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Fucosylated surfactant protein-D is a biomarker candidate for the development of chronic obstructive pulmonary disease☆

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ABSTRACT

We previously reported that knockout mice for α1,6-fucosyltransferase (*Fut8*), which catalyzes the biosynthesis of core-fucose in *N*-glycans, develop emphysema and that *Fut8* heterozygous knockout mice are more sensitive to cigarette smoke-induced emphysema than wild-type mice. Moreover, a lower FUT8 activity was found to be associated with a faster decline in lung function among chronic obstructive pulmonary disease (COPD) patients. These results led us to hypothesize that core-fucosylation levels in a glycoprotein could be used as a biomarker for COPD. We focused on a lung-specific glycoprotein, surfactant protein D (SP-D), which plays a role in immune responses and is present in the distal airways, alveoli, and blood circulation. The results of a glycomic analysis reported herein demonstrate the presence of a core-fucose in an *N*-glycan on enriched SP-D from pooled human sera. We developed an antibody–lectin enzyme immunoassay (EIA) for assessing fucosylation (core-fucose and α1,3/4 fucose) in COPD patients. The results indicate that fucosylation levels in serum SP-D are significantly higher in COPD patients than in non-COPD smokers. The severity of emphysema was positively associated with fucosylation levels in serum SP-D in smokers. Our findings suggest that increased fucosylation levels in serum SP-D are associated with the development of COPD.

Biological significance: It has been proposed that serum SP-D concentrations are predictive of COPD pathogenesis, but distinguishing between COPD patients and healthy individuals to establish a clear cut-off value is difficult because smoking status highly affects circulating SP-D levels. Herein, we focused on *N*-glycosylation in SP-D and examined whether or not *N*-glycosylation patterns in SP-D are associated with the pathogenesis of COPD. We performed an *N*-glycomic analysis of human serum SP-D and the results show that a core-fucose is present in its *N*-glycan. We also found that the *N*-glycosylation in serum SP-D was indeed altered in COPD, that is, fucosylation levels including core-fucosylation are significantly increased in COPD patients compared with non-COPD smokers. The severity of emphysema was positively associated with fucosylation levels in serum SP-D in smokers. Our findings shed new light on the discovery and/or development of a useful biomarker based on glycosylation changes for diagnosing COPD.

This article is part of a Special Issue entitled: HUPO 2014.

1. Introduction

Chronic obstructive pulmonary disease (COPD) is characterized by a persistent airflow limitation that is generally progressive and is not entirely reversible [1]. The disease conditions are quite complex and are pathologically characterized by a mixture of obstructive bronchiolitis and emphysema that are principally caused by exposure to a long-term smoking and/or noxious air pollutants [2]. COPD is exacerbated in some patients and that is typically caused by acute airway inflammation in response to inhaled bacteria or viruses [3,4]. COPD is currently the fourth-leading cause of death worldwide and is a serious health problem that results in a substantial economic and social burden [1]. The disease has thus received wide-spread social attention and inquiries in relation to both the onset and severity of the disease. COPD can be objectively diagnosed by spirometry, but the method has a diagnostic drawback in terms of specificity and sensitivity, and hence the disease has been considerably underdiagnosed in some countries, including Japan [5,6]. The introduction of a quantifiable serum biomarker for COPD would thus be a great benefit for physicians, especially those who are not as familiar with the disease as pulmonologists, in assessing patients, predicting risk levels, and the type of treatment [5,7].

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Several potential biomarkers for COPD have been reported under different conditions [7,8]. These include local inflammatory biomarkers such as surfactant protein D (SP-D), club cell protein 16 (CC-16), and pulmonary and activation-regulated chemokine (PARC/CCL-18), and systemic inflammatory biomarkers such as the C-reactive protein (CRP) and fibrinogen [7]. Among them, SP-D (43 kDa) belongs to the collectin subgroup of the C-type lectin superfamily and consists of a short N-terminal domain, a collagen-like domain, a neck domain and a C-terminal carbohydrate recognition domain [8,9]. The protein structure is stabilized by assembling into a dodecamer (four trimers) via two conserved cysteine residues (Cys-35 and Cys-40) in the N-terminal domain [10,11]. SP-D is principally produced in and secreted from type II pneumocytes in the lung and is found in the distal airways and alveoli, and also in the blood circulation [7,8]. The protein contains one potential *N*-glycosylation site at Asn-90 in the primary sequence, and the site is indeed *N*-glycosylated albeit the precise glycan structures still remain unclear [9,11]. SP-D plays an important role in regulating immune and inflammatory responses in the lung, and hence, it has been linked to a variety of lung diseases, including COPD [7,8,12]. SP-D knockout mice develop emphysema [13]. In addition, our previous genetic association study showed that single nucleotide polymorphisms (SNPs) in SP-D (Met11Thr) are associated with a susceptibility to emphysema [14]. Serum SP-D was reported to be a candidate biomarker for subclinical cigarette smoke-induced lung damage, possibly including COPD [15]. Moreover, increases in serum SP-D concentrations have a diagnostic value for assessing patients with interstitial pneumonia [16,17]. Several lines of evidence indicate that serum SP-D concentrations are also increased in COPD patients and that it could be a useful biomarker for determining COPD patients' subtypes and a useful therapy to decrease exacerbation and disease progression [7,12]. Nevertheless, the difference in serum SP-D concentrations between healthy individuals and COPD patients is not sufficient to permit a clear cut-off value to be established because smoking status has a substantial effect on circulating SP-D levels [5,18–20].

