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α1,6-Fucosyltransferase (Fut8) is implicated in vulnerability to elastase-induced emphysema in mice and a possible non-invasive predictive marker for disease progression and exacerbations in chronic obstructive pulmonary disease (COPD)

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ABSTRACT

Fut8 (α1,6-Fucosyltransferase) heterozygous knock-out (*Fut8*$^{+/-}$) mice had an increased influx of inflammatory cells into the lungs, and this was associated with an up-regulation of matrix metalloproteinases, MMP-2 and MMP-9, after treatment with porcine pancreatic elastase (PPE), exhibiting an emphysema-prone phenotype as compared with wild type mice (*Fut8*$^{+/+}$). The present data as well as our previous data on cigarette-smoke-induced emphysema [8] led us to hypothesize that reduced Fut8 levels leads to COPD with increased inflammatory response in humans and is associated with disease progression. To test this hypothesis, symptomatic current or ex-smokers with stable COPD or at risk outpatients were recruited. We investigated the association between serum Fut8 activity and disease severity, including the extent of emphysema (percentage of low-attenuation area; LAA%), airflow limitation, and the annual rate of decline in forced expiratory volume in 1 s (FEV_1). Association with the exacerbation of COPD was also evaluated over a 3-year period. Serum Fut8 and MMP-9 activity were measured. Fut8 activity significantly increased with age among the at risk patients. In the case of COPD patients, however, the association was not clearly observed. A faster annual decline of FEV_1 was significantly associated with lower Fut8 activity. Patients with lower Fut8 activity experienced exacerbations more frequently. These data suggest that reduced Fut8 activity is associated with the progression of COPD and serum Fut8 activity is a non-invasive predictive biomarker candidate for progression and exacerbation of COPD.

1. Introduction

COPD is characterized by a combination of emphysema and chronic bronchitis, and the patients suffer a progressive loss of lung function caused by the inhalation of noxious gases, most commonly from tobacco but also from occupational exposures [1].

α1,6-Fucosyltransferase (Fut8) catalyzes the addition of a core fucose to *N*-glycans of glycoproteins (Supplementary Figure E1), and has a variety of physiological functions [2–6]. Our previous studies indicated that *Fut8*$^{-/-}$ mice develop an emphysematous phenotype due to dysregulation of the TGF-β1 receptor [7]. And a decrease in Fut8 activity is observed in cigarette smoke-exposed mice [8], which is thought to be related to the development of pathological process in COPD. Moreover, Fut8 affects the function of various growth factor receptors, such as epidermal growth factor (EGF) receptor [9], platelet derived growth factor (PDGF) receptor [10], insulin-like growth factor-1 (IGF-1) [11], and vascular endothelial growth factor receptor (VEGFR) – 2 [12], whose deficits are also related to emphysema formation [13]. Because increased VEGF level has been reported in exacerbation of COPD as a favorable response [14,15], decreased Fut8 activity is considered to be related to attenuated response to VEGF resulting in the deterioration of lung function. Furthermore, failure in alveologenesis has been reported in PDGF deficient mice [16]. Thus, signal transduction mediated by Fut8 catalytic activity is considered to be crucial in avoiding pathological conditions. However, investigations regarding Fut8 activity in humans are incomplete, especially among patients with COPD.

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Matrix metalloproteinases (MMPs) have been implicated in the pathogenesis of COPD [17], and increased expression of these proteinases has been reported in lungs of *Fut8*$^{-/-}$ mice [7]. Among MMPs, several studies have implicated MMP-9 in COPD pathogenesis [18–20] although there has been a debate concerning this issue [21]. Moreover, it has been reported that increased MMP-9 is associated with not only a rapid decline of lung function [22] but also exacerbation [23]. Considering these observations, increased MMP-9 through decreased Fut8 activity is thought to be involved in both the pathogenesis and progression of COPD. However, no reports concerning MMP-9 in association with Fut8 activity in humans have appeared.

