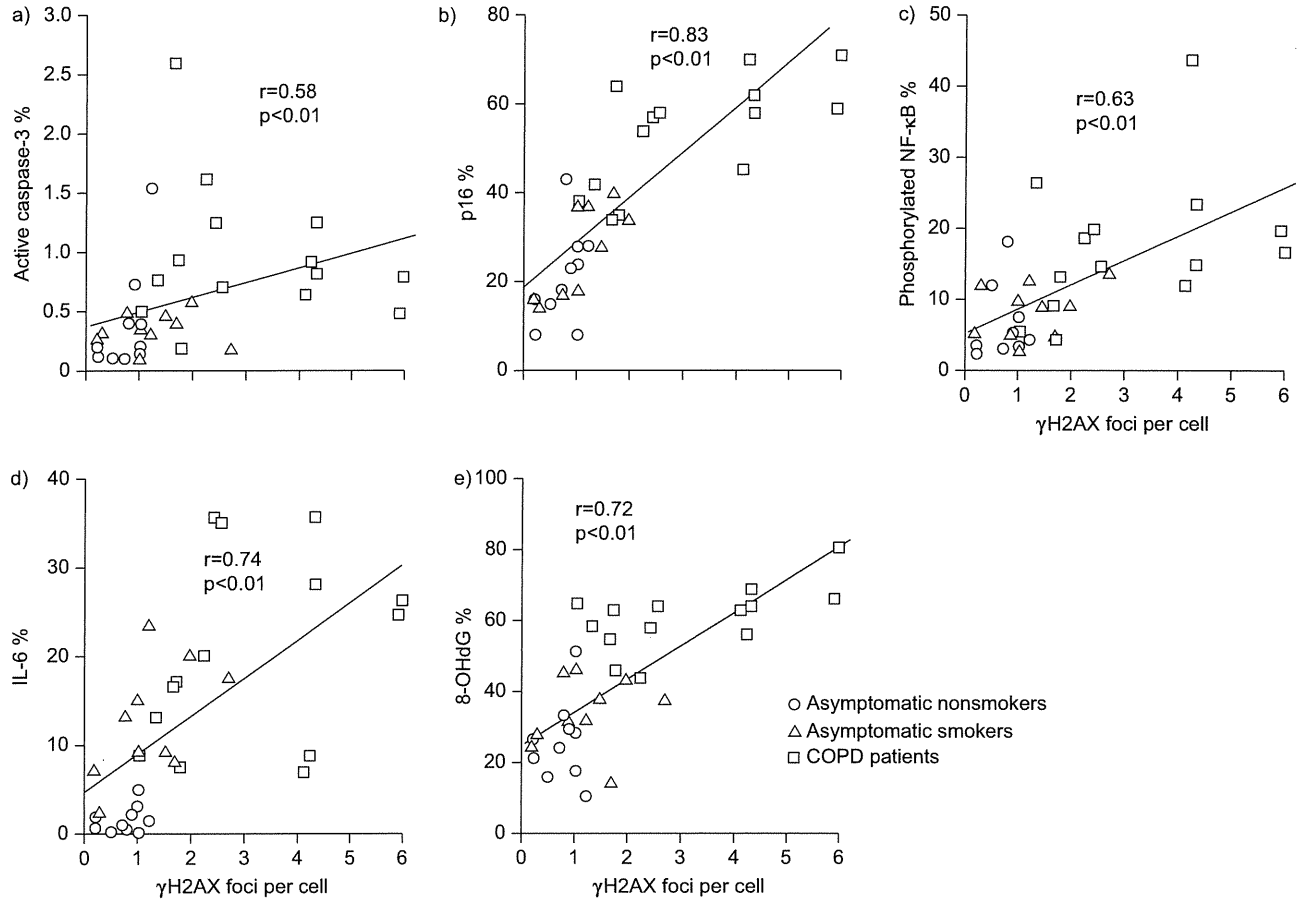

FIGURE 5. Tissue-level analyses of the relationships between the numbers of phosphorylated histone 2AX (γH2AX) foci per cell and the percentages of cells that stained positive for markers of apoptosis, cell senescence, pro-inflammatory phenotypic changes and oxidative stress in the entire group of subjects. The panels show positive correlations between the numbers of γH2AX foci per cell in type II cells and percentages of a) alveolar wall cells that expressed active caspase-3, and between the number of γH2AX foci per cell in type II cells and percentages of type II cells that expressed b) $p16^{INK4a}$ (p16), c) phosphorylated nuclear factor (NF)-κB, d) interleukin (IL)-6 and e) 8-hydroxy-2-deoxyguanosine (8-OHdG). COPD: chronic obstructive pulmonary disease.

alveolar wall cells of the lungs of guinea pigs with cigarette smoke-induced emphysema than in the lungs of sham-exposed animals (fig. S4). Our findings are consistent with those of others [26–28], who detected DNA lesions in the form of microsatellite instability (MSI) and loss of heterozygosity (LOH) in the sputum cells and bronchoalveolar fluid (BALF) cells obtained from COPD patients. Cigarette smoke contains many genotoxins, including benzo(a)pyrene, nitrosamines, aldehydes and oxidants, that induce various forms of DNA adducts [5]. The DNA damage accumulates at multiple genetic loci and sometimes induces DSBs, the most cytotoxic damage; these DSBs trigger the formation of γH2AX foci at the break site [29]. The results of the present study revealed significantly higher numbers of γH2AX foci per cell in the alveolar wall cells of COPD smokers, but not of non-COPD smokers, than in nonsmokers. This finding corroborates the findings of others [26–28], who detected MSI and LOH in sputum cells and BALF cells of COPD smokers but not of non-COPD smokers. Their findings, together with our own, strongly suggest that the DNA damage in the lungs of COPD smokers is amplified and/or remains unrepaired, and this results in gradual accumulation of DNA damage in their lungs.

The current theory of the pathogenesis of COPD suggests that alveolar destruction is caused by interactions between several pathobiological processes, including inflammation, apoptosis, cell senescence and oxidative stress [1]. By carefully analysing correlations at both the tissue and cellular levels, we found that DNA damage is correlated with apoptosis, cell senescence and pro-inflammatory phenotypic changes in the lungs of COPD patients, thereby underscoring DNA damage as a molecular link between the pathobiological processes thought to be involved in the alveolar destruction in COPD.