α1,6-Fucosyltransferase (*Fut8*) catalyzes the transfer of a fucose residue from GDP-L-fucose to the reducing terminal *N*-acetylglucosamine residue in an *N*-glycan core structure via an α1,6-linkage (termed as core-fucose) [21,22]. Our previous studies indicated that *Fut8* knockout mice ($Fut8^{-/-}$) spontaneously develop emphysema [23] and that *Fut8* heterozygous knockout mice ($Fut8^{+/-}$) easily develop emphysema when chronically exposed to cigarette smoke compared to wild-type mice ($Fut8^{+/+}$) [24]. Additionally, another of our previous studies indicated that a lower FUT8 activity is closely associated with a faster decline in lung function, as evidenced by the forced expiratory volume in one second (FEV1) among COPD patients and that patients with lower FUT8 activity suffer from more-frequent exacerbations [25]. Furthermore, a recent report on SNPs in human FUT8 (Thr267Lys) revealed its association with emphysema [26], suggesting that FUT8 might be relevant to development of COPD. These results led us to hypothesize that core-fucosylation levels in a lung-specific glycoprotein, such as SP-D, might be associated with the pathogenesis of COPD.

In the present study, we examined the issue of whether changes in *N*-glycosylation patterns in serum SP-D would have a predictive value for assessing COPD pathogenesis, especially in terms of disease development. To accomplish this, we first performed an *N*-glycan structural analysis of SP-D obtained from pooled human sera by using the high-sensitive AQ-labeling method for glycans [27] and demonstrated the presence of a core-fucose in the SP-D *N*-glycan. We next constructed a new antibody–lectin enzyme immunoassay (EIA) for fucosylated SP-D based on the sandwich technique with an anti-SP-D polyclonal antibody and a fucose-specific *Aleuria aurantia* lectin (AAL). Using the developed assay, we assessed the fucosylation levels in serum SP-D from COPD patients and compared the findings with those for non-COPD smokers and subjects who were never smokers. The potential of fucosylated SP-D as a biomarker candidate for COPD development is discussed.

2. Materials and methods

2.1. Materials

Materials were obtained from the following suppliers: Maltose agarose for affinity chromatography and biotinylated AAL from Seikagaku Biobusiness (Tokyo, Japan); pooled human serum from Kohjin-Bio (Saitama, Japan); HRP-conjugated streptavidin, SuperBlock T20 Blocking Buffer, SuperSignal West Femto, and CBB from Thermo scientific (Waltham, MA); sodium metaperiodate ($NaIO_4$), borane-dimethylamine complex, iodoacetamide (IAA), and ammonium bicarbonate (NH_4HCO_3) from Wako Pure Chemicals (Osaka, Japan); α-cyano-4-hydroxycinnamic acid (CHCA), and 2,5-dihydroxybenzoic acid (DHB) from Shimadzu Biotech (Kyoto, Japan); 3-aminoquinoline (3-AQ), ammonium dihydrogen phosphate ($NH_4H_2PO_4$), peptide-*N*-glycosidase F (PNGase F), and DTT from Sigma-Aldrich (ST. Louis, MO); and trypsin (mass spectrometry grade) from Promega Corp. (Madison, WI). All solvents used for MS analyses were of HPLC grade.

2.2. Ethical considerations

The current study was approved by the ethics committee of the Nippon Medical School (approval number: 18-11-31). Written informed consent was obtained from all subjects. The study was also approved by the ethics committee of RIKEN.

2.3. Classification of human subjects and sampling of sera

Outpatients who were current or ex-smokers; had cough, expectoration, and/or dyspnea; and who visited the Respiratory Care Clinic of the Nippon Medical School for ambulatory treatment between April 2007 and April 2009 were invited to participate in the study. Subjects who were diagnosed with COPD according to the Global initiative for chronic Obstructive Lung Disease (GOLD) criteria [28] and were classified into the COPD group (n = 20). The others were classified into the "non-COPD smokers" group (n = 15). Subjects who were never smokers with no airflow limitations who visited our clinic during the same period were also invited to participate as control subjects (n = 18). The basic characteristics of the subjects are shown in Table 1. Serum samples were collected from these subjects when they were stable. A stable state was defined as being "free from an exacerbation for at least 8 weeks".

2.4. COPD-related clinical parameters

2.4.1. Lung function tests

Post-bronchodilator forced expiratory volume in 1 s (FEV1), carbon monoxide-diffusing capacity (diffusing capacity divided by alveolar volume [DLCO/VA]), vital capacity (VC), and forced vital capacity (FVC) were measured according to the American Thoracic Society guidelines [29] using equipment for lung function testing (CHESTAC; CHEST Co., Tokyo, Japan). We used the reference values for post-bronchodilator FEV1 and VC specified by the Japanese Respiratory Society [30].

2.4.2. High-resolution computed tomography (HRCT)

We performed helical high-resolution computed tomography (HRCT) scans at 1.25-mm collimation, 0.8 s scan time (rotation time), 120 kV, and 100–600 mA with a Light Speed Pro16 CT scanner (GE Co., Tokyo, Japan). The percentage of low attenuation area in the upper lung field (LAA%-U), reflecting the severity of emphysema, was calculated as described previously [31,32].

2.5. Enrichment of human SP-D from pooled serum

Human serum SP-D was enriched according to a method described by Lu et al. [11] with minor modifications. To enrich SP-D for *N*-