Our recent study indicated that *Fut8*$^{+/-}$ mice are sensitive to cigarette-smoke induced emphysema and at early stage of cigarette smoke, an activation of MMP-9 was observed [8]. This study led us to hypothesize that reduced Fut8 enhances the onset or progression of COPD with increased inflammatory response in both mice and humans.

We first attempted to determine whether or not *Fut8*$^{+/-}$ mice are also emphysema-prone by using a porcine pancreatic elastase (PPE) – induced model. We next measured Fut8 activity and MMP-9 activity in sera from symptomatic ex- or current smokers with COPD and at risk outpatients in a stable state and compared it with the data for the rate of decline of airflow limitation assessed by pulmonary function tests, frequencies of exacerbation, and the extent of emphysema which was evaluated by helical-resolution computed tomography (HRCT) of the chest. The current study is the first to have demonstrated the involvement of Fut8 activity in the exacerbation and progression of human COPD.

Preliminary studies have been previously reported in an abstract form at the American Thoracic Society meeting [24].

2. Materials and methods

2.1. Mice

Fut8$^{+/-}$ mice were generated on a pure C57BL/6J background (>10 backcrosses). Age- and sex-matched WT C57BL/6J mice were used as controls. Their genotypes were determined by the polymerase chain reactions. All experimental protocols and procedures were approved by the Ethical Committee on Animal Research of Hokkaido University School of Medicine.

2.2. PPE-induced emphysema mouse model

Nine-week-old mice were anesthetized by intraperitoneal injection of ketamine and xylazine. Five units of PPE (EC134; Elastin Products, Owensville, MO) dissolved in 50 ml of sterile saline or 50 ml of saline alone was then injected into the trachea with a MicroSprayer drug delivery device (Penn-Century, Philadelphia, PA) (24Bivas).

2.3. Sampling of mouse lung tissue

On completion of the protocol, the right ventricle was perfused with saline and the mice were sacrificed by CO_2 inhalation. The lungs were inflated with diluted Tissue-Tek OCT (Sakura Finetek USA, Torrance, CA) [50% (vol/vol) in RNase-free phosphate-buffered saline containing 10% sucrose], removed from the thoracic cavity, immediately frozen on dry ice, and stored at −80 °C as previously described [25].

2.4. Brochoalveolar lavage (BAL) fluid analysis

The lungs were lavaged with 0.75 ml of PBS × 4 to obtain the BAL fluid, by inserting a 22-g i.v. catheter into the trachea. Total cell counts of the BAL fluid were determined with a hemocytometer after lysis of red blood cells. Cell differentials in BAL fluid were examined by cytospin preparation stained with Diff-Quick reagent (Sysmex International Reagents, Kobe, Japan). Differential counts were performed by examining >250 cells with a standard light microscope.

2.5. Morphometric assessment

After fixation, midsagittal sections of the lungs (4 μm) were stained with hematoxylin and eosin. Alveolar size of the lung was assessed by the determination of the mean linear intercepts (Lm). Lm were calculated based on 20 randomly selected fields in each section (in total 80 fields/mouse) at 200× magnification with two crossed test lines. The intercepts of alveolar walls with these lines were counted. Images with bronchi, blood vessels, and compression of alveolar space were excluded. Adjustment by shrinkage of samples accompanied with processing has not been performed.

2.6. Gelatin zymography

We assessed the gelatinolytic activities of BAL fluid by gelatin zymography. To semi-quantify the gelatinolytic activities, zymograms were captured using a Scan Jet II cx/T (Hewlett Packard, Palo Alto, CA) and the band intensity was calculated using NIH Image software (version 1.54, ML) [26]. The results were presented in arbitrary units (AU).

2.7. Human subjects

164 consecutive symptomatic ex- or current smokers with COPD and 62 at risk outpatients, presenting from July 2004 to June 2007 at the Respiratory Care Clinic, Nippon Medical School, Tokyo, Japan, which is a secondary care clinic specialized for COPD managements, were recruited as shown in Supplementary Table E1. See Note #E1 in the online supplement for recruitment criteria of patients.

The current study was approved by the ethical committee of the Nippon Medical School (approval number: 22-1). Written informed consent was obtained from each subject.

2.8. Pulmonary function tests (PFTs) and extent of emphysema on helical computed tomorgraphy test (HRCT)

See Note #E2 in the online supplement for detailed information.

2.9. Definition of exacerbation of COPD and follow-up study

Exacerbations were diagnosed following American Thoracic Society (ATS)/European Respiratory Society (ERS)-defined criteria. See Note #E3 in the online supplement for detailed definition.

2.10. Blood collection for the measurement of serum Fut8 and MMP-9 activity, and platelet count

See Note #E4 in the online supplement for detailed information.

2.11. Statistical analysis

Data obtained from mice are expressed as the mean value ± SEM, and differences between groups were assessed by analysis of variance (ANOVA). Patient characteristics recorded at recruitment are presented as mean ± SD or median with interquartile

range unless otherwise stated. Differences in patient characteristics between groups were evaluated by Student's t test or χ^2 test. Data were analyzed using JMP 8 software version 8.0.1 (SAS Institute Inc., Cary, NC, USA). P values < 0.05 were considered significant. See Note #E5 in the online supplement for further details of statistical analysis.

3. Results

3.1. Fut8 $^{+/-}$ mice are vulnerable to PPE-induced emphysema

Fut8$^{+/-}$ mice showed significant increases of inflammatory cells such as neutrophils and macrophages as compared to wild type mice after administration of intratracheal PPE. The total cell numbers (Fig. 1A), macrophages (Fig. 1B) and neutrophils (Fig. 1C) were markedly increased at 3 h, on day 1 and on day 7 after PPE administration (P < 0.05 versus untreated group).

MMP-2 and MMP-9 in BAL fluid from all mice were analyzed by gelatin zymography and it was found that PPE administration induced a remarkable increase in gelatinolytic activities of MMP-2 and MMP-9 in BAL fluid of *Fut8*$^{+/-}$ mice (Fig. 2). A semi-quantitative analysis indicated that the gelatinolytic activity was increased by 3 times for MMP-9 in heterozygous mice (P < 0.05 versus wild type mice). Activation of MMP-2 and MMP-9 may lead to the progression of increased alveolar destruction and decline in lung function.

Morphometric assessments were then performed on day 14 and day 21 after PPE administration. Intratracheal PPE administration resulted in a remarkable airspace enlargement in *Fut8*$^{+/-}$ mice at day 21 (Fig. 3). Mean linear intercept (Lm), a commonly used index for airspace enlargement (emphysema) and the severity associated with structural destruction in the lung, was significantly increased (52 ± 1.2 to 75 ± 7.1, P < 0.05 versus saline treated mice) compared with wild type mice (51 ± 0.6 to 58 ± 1.5). More importantly, *Fut8*$^{+/-}$ mice having only half of the Fut8 enzyme activity developed emphysematous changes from day 14 after PPE treatment, which is a much more rapid progress than that of wild type mice, most of which exhibit emphysema at 21 days after the treatment. This indicates that *Fut8*$^{+/-}$ mice are again vulnerable to emphysema as we observed in cigarette-smoke induced emphysema in a previous study [8].

3.2. Correlation of Fut8 activity with age

In all cases including 164 COPD patients and 62 at risk patients, Fut8 activity was associated with platelet counts (P = 0.0022, n = 226, data not shown) which is consistent with a report by Koscielak et al. [27]. For this association, log-transformed Fut8 activity divided by platelet count was used in the subsequent analysis. Among 62 at risk patients, Fut8 activity significantly increased with age (Supplementary Figure E2A, P = 0.043, R^2 = 0.079), while Fut8 activity appeared to decrease or did not change with age among 164 COPD patients although statistical significance was not achieved (Figure E2B).