Since this was an observational study of human lung tissue specimens, it was impossible to establish a causal connection between DNA damage and the development of apoptosis, cell senescence and pro-inflammatory phenotypic changes. Some of the DNA damage observed in this study may have been the result of apoptosis, cell senescence and inflammation rather than their cause. However, it is well established that DNA

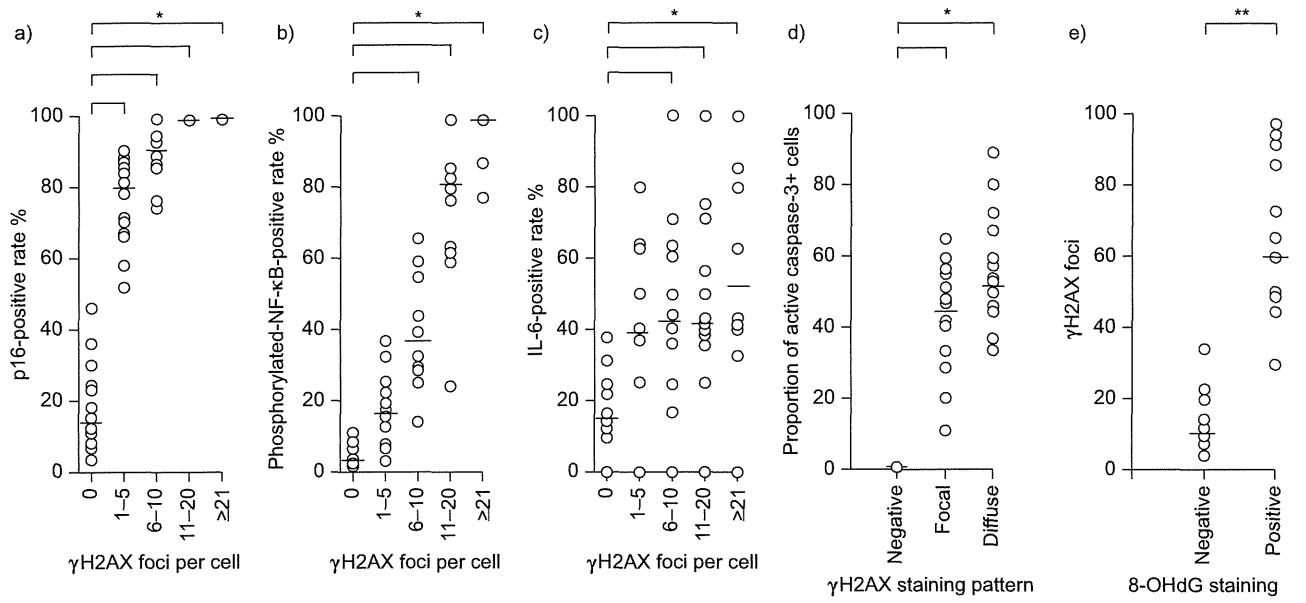

FIGURE 6. Cellular level analyses of the relationships between the numbers of phosphorylated histone 2AX (γH2AX) foci per cell and the percentages of cells that stained positively for markers of apoptosis, cell senescence, pro-inflammatory phenotypic changes and oxidative stress in chronic obstructive pulmonary disease (COPD) patients. Percentages of type II cells that expressed a) $p16^{INK4a}$ (p16), b) phosphorylated nuclear factor (NF)-κB and c) interleukin (IL)-6 according to the numbers of γH2AX foci per cell. d) Proportion of active caspase-3-positive alveolar wall cells that exhibited no γH2AX staining, focal γH2AX staining and diffuse γH2AX staining (see text for explanation). e) Number of γH2AX foci per cell in type II cells that expressed or did not express 8-hydroxy-2-deoxyguanosine (8-OHdG). Mean values obtained in an individual COPD patient are shown. —: median. *: $p<0.05$. **: $p<0.01$.

damage, in particular DSBs, is a strong inducer of apoptosis, cell senescence and pro-inflammatory responses in various types of cells and tissues [6–12]. In fact, many studies have shown that activation of an ATM/γH2AX-mediated signal transduction pathway in response to unrepaired DSBs causes apoptosis and cell senescence, thereby eliminating the DNA-damaged cells from the tissue and preventing their oncogenic transformation [6, 9]. Furthermore, a novel ATM/γH2AX-mediated pro-inflammatory signal transduction pathway in response to DSBs has recently been discovered that activates NF-κB and CCAAT/enhancer-binding protein-β transcriptional activity and stimulates secretion of pro-inflammatory cytokines, including IL-6, which in turn acts in an autocrine feedback loop to reinforce the senescence growth arrest [11, 12]. In the present study, we showed that DSBs caused by X-irradiation of cultured lung microvascular endothelial cells induced pro-inflammatory responses, such as NF-κB phosphorylation and IL-6 production, as well as caspase-3 activation and p16 expression, suggesting that DSBs are direct causes of pro-inflammatory responses, apoptosis and cell senescence (fig. S2). We also showed that treatment of A549 cells with bleomycin, which induced γH2AX focus formation, caused NF-κB phosphorylation and IL-6 production, as well as caspase-3 activation and p21 expression (fig. S3). We also recently found that persistent DNA damage in Clara cells induced by repeated injection of mice with naphthalene and bromo-2-deoxyuridine caused airway epithelial senescence accompanied by a p38 mitogen-activated protein kinase (MAPK)-dependent airway inflammatory response [30]. These lines of evidence, although not wholly conclusive, support the hypothesis that DNA damage plays a causative role in the apoptosis, cell senescence and pro-inflammatory responses observed in the lungs of COPD patients. However, future studies on animal models of COPD will be needed to show whether this view is correct.

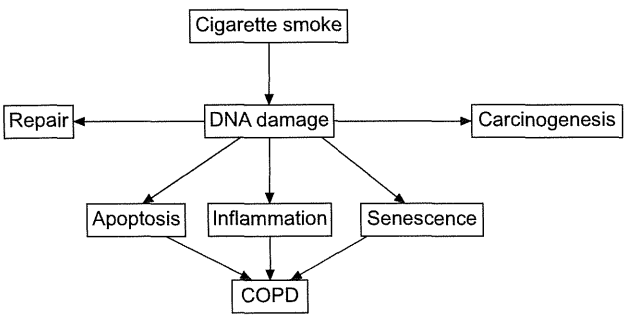

FIGURE 7. A DNA damage hypothesis for the pathogenetic mechanism of chronic obstructive pulmonary disease (COPD) in smokers. Cigarette smoke causes DNA damage, at least in part through oxidative stress. If left unrepaired, DNA damage induces apoptosis, cell senescence and a pro-inflammatory response of the alveolar wall cells, all of which contribute to the development of COPD. Unrepaired DNA damage also promotes carcinogenesis.

Oxidative stress is among the major causes of DSBs [23]. In the present study, we demonstrated an association between the presence of γH2AX foci and the presence of 8-OHdG, suggesting that oxidative stress is responsible for the DSBs in the lungs of COPD patients. It has been proposed that oxidative stress contributes to apoptosis, cell senescence and inflammation in COPD [1, 31], and recent evidence suggests

that oxidative stress induces inflammation *via* multiple mechanisms that involve oxidative activation of c-Jun activated kinase, p38-MAPK, NF-κB and activator protein-1, and oxidative inhibition of histone deacetylase activity [31]. Our findings in this study show that oxidative DNA damage may also contribute to the mechanism of persistent inflammation in COPD.

The results of the present study showed that γH2AX focus formation was also associated with IL-6 production by type II cells. This finding corroborates recent evidence that "danger signals" from injured cells elicit an immune response [32], and that IL-6 is produced by DNA-damaged cells and is a major secreted factor in their pro-inflammatory phenotype [11, 12, 33]. It has been shown that IL-6 is involved in pulmonary and systemic inflammation in COPD [34] and that higher serum IL-6 levels are associated with lower forced expiratory volume in 1 s [35]. Overexpression of IL-6 in mice was found to result in the development of emphysema and airway fibrosis [36]. In view of these findings, we suggest that IL-6 may be a key mediator that links DNA damage to the inflammatory responses in COPD.

There were several limitations to this study. First, the COPD patients had a 1.6-fold greater smoking pack-yr history than the non-COPD smokers, although the difference between the two groups was not statistically significant (table 1) ($p=0.12$ by unpaired t-test). This possible mismatch regarding smoking intensity, however, is unlikely to have accounted for the higher numbers of γH2AX foci per cell in the COPD smokers, because no significant correlations were found between the smoking pack-yrs and the numbers of γH2AX foci per cell in type I cells ($r=0.16$, $p=0.58$), type II cells ($r=0.001$, $p=0.99$) or endothelial cells ($r=0.18$, $p=0.22$). Secondly, it remains uncertain whether DNA damage is an early pathobiological event in COPD, because most of the COPD patients included in the present study had advanced COPD that received lung volume reduction surgery. A further study evaluating the number of γH2AX foci in all COPD stages, in subjects with different degrees of smoking exposure and in COPD patients treated with different medications would enable us to better elucidate the DNA damage in COPD. Thirdly, this study may have overestimated the level of DNA damage in the lungs of the non-COPD smokers and nonsmokers, because a comorbid cancer might have promoted the formation of γH2AX foci in the surrounding lung tissue through the bystander effect [37]. Finally, it remains unknown whether DNA damage in nonparenchymal cells, such as airway epithelial cells and pulmonary arterial endothelial cells, is also more severe in COPD patients.

Based on the results of the present study, we hypothesise that the apoptosis, cell senescence and inflammation, which are thought to represent the pathobiological processes of COPD [1], are at least partly attributable to DNA damage (fig. 7). The traditional theory of the pathogenesis of COPD suggests that activation of inflammation by inhaled cigarette smoke and other pollutants plays a central role in airway wall thickening, alveolar destruction, airspace enlargement and vascular remodelling [38]. Our hypothesis that DNA damage underlies the molecular mechanism of COPD seems to suggest answers to several important questions that the traditional theory does not address. The first question is, why does COPD take decades to develop? The answer based on our DNA damage hypothesis would be that the DNA damage caused by long-term smoking needs to accumulate over several decades before COPD develops, by analogy to the development of lung cancer. The second question is, why does inflammation persist after ceasing to smoke? The answer is that it probably persists because smoking-induced DNA damage persists long after smoking cessation, as is reported previously [3, 4]. The third question is, why do corticosteroids have little impact on the inflammation in COPD? The answer may be that corticosteroids do not restore the DNA damage. Finally, why is it that some smokers develop COPD while others do not, and, why are COPD smokers more prone to develop lung cancer than non-COPD smokers? The answer to the last two questions would be that the greater susceptibility to DNA damage due to smoking may be genetically determined just as greater susceptibility to smoking-induced lung cancer, so that smokers who are more susceptible to DNA damage may be predisposed to both COPD and lung cancer. However, the results of the current study do not answer all these question; longitudinal studies will be needed that include a larger number of COPD patients with different stages of disease severity.

Two independent groups of investigators have recently proposed a somatic mutation hypothesis of COPD, which differs from our hypothesis in requiring a "somatic gene mutation" to explain enhanced inflammatory responses in COPD. In 2003, ANDERSON and BOZINOVSKI [39] proposed that acquired somatic mutations in the genes encoding p53, Ras, epidermal growth factor receptor and PTEN (phosphatase tensin homologue) induced by cigarette smoke carcinogens may contribute to the pathogenesis of COPD by causing aberrant inflammatory responses. In 2009, TZORTZAKI and SIAFAKAS [40] proposed that acquired somatic mutations, such as MSI and LOH, may lead to altered lung epithelial barrier cells, which are in turn misinterpreted by the host immune system as "nonself", leading to an aberrant immune response and the clonal expansion of cytotoxic CD8+ cells, ultimately resulting in apoptosis and/or necrosis. Although both our DNA damage hypothesis and the somatic mutation hypothesis require further experimental support, we think that both of these hypotheses suggest a new, previously overlooked role of genetic damage in the pathogenesis of COPD.

In conclusion, the results of the present study strongly suggest that DNA damage underlies the molecular pathogenesis of COPD. The DNA damage hypothesis may help to understand better the pathogenetic mechanism of COPD and to target new drugs, such as drugs to prevent DNA damage and to modulate responses to the DNA damage that leads to the pathobiological processes of COPD.

SUPPORT STATEMENT

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STATEMENT OF INTEREST

None declared.

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Original Article

Changes of ghrelin and leptin levels in plasma by cigarette smoke in rats

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ABSTRACT — Cigarettes smoke (CS) limits food intake and body weight increase. Ghrelin and leptin are hormones regulating appetite and energy balance. While ghrelin increases food intake and causes a positive energy balance, leptin decreases food intake and enhances a negative energy balance. To investigate the possible role of ghrelin and leptin regarding the negative energy balance caused by CS, 10-week old male Wistar rats (n = 10) were exposed to CS from 30 cigarettes twice a day for 5 days a week for four weeks. In the smoking group, food intake and body weight gain were less than those in the non-smoking group (n = 10) during the entire CS exposure. In the smoking group, the plasma levels of acyl ghrelin were significantly higher (75.9 ± 5.1 fmol/ml versus 46.5 ± 3.3 fmol/ml, $p < 0.01$), while those of leptin were significantly lower than those in the non-smoking group (434.9 ± 41.1 ng/ml versus 744.0 ± 45.4 ng/ml, $p < 0.01$) after the final CS exposure. However, the plasma des-acyl ghrelin levels were not affected by CS exposure. These results suggested that acyl ghrelin and leptin levels in plasma may change to compensate for the negative energy balance by CS.

Key words: Cigarette smoke, Energy balance, Food intake, Ghrelin, Leptin

INTRODUCTION

Epidemiologic studies have demonstrated that cigarette smokers weighted less than nonsmokers of same age and gender, and also that anorexia is commonly observed among smokers (Albanes *et al.*, 1987; Klesges *et al.*, 1989). Both a decrease in food intake (Fulkerson and French, 2003) and an increase of energy expenditure (Chen *et al.*, 2007) are thought to contribute to the negative energy balance caused by cigarette smoke. However how cigarette smoke causes negative energy balance has not been fully elucidated.

Energy homeostasis is closely regulated by a complex network of peripheral mediators, such as hormones, neuropeptides and cytokines. Ghrelin and leptin are hormones linked to these mediators. Ghrelin has been shown to elicit the potency, namely, the long-lasting stimulation of food intake through the activation of neuropeptides Y (NPY) neurons in the hypothalamic arcuate nucleus in rats and mice (Shintani *et al.*, 2001; Tschop *et al.*, 2000; Wren *et al.*, 2000). Leptin, is one of the peptides derived from the adipocytes. It is produced in differentiated adipocytes and causes the inhibition of both NPY and agouti-related peptide (AgRP) neurons followed by a suppression of appetite (Halaas *et al.*, 1995) and an enhancement of energy expenditure (Collins *et al.*, 1996).

In underweight patients with chronic obstructive pulmonary disease (COPD), anorexia nervosa, and cancer cachexia, plasma ghrelin levels increased (Itoh *et al.*, 2004; Otto *et al.*, 2001; Shimizu *et al.*, 2003), while plasma leptin levels decreased (Takabatake *et al.*, 1999; Schols *et al.*, 1999; Grinspoon *et al.*, 1996; Simons *et al.*, 1997). COPD is mainly caused by cigarette smoke and has been recognized as a systemic disease. Malnutrition is one of the systemic effects in COPD (Takabatake *et al.*, 1999; Schols *et al.*, 1999). However it has not been fully elucidated how the malnutrition in COPD develops. Cigarette smoke contributes to the systemic effects in COPD (Fabbri *et al.*, 2007). Negative energy balance caused by cigarette smoke may contribute to malnutrition in COPD but the

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relationship is unclear.

In the present study, in order to investigate the role of ghrelin and leptin regarding the negative energy balance induced by cigarette smoke we measured plasma levels of ghrelin and leptin in rats after four weeks exposure to cigarette smoke.

MATERIALS AND METHODS

All procedures performed during these animal experiments were approved by our Instititional Ethics Committee in accordance with The Guidelines for Animal Experiments in Nara Medical University and the Guiding Principles for the Care and Use of Laboratory Animals approved by The Japanese Pharmacological Society.

Experimental animals and cigarette smoke exposure

Ten-week-old, male Wistar Kyoto (WKY/Izm) rats were purchased from Japan SLC, Inc. (Shizuoka, Japan), and fed with commercial solid diet (CE-2; CLEA Japan, Inc., Tokyo, Japan) and water *ad libitum* throughout the preconditioning and experimental periods in the laboratory animal research center at Nara Medical University. Animals were kept in a limited-access barrier housing maintained at a room temperature of 22 ± 1°C, within humidity level of 55 ± 10%, and a 12 hr light/dark cycle, with illumination from 08:00 to 20:00.

Animals were compulsively exposed to cigarette smoke using a Hamburg II smoking apparatus (Borgwaldt, Germany) according to the method the present authors have reported (Tomoda *et al.*, 2011). All smoke exposure experiments were carried out using Hi-lite® filter cigarettes (Japan Tobacco Industry Co., Ltd., Tokyo, Japan), which have nicotine and tar contents 1.4 mg and 17 mg per cigarette, respectively. The cigarette was smoked at a rate of 15 puffs per minute with an inhalation of 2 sec of smoke, mixed with 7 volumes of air, followed by 2 sec of air in the chamber. The mixture of air and smoke was moved to the 10 holders each containing one animal connected through the chamber. Preliminarily, the percent carboxyhemoglobin (CO-Hb) was determined spectrophotometrically with CO-Oxymeter (GEM Premier 4000, Nihon Medi. Science Co., Ltd., Gunma Pref., Japan) on fresh heparin-anticoagulated blood aliquots (100 UI heparin/ml blood) taken before and at defined intervals after cigarette smoke exposure from the middle caudal artery in animals under anesthesia with pentobarbital sodium (Nembutal®, 50 mg/kg i.p.; Abbott Laboratories, Abbott Park, IL, USA). Seven animals were used for the determination of the % CO-Hb at each time point (Table 1).

Animals were randomly divided into two groups (10 animals per group) and 10 animals in the smoking group were exposed to smoke from 30 cigarettes twice a day for 5 days a week, (Monday to Friday) for four weeks. Ten animals in the non-smoking group were also kept in the Hamburg II apparatus holders but without exposure to cigarette smoke. Body weight was measured every Saturday. The food intake was calculated from the feeding volume beginning on Monday and subtracting the residual volume on Saturday for each individual animal.

Anti-oxidant/oxidant balance in plasma

At 12 hr after the final cigarette-smoke exposure, whole blood was collected from the abdominal artery of each animal in each group, under anesthesia with pentobarbital sodium (50 mg/kg i.p.). The plasma was separated by centrifugation and stored at -80°C until determination of anti-oxidant/oxidant balance in plasma by evaluation total anti-oxidant capacity and hydroperoxides levels in plasma (OXY-adsorbent and Diacron-Reactive Oxygen Metabolites [d-ROMs] tests, Diacron, Grosseto, Italy).

Total anti-oxidant capacity was measured by a spectrophotometric assay, OXY-adsorbent Test (OXY) of a plasma sample (Vassalle *et al.*, 2008). This test is based on the capacity of hypochlorous acid (HClO) to oxidize physiological antioxidants. Total antioxidant capacity can be obtained by evaluating the capacity to inactivate the oxidant solution (HClO) added in excess to the sample. As HClO reacts with a chromogenic substrate (N, N-diethyl-paraophenylendiamine), a colored complex devel-

Table 1. Influences of cigarette smoke exposure on carboxyhemoglobin levels in arterial blood

Time (min) after exposure	Before exposure	20 min	40 min
CO-hemoglobin (%)	1.0 ± 0.2	18.6 ± 3.4**	14.0 ± 2.9**

**p < 0.01 vs. Before exposure; Values are expressed as means ± S.D. Carboxyhemoglobin were determined spectrophotometrically on fresh blood samples taken from 6 or 7 rats. Measurement were performed before and 20 min and 40 min after cigarette smoke exposure according to Materials and Methods. Date given in the table are means ± S.D.. **p < 0.01 versus baseline values analyzed by one-way analysis of variance.

ops that can be measured photometrically. The spectrophotometric measurement was determined within 1 min of incubation at room temperature, at a wavelength of 540 nm. The concentration of the colored complex is directly proportional to the concentration of HClO and indirectly proportional to the anti-oxidant capacity. The results were expressed as μmol of HClO consumed by 1 ml of the sample (μmol HClO/ml).

The oxidative status in plasma was evaluated as hydroperoxide levels measured by the [d-ROMs] test (Cesarone *et al.*, 1999; Alberti *et al.*, 2000). The hydroperoxides are the products of dehydrogenation and peroxidation of several cellular components including proteins, peptides, amino acids, lipids and fatty acids. When samples are dissolved in an acidic buffer, the hydroperoxides react with the transitional metal iron ions liberated from the proteins in the acidic medium and are converted to alkoxy and peroxy radicals. They can oxidize an additive (N, N-diethyl-paraophenylendiamine) to the corresponding radical cation. The concentrations can be easily determined through spectrophotometric procedures (absorption at 505 nm).

Estimation of ghrelin levels and leptin levels in plasma

At 12 hr after the final cigarette-smoke exposure, whole blood was collected from the abdominal aorta of each animal in each group, under anesthesia with pentobarbital sodium (Nembutal®, 50 mg/kg i.p.). Until the blood collection all animals were fed *ad libitum*. Whole blood samples were immediately transferred to chilled polypropylene tubes containing EDTA-2Na (1 mg/ml) and aprotinin (1000 kallikrein inactivator units per milliliter) and were immediately separated to plasma samples by centrifugation at 4°C. Hydrogen chloride was immediately added to plasma samples, which were adjusted to a final concentration of 0.1 N. These procedures were needed to avoid any fragmentation or inactivation of ghrelin because ghrelin is very unstable. These plasma samples were stored at -80°C for subsequent determination of ghrelin levels. Acyl ghrelin and des-acyl ghrelin levels in plasma were measured by enzyme-linked immunosorbent assay (ELISA) kits (SCETI, Tokyo, Japan). The detection limits of the kit for acyl ghrelon and des-acyl ghrelin were 2.5 fmol/ml and 12.5 fmol/ml respectively.

Leptin levels in plasma were measured by ELISA (Yanaihara Institute, Shizuoka, Japan, Ohtsuka Institute, Tokyo, Japan respectively). The detection limit of the kit was 312.5 pg/ml.

Statistics

Data were expressed as the means ± S.D.. Comparisons of values between the two groups were analyzed by the Mann-Whitney U test. Comparison of % CO-Hb level before and 20 min or 40 min after exposure to cigarette smoke was performed by one-way analysis of variance, while comparison of body weight and food intake between the smoking and non-smoking groups were performed with two-way analysis of variance. A p-value of less than 0.05 was considered to indicate a statistically significant difference.

RESULTS

Effect of cigarette smoke exposure on % CO-Hb

Table 1 shows that the % CO-Hb level in plasma 20 min after cigarette smoke exposure was significantly higher than the levels before exposure and remained higher the entire 40 min follow-up period, and then returned to the baseline 12 hr later (unpublished data). The recorded data correspond to measurements in human subjects, where plasma CO-Hb levels of 18% are common in heavier smokers and 20% in pipe smokers (Cole, 1981) (Table 1). In this study the peak % CO-Hb level was 18.6% on average, which may be equivalent to those of heavier smokers.

Based on these results, we measured ghrelin and leptin levels as well as anti-oxidant/oxidant balance in plasma 12 hr after the last exposure to cigarette smoke when there were only minimal direct effects by cigarette smoke.

Effect of cigarette smoke exposure on food intake and body weight gain

Figure 1 shows food intake at every week in the smoking and non-smoking groups. The food intake was measured as the amount of chow eaten by each animal twice a day for 5 days, from Monday to Friday. Food intake in the smoking group was significantly lower than that in the non-smoking group from the first week to the final week of the cigarette smoke exposure period ($p < 0.0001$).

Figure 2 shows body weight at every week in the smoking and non-smoking groups. The body weight gain in the smoking group was significantly lower than that in the non-smoking group from the first week to the fourth week of cigarette smoke exposure ($p < 0.0001$).

Anti-oxidant/oxidant balance in plasma

In the smoking group, at 12 hr after final smoke exposure d-ROM levels were lower and OXY levels were higher than those in the non-smoking group but without statistically significant differences. However the ratio of

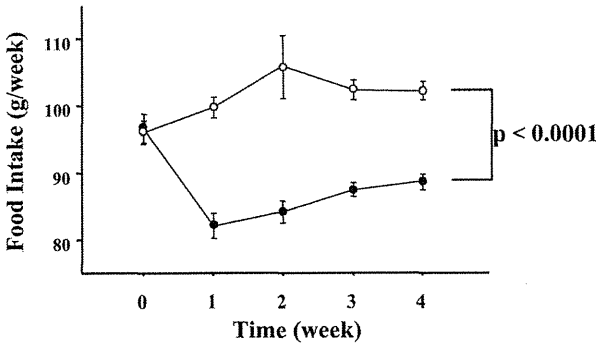

Fig. 1. Effects of cigarette smoke exposure on food intake in WKY rats. Circles show the mean values of the group not exposed to cigarette smoke, while solid dots are those of the smoke-exposed group. Each point indicates the mean ± S.D. of 10 animals. Data were analyzed by two-way analysis of variance (ANOVA). The cigarette smoke-exposed group significantly differed from the cigarette smoke-unexposed group ($p < 0.0001$).

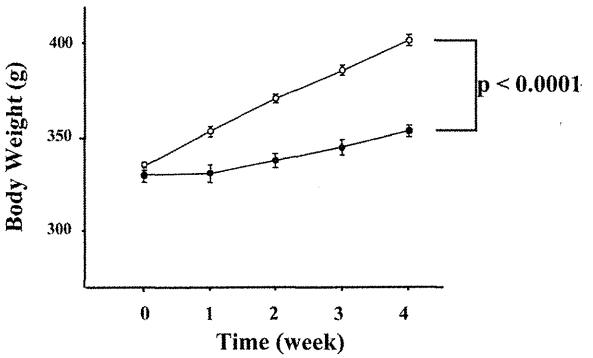

Fig. 2. Effects of cigarette smoke exposure on body weight in WKY rats. Circles show the mean values of the non-exposed group, while solid dots are those of the smoke-exposure group. Each point indicates the mean ± S.D. of 10 animals. Data were analyzed by two-way ANOVA. The cigarette smoke-exposure group significantly differed from the cigarette smoke-unexposed group ($p < 0.0001$).

OXY levels with regard to d-ROM levels in the smoking group was significantly higher than that in the non-smoking group ($p = 0.028$) (Table 2).

These findings suggest that anti-oxidant/oxidant balance in plasma is changed at 12 hr after final cigarette smoke exposure.

Effect of cigarette smoke exposure on plasma levels of ghrelin and leptin

The plasma concentrations of acyl ghrelin, des-acyl ghrelin and leptin were evaluated 12 hr after the final cigarette-smoke exposure. Plasma acyl ghrelin levels in the smoking group were significantly higher than those in the non-smoking group (75.9 ± 5.1 fmol/ml versus 46.5 ± 3.3 fmol/ml, $p = 0.0046$). However there was no significant difference in des-acyl ghrelin levels between the smoking group and the non-smoking group (433.7 ± 93.9 fmol/ml versus 417.8 ± 60.3 fmol/ml, $p = 0.326$) (Fig. 3). However, plasma leptin levels in the smoking group was significantly lower than those in the non-smoking group (434.9 ± 41.1 ng/ml versus 744.0 ± 45.4 ng/ml, $p = 0.0003$) (Fig. 4).

DISCUSSION

The present study demonstrated that both food intake and body weight gain were significantly suppressed from the first week to the final week of cigarette smoke exposure. At the end of exposure in the smoking group plasma acyl ghrelin levels were significantly higher while the plasma leptin levels were significantly lower than those in the non-smoking group. However, there was no difference in the plasma des-acyl ghrelin levels in both groups.

Cigarette smoke decreases food intake and body weight gain across species humans, rats, mice, and hamsters. It has been suggested that during exposure to cigarette smoke decreased NPY levels in the hypothalamus partially contributed to anorexia (Chen *et al.*, 2005, 2006) while an increased basal metabolic rate suppressed body weight gain in cigarette smokers (Moffatt and Owens, 1991). Additionally, some studies have demonstrated that nicotine administration decreases body weight and caloric intake (Wager-Srdar *et al.*, 1984; Grunberg, *et al.*, 1986; Hajek *et al.*, 1988; Belliger *et al.*, 2010), which were related to a decrease of NPY concentration in the hypothalamus (Frankish *et al.*, 1995). In the present study, animals were exposed with cigarette smoke twice a day, followed by evaluation by the method one of the present authors Kubo and his colleagues (Tanaka *et al.*, 2004) have reported. The report demonstrated that the plasma nicotine levels in the rats exposed to cigarette smoke were elevated to similar nicotine levels of smokers (Tanaka *et al.*, 2004). Therefore, the decreased food intake observed in the present study is thought to represent an inhibition of appetite loss in smokers.

Energy homeostasis is closely regulated by a complex network of peripheral mediators, neuropeptides, cytokines, and hormones, such as ghrelin and leptin. Ghrelin, an endogenous growth hormone (GH)-releasing peptide, was isolated from the stomach (Kojima *et al.*, 1999) and

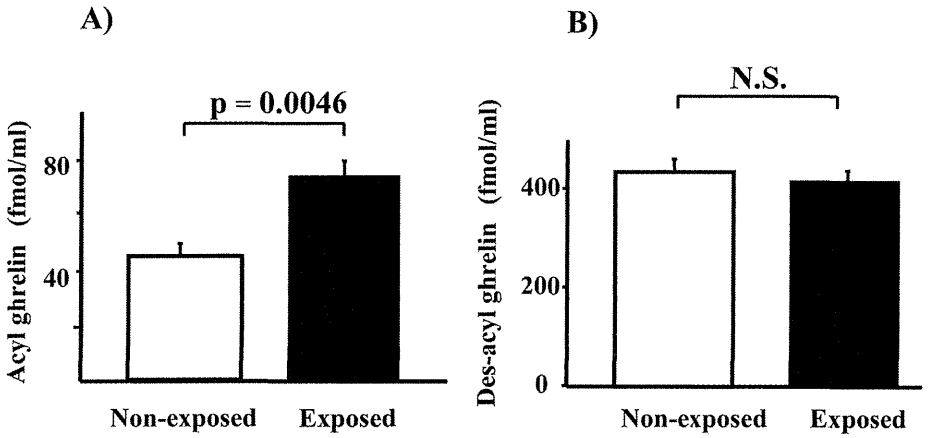

Fig. 3. Influences of cigarette smoke exposure on ghrelin levels in plasma. The outlined bars show the group not exposed to cigarette smoke, while the solid bars show those the smoke-exposed group. Each value indicates the mean ± S.D. of 10 animals. Data were analyzed by the Mann-Whitney U test. A) In acyl ghrelin levels the cigarette smoke-exposed group significantly differed from the cigarette smoke-unexposed group, p = 0.0046. B) in des-acyl ghrelin levels there was no significant difference in both groups.

was shown to cause a positive energy balance by reducing fat utilization through GH-independent mechanisms (Nakazato *et al.*, 2001). In addition, an administration of ghrelin has been shown to elicit the potency, namely, the long-lasting stimulation of food intake through stimulating NPY/AgRP and pro-opiomelanocortin (POMC) neurons in the hypothalamic arcuate nucleus in human and animals (Tschop *et al.*, 2000; Wren *et al.*, 2000; Shintani *et al.*, 2001). Ghrelin has been proved to circulate in both acylated and desacylated form. Of the circulating ghrelin forms, the acylated one (acyl ghrelin) is thought to be essential for ghrelin biological activity (Hosoda *et al.*, 2003), although the function of the desacylated one (des-acyl ghrelin) has not been fully elucidated. Leptin, one of the peptides derived from adipocytes, is produced in differentiated adipocytes and suppresses NPY neurons resulting in an inhibition of appetite (Halaas *et al.*, 1995). An enhancement of energy expenditure was shown to cause a negative energy balance (Collins *et al.*, 1996). Ghrelin and leptin have shown to antagonize each other on the hypothalamic NPY-Y1 receptor pathway in animal experiments (Shintani *et al.*, 2001).

Therefore, the present study suggests that during exposure to cigarette smoke, acyl ghrelin and leptin levels may change to compensate for negative energy balance caused by cigarette smoke. Additionally, the present study suggests that the plasma levels of both acyl ghrelin and leptin may change to stimulate the suppressed NPY pathway during exposure to cigarette smoke. Further investigation regarding the relationship of ghrelin and leptin in the regulation of food intake during exposure to cigarette smoke is needed.

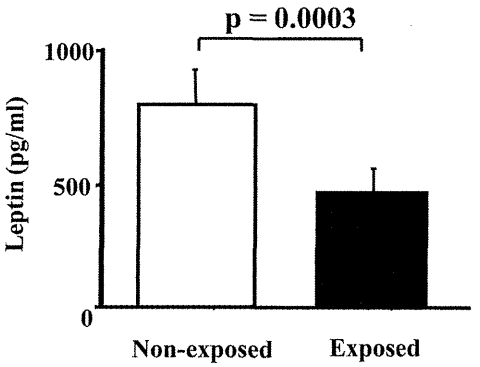

Fig. 4. Influences of cigarette smoke exposure on leptin level in plasma. The outlined bars show the group not exposed to cigarette smoke, while the solid bars show the smoke-exposed group. Each value indicates the mean ± S.D. of 10 animals. Data were analyzed by the Mann-Whitney U test. The cigarette smoke-exposed group significantly differed from the cigarette smoke-unexposed group, p = 0.0002.

Besides the negative energy balance cigarette smoke itself may contribute to the changes in plasma acyl ghrelin and leptin levels. In this study there may be only few direct effects of cigarette smoke because we measured the plasma levels 12 hr after final exposure when % CO-Hb returned to the baseline as shown at Table 1. However nicotine may possibly contribute to the changes in plasma

Table 2. Effects of cigarette smoke exposure on anti-oxidant/ oxidant balance in plasma.

	Non-exposed	Exposed
OXY (HCLO μmol/ml)	322 ± 9.1	359 ± 14.8
d-ROM (Carr unit)	356 ± 17.5	325 ± 18.6
OXY/d-ROM	0.91 ± 0.03	1.11 ± 0.06*

OXY: Oxy-adsorbent assay, index of anti-oxidant capacity. d-ROM: Diacron reactive oxygen metabolites, index of oxidative stress. : $p < 0.05$ vs. controls; Values are expressed as means ± S.D.. The oxidative status and total anti-oxidant capacity were determined by Oxy-absorbent test and d-ROMs test on fresh blood samples, taken from 10 rats, 12 hr after the final cigarette smoke exposure. Date given in the table are means ± S.D.. *$p < 0.05$ versus baseline values analyzed by the Mann-Whitney U test.

acyl ghrelin and leptin levels. The effect of nicotine on circulating leptin levels is controversial. Administration of nicotine to rats decreases plasma leptin levels (Li and Kane, 2003) while plasma leptin levels in long-term user of nicotine gum are elevated (Eliasson and Smith, 1991). The effects of nicotine on plasma ghrelin levels have not been studied yet. Further investigation about effects of nicotine on production of ghrelin and leptin is needed.

The function of des-acyl ghrelin has not been cleared, because it has been reported that des-acyl ghrelin might activate orexin and stimulate appetite (Toshinai *et al.*, 2006) while des-acyl ghrelin has been proved to not only enhance peristaltic movements but also suppress food intake (Asakawa *et al.*, 2005). The present study demonstrated that the des-acyl ghrelin levels were unchanged after the exposure to cigarette smoke. There was no significant relationship between the des-acyl ghrelin levels and food intake, suggesting that they are not related to changes in food intake during a 4-week exposure to cigarette smoke.

In underweight patients with COPD, anorexia nervosa, and cancer cachexia, plasma ghrelin levels increased (Itoh *et al.*, 2004; Otto *et al.*, 2001; Shimizu *et al.*, 2003), while plasma leptin levels decreased (Takabatake *et al.*, 1999; Schols *et al.*, 1999; Grinspoon *et al.*, 1996; Simons *et al.*, 1997). Malnutrition has been recognized as one of the systemic effects in COPD, because it has been proved to be not only related with clinical findings but also to be an independent prognostic factor (Agusti *et al.*, 2002). However while it has not been fully elucidated how malnutrition develops in COPD (Agusti *et al.*, 2002), the systemic effects by cigarette smoke are thought to partially contribute to the development of COPD and its systemic effects (Fabbri *et al.*, 2007). In present study emphysematous lesions have not been found after four weeks exposure of cigarette smoke (unpublished data). However negative energy balances with changes in plasma ghrelin and leptin levels were similar in those with underweight patients with COPD. These results may support the hypothesis that the systemic rather than only intrapulmonary effects of cigarette smoke may contribute to development of COPD and its systemic effects.

The present study did not clarify the effects of changes in plasma ghrelin and leptin, but after 4 weeks of exposure the ratio of antioxidant to oxidant increased. Some reports indicate that ant-oxidants in smokers might be enhanced compared with non-smokers, from the results of measuring the rates of accumulation of ascorbic acid and dehydroascorbate in alveolar macrophages (McGowan *et al.*, 1984) and contents of glutathione and catalase and protection endothelial cells from hydrogen peroxide in erythrocytes in smokers (Toth *et al.*, 1986). It has been not fully elucidated how anti-oxidant activities are increased in smokers. Recently ghrelin has been proved to have anti-inflammatory effects (Ersahin *et al.*, 2010). Elevated ghrelin levels may be related to an increased ratio of antioxidant to oxidant. Further investigations are needed to determine the relationship between ghrelin and systemic inflammation during exposure to cigarette smoke.

In summary, during 4 weeks of exposure to cigarette smoke in WKY rats, food intake and body weight gain were suppressed, while plasma acyl ghrelin levels increased and plasma leptin levels decreased. However, the plasma des-acyl ghrelin levels were not affected by cigarette smoke exposure. Acyl ghrelin and leptin levels may change to compensate for negative energy balance induced by cigarette smoke.

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Different *MUC1* gene polymorphisms in German and Japanese ethnicities affect serum KL-6 levels

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KEYWORDS
Krebs von den lungen-6 (KL-6);
Biological marker;
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Mucin-1;
Single nucleotide polymorphism

Summary
Background: KL-6 is a high-molecular-weight glycoprotein classified as human Mucin-1 (MUC1). KL-6 has been reported to be a sensitive biomarker for interstitial lung diseases (ILDs) in the Japanese population. It is also known that polymorphisms in the MUC1 gene affect serum levels of KL-6. This study was conducted to evaluate serum levels of KL-6 and MUC1 polymorphisms in both German and Japanese populations.
Methods: Serum levels of KL-6 were measured in 267 patients with ILDs (152 German and 115 Japanese) and 186 healthy subjects (HS) (76 German and 110 Japanese). In addition, rs4072037 single nucleotide polymorphisms (SNPs) were genotyped by polymerase chain reaction. The optimal cutoff values for discriminating patients with ILDs from HS was determined by receiver operating characteristic analysis based on ethnicity and rs4072037 genotypes.
Results: The serum KL-6 levels in patients with ILDs were significantly higher compared with HS in both the German and the Japanese cohorts (both $p < 0.001$). The discriminating cutoff value of serum KL-6 in the German cohort was significantly higher than the value in the Japanese cohort. The difference in the serum levels of KL-6 was significantly associated with the rs4072037 genotype distribution.
Conclusions: Even in the German cohort, the serum KL6 levels were significantly higher in patients with ILDs than HS. Because of differences in the genotype distribution of

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rs4072037, the KL-6 cutoff value for the German cohort that discriminated patients with ILDs from HS was significantly higher than the value in the Japanese cohort.

Background

The idiopathic interstitial pneumonias (IIPs) are a group of diffuse parenchymal lung diseases characterized by interstitial involvement resulting from various patterns of inflammation and fibrosis of unknown cause. The prevalence of IIPs has been generally reported to be 5–20 per 100,000 persons.[1–4] Since therapy with corticosteroids and/or immunosuppressants is largely ineffective for advanced stages of interstitial lung diseases (ILDs), early diagnosis is of utmost importance.[5,6] High-resolution computed tomography (HRCT) and/or surgical lung biopsy (SLB) are at present the fundamental modalities for definitive diagnosis of IIPs.[7,8] Compared to these diagnostic methods, an optimal biomarker for discriminating patients with IIPs from healthy subjects should be less invasive, more rapid and reproducible, and easier to obtain from patients.[9]

We previously developed a murine IgG1 monoclonal antibody that recognized a sialylated sugar chain, designated Krebs von den lungen-6 (KL-6). KL-6 is a high-molecular-weight glycoprotein and has been classified as a human Mucin-1 (MUC1).[10–13] We have demonstrated that KL-6 is a useful biomarker for discriminating ILDs from other benign lung diseases, evaluating disease activity, predicting disease outcome, and monitoring the clinical course.[10,13,14] Because of these findings, KL-6 was approved in 1999 by the Japanese Health Insurance Program as a diagnostic biomarker for ILDs, and has been measured in over 1,800,000 samples per year in Japan. In most countries, however, KