2$; range, 6–30) was significantly lower than that determined in the nonsmokers (48 cells/mm$^2$; range, 12–140) ($P=0.0004$ and $P=0.002$, respectively). However, there was no difference in sirtuin expression between the smokers with and without COPD. The findings of Western blot analysis confirmed immunohistochemical data (Fig. 1B).

Fig. 2A–C shows the presence of sirtuin-positive cells in large airways.

When all patients were analyzed together, a positive correlation was found between the number of sirtuin-positive cells and airways obstruction, as judged from FEV$_1$% (Rho=0.64, $P<0.0001$)
There was a negative correlation between sirtuin expression in large airways and smoked pack-years (Rho=–0.63; \(P<0.0001\)) (Fig. 4A).

**Sirtuin Expression in Small Airways.** Fig. 5 shows the number of sirtuin-positive cells and protein expression in small airways. Sirtuin was expressed by epithelial cells, macrophages, and lymphocytes. The number of sirtuin-positive cells in small airways in the patients with COPD (11 cells/mm\(^2\); range, 2–21) was significantly lower than that determined in the smokers with normal lung function (26 cells/mm\(^2\); range, 3–60) and the nonsmokers (36 cells/mm\(^2\); range 10–130) (\(P=0.007\) and \(P=0.0001\), respectively). In addition, the patients with COPD had the decreased numbers of SIRT-1-positive cells compared with the asymptomatic smokers. The findings of Western blot confirmed immunohistochemical data (Fig. 5B).

Fig. 2D–F shows the presence of sirtuin-positive cells in small airways.

When all the patients were analyzed together, there was a weak positive correlation between the number of sirtuin-positive cells and airflow limitation, as judged from FEV\(_1\)% (Rho=0.62,
However, there was no significant correlation between sirtuin expression in small airways and smoked pack-years (Rho=–0.28; P=0.08, Fig. 4B).

Discussion

This study has demonstrated that the nonuniform expression of sirtuin exists throughout the bronchial tree. The decreased sirtuin expression in large airways of both smokers with and without COPD was observed, which correlated with airflow limitation.

In addition, the smokers with preserved lung function showed an unchanged number of sirtuin-positive cells, whereas the patients with COPD had decreased sirtuin expression in small airways.

A decrease in the sirtuin levels may partially be explained by oxidative/nitrosative/aldehydes alterations. Tobacco smoke may influence the expression of the SIRT1 and HDAC2 proteins. Such oxidative/nitrosative modifications may in turn render HDAC ineffective for the interaction with other signaling components. Similarly, the covalent modification of other redox-sensitive transcription factors has been recently shown (7, 8).

It has recently been demonstrated that exposure of MonoMac6 cells to cigarette smoke decreased sirtuin levels (involved in deacetylation process) associated with the increased activation of RelA/p65 subunit of NF-κB and increased proinflammatory cytokine release (7).

McBurney et al. reported that SIRT1-deficient mice had severe phenotype changes such as a small size, delay in eyelid opening, cardiac defect, and sterility; in addition, the lungs were consistently affected in the SIRT1 mutants (14). Increased neutrophil infiltration in the lungs of these mice after chronic pulmonary infection led to pneumonitis, pulmonary edema, and right ventricular hypertrophy. Thus, it is possible that the cigarette smoke-mediated reduction of SIRT1 is responsible for neutrophil influx and inflammatory response seen in rat lungs (7).

Rajendrasozhan et al. recently showed that SIRT1-deficient mice had severe phenotype changes such as a small size, delay in eyelid opening, cardiac defect, and sterility; in addition, the lungs were consistently affected in the SIRT1 mutants (14). Increased neutrophil infiltration in the lungs of these mice after chronic pulmonary infection led to pneumonitis, pulmonary edema, and right ventricular hypertrophy. Thus, it is possible that the cigarette smoke-mediated reduction of SIRT1 is responsible for neutrophil influx and inflammatory response seen in rat lungs (7).

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It has been demonstrated that sirtuins play an important role in regulating autophagy in response to cigarette smoke (15).

However, the low expression of SIRT1 cannot be explained only by cigarette smoke-induced suppression. Our data showed that sirtuins were much more suppressed in the patients with COPD than simply in the smokers. Besides this, a negative correlation between smoked pack-years and SIRT1 expression indicates the role of smoking. Similarly, the correlation between FEV₁ % and SIRT1 expression also indicates the link between the severity of the disease process and expression of sirtuins.

Our results about sirtuin expression in small airways are consistent with the previous study, which demonstrated that SIRT1 levels were significantly decreased in the peripheral lungs of patients with COPD, and this was inversely correlated with matrix metalloproteinase-9 (MMP-9) activity (16). MMP-9 is closely related to the pathogenesis of COPD (17). Our data showed that sirtuins were much more suppressed in the patients with COPD than simply in the smokers. Besides this, a negative correlation between smoked pack-years and SIRT1 expression indicates the role of smoking. Similarly, the correlation between FEV₁ % and SIRT1 expression inversely correlated with matrix metalloproteinase-9 activity. Our results about sirtuin expression in small airways are consistent with the previous study, which demonstrated that SIRT1 levels were significantly decreased in the peripheral lungs of patients with COPD, and this was inversely correlated with matrix metalloproteinase-9 (MMP-9) activity (16). MMP-9 is closely related to the pathogenesis of COPD (17). It is intriguing why sirtuin was downregulated in the small airways in the patients with COPD only.

Firstly, it is possible that sirtuin expression is associated with the number of inflammatory cells in the airways. This hypothesis in some extent is consistent with the previous study, which demonstrated that patients with COPD had the greater numbers of inflammatory cells in small airways than large ones (18, 19).

In addition, SIRT1 plays a key role in the regulation of NF-κB-dependent proinflammatory cytokine release (20). Secondly, the decreased sirtuin expression in small airways in patients with COPD leads to the increased MMP-9 expression with subsequent lung remodeling that is characteristic of COPD (16, 17).

To conclude, asymptomatic smokers had decreased sirtuin expression in large airways; however, sirtuin expression was not suppressed in small airways. COPD is characterized by more severe suppression of sirtuin expression with predominance in small airways, which results in subsequent persistent inflammation and lung tissue remodeling.

Statement of Conflict of Interest
The authors state no conflict of interest.

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