3.3. Association of Fut8 activity with rate of decline of FEV_1 among COPD patients

In order to know if the altered activity of Fut8 was associated with pathogenesis of COPD, especially in terms of disease progres-

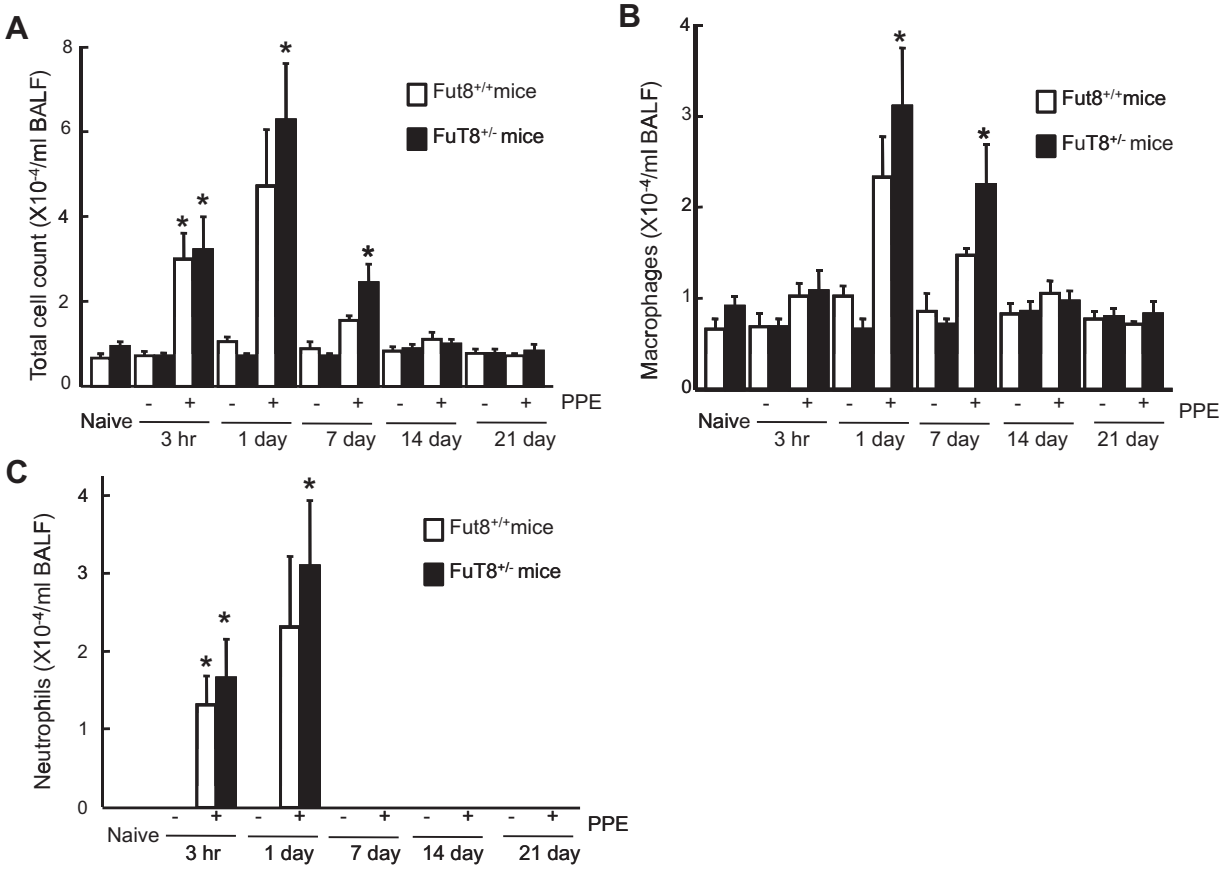

Fig. 1. *Fut8*$^{+/-}$ mice are vulnerable to PPE treatment. *Fut8*$^{+/-}$ mice showed increased lung inflammation induced by PPE. Bronchoalveolar lavage (BAL) fluid was collected at the indicated days after PPE instillation. Numbers of total cells (A), macrophages (B) and neutrophils (C) were counted. Data are the mean ± SE (n = 6). (*P < 0.05 compared with untreated group).

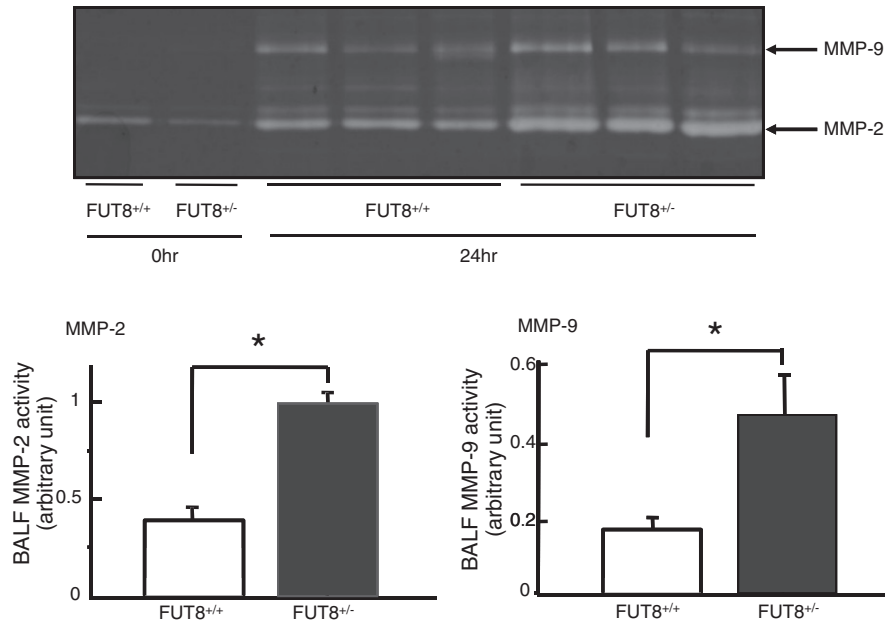

Fig. 2. PPE administration induced high activity of MMP-2 and MMP-9 in *Fut8*$^{+/-}$ mice. Gelatin zymography was performed on BAL fluid collected from all the mice at 24 h after PPE administration. The band intensity of MMP-2 and MMP-9 were semi-quantified. (*$P < 0.05$ compared with the wild mice).

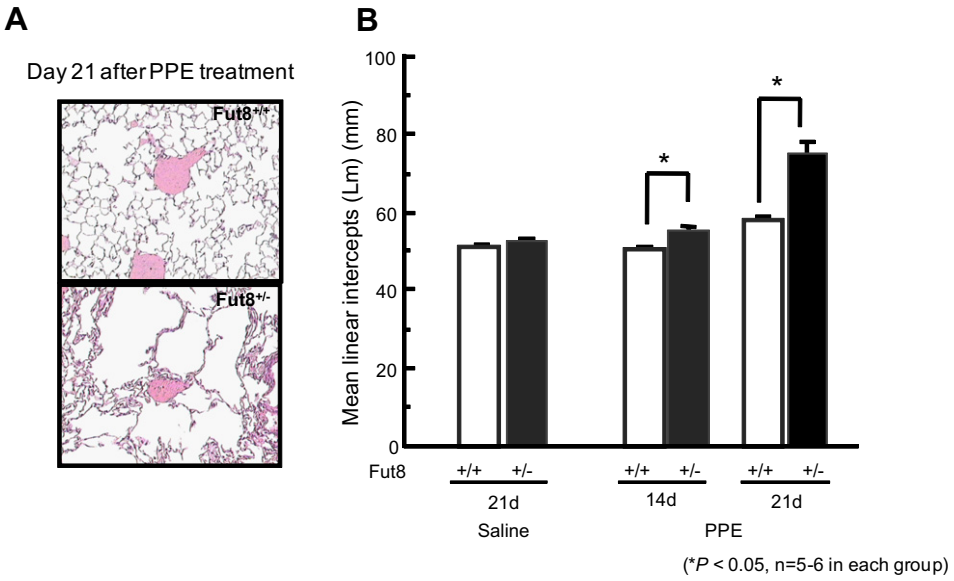

Fig. 3. Fut8$^{+/-}$ mice rapidly develop emphysema after PPE administration. (A) Representative H and E stained sections are shown. Mean linear intercept (Lm), which is proportional to emphysema, was quantified. Data are the mean ± SE ($n = 10$, CS: $n = 5$, PPE treatment. (B) The mean linear intercepts were significantly increased in *Fut8*$^{+/-}$ mice in response to PPE treatment at 14 and 21 days after the treatment. (*$P < 0.05$ compared with wild type).

sion, we analyzed the association of Fut8 activity with the annual rate of decline in FEV_1 obtained from 95 COPD patients. The FEV_1 is the volume exhaled during the first second of a forced expiratory maneuver started from the level of total lung capacity, and the annual decline of FEV_1 is faster in patients with COPD when compared with healthy individuals [1]. The median value for the rate of decline of FEV_1 was −24.6 ml/year (IQR -49.5–0.7) with a normal distribution (data not shown). Major characteristics of these patients are listed in Supplementary Table E2. Subsequently, we divided the population into two groups: "fast decliners", whose rate of decline of FEV_1 was less than median value −24.6 ml/year, and "slow decliners", whose rate of decline of FEV_1 was more than −24.6 ml/year. As shown in Fig. 4, Fut8 activities among fast decliners were significantly decreased when compared with those of slow decliners ($P = 0.0131$, $R^2 = 0.096$) suggesting that Fut8 enzyme activity is associated with the progression of COPD.

3.4. Association of Fut8 activity with exacerbation of COPD

Among 95 patients with COPD whose annual rate of decline in FEV_1 was obtained, 78 patients were followed up for exacerbation during the study period. The basic characteristic differences between non-exacerbators and exacerbators are listed in Supplementary Table E3. 24 patients who experienced frequent exacerbation had more deteriorated vital capacity (VC), FEV_1 and FEV_1% predicted.

Fig. 4. Association between Fut8 activity and annual rate of decline of forced expiratory volume in one second (FEV_1) among COPD patients. Fast and slow decliners indicate whose annual rate of decline of FEV_1 was either less than median value −24.6 ml/year or more than that, respectively. Fut8 activities among fast decliners were significantly decreased when compared with those of slow decliners (*$P = 0.0131$, $R^2 = 0.096$, $n = 95$).

In an attempt to elucidate whether Fut8 activity is one of the independent predictors for exacerbation, we used logistic regression model between exacerbators and non-exacerbators. Because changes in FEV_1 and increase in white-cell counts have been reported to associate with increased exacerbation frequency [28], these two variables were incorporated into the model, together with age, sex, smoking status, pack-years, $FEV_{1\%}$ predicted and log-transformed ratio of Fut8 activity to platelet count. As a result, higher Fut8 activity was an independent predictor for non-exacerbators when compared with exacerbators (Supplementary Table E4) (Odds Ratio 7.92, 95% CI; 1.94 – 49.5, $P = 0.0024$).

3.5. Correlation of percentage of low attenuation area (LAA%) with Fut8 activity or MMP-9 activity

Since *Fut8*$^{+/-}$ mice are prone to develop emphysema which is induced by not only cigarette smoke but also PPE, we investigated whether lower Fut8 activity was associated with LAA% in human patients. LAA on CT scans reflects emphysema in the lungs which is one of the pathological features of COPD, and percentage of LAA (LAA%) increases as emphysema becomes more severe [29]. Even though there was no correlation between Fut8 activity and LAA%, MMP-9 activity was significantly correlated with LAA% (Supplementary Figure E3, $P = 0.0252$, $R^2 = 0.083$).

4. Discussion

Glycosylation of glycoproteins by Fut8 and its enzymatic product designated as a core fucose play a pivotal role in various cellular functions including cell surface receptors such as TGF-β1, EGFR, VEGFR, and membrane receptors such as integrins and their signaling [2,3]. Among these signals, TGF-β1 plays important roles in the maintenance of injury-repair balance by controlling the expressions of MMPs. *Fut8*$^{-/-}$ mice develop emphysema through dysregulation of TGF-β1 signaling [7]. Fut8 activity has been reported to increase with aging in mice [11]; however, the effect of cigarette smoke exposure on Fut8 activity remains unclear.

Our recent *in vivo* and *in vitro* studies showed that an impaired protein-core fucosylation enhanced the susceptibility to cigarette smoke and is involved in at least a part of disease process of emphysema, in which TGF-β1-Smad signaling was impaired and MMP-mediated destruction of lung parenchyma was upregulated because cigarette smoke exposure inhibits Fut8 activity in mice [8]. Taking these observations into consideration, it is possible that altered Fut8 activity in some conditions could modulate the pathological changes observed in emphysema formation in the lung. In the present study, we found that tracheal inhalation of PPE to the *Fut8*$^{+/-}$ mice had the similar response as cigarette smoking exposure, and Fut8 might be contributing to the development of COPD.

Recently, pathogenesis of COPD is estimated to be associated with abnormal aging process with accelerated deterioration in lung function [30]. Like sirtuin 1 [31], which is considered to be an anti-aging molecule, Fut8 might be also working as an anti-aging molecule. However, our knowledge regarding the prediction of Fut8 in the pathogenesis of COPD in humans is incomplete. In this study, lower Fut8 activity was observed among COPD patients with faster annual decline of FEV_1. Annual rate of decline of FEV_1 is affected by smoking status [32,33], frequency of exacerbation [34], and some pharmacotherapies [35,36]. Recently, Nishimura and colleagues reported that emphysema severity also affects the annual change of FEV_1 [37]. Although decrement in Fut8 activity among COPD patients with faster decline in FEV_1 is the first to have been reported in the current study, the precise mechanisms by which Fut8 activity is affected are still unknown. Previously we have reported that a polymorphism within *Fut8* gene is associated with emphysema [38]. Together with cigarette smoke exposure which reduces Fut8 activity and PPE-induced emphysema model by using *Fut8*$^{+/-}$ mice in this study, multiple mechanisms are considered to be involved in the regulation of Fut8 activity and in the development and progression of COPD through modification of Fut8 activity.

In this study, *Fut8*$^{+/-}$ was exposed to PPE, and MMP-2 and MMP-9 were significantly increased in BAL fluid after exposure when compared with those of wild type mice. Moreover, increase in the mean linear intercepts was observed earlier than usual, suggesting that the Fut8 enzyme activity is important in the pathogenesis of emphesema in this mice model. In human studies, an association between Fut8 activity and exacerbation was clearly observed among patients with COPD. However, this was not true with MMP-9 activity although the activity of MMP-9 was significantly correlated with the severity of emphysema. Despite of these observations, we failed to find an association between Fut8 and MMP-9 activity. This might be because blood sampling was done at when COPD was in a stable state. In addition, MMP-9 activity is affected by releasing process from cells in response to inflammatory mediators such as CXCL8 [39], TNF-α [40] and endotoxin [41]. It is likely that decreased Fut8 activity might increase the expression of MMPs at exacerbation, and then accelerate exacerbation frequency. As a result of this, frequent exacerbation could lead to decline in FEV_1 [34].

In conclusion, reduced Fut8 activity might be closely associated with disease progression of COPD through the increased exacerbation frequency and accelerated decline in FEV_1. Serum Fut8 is a non-invasive surrogate marker candidate for progression and exacerbation of COPD. Even though underlying precise mechanisms by which reduced Fut8 leads to COPD are still under investigation, possible explanation for this is probably the low levels of Fut8 may down-regulate the receptor molecules which then dysregulate the down stream signaling just like TGF-beta signaling observed in the mice model.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2012.06.081.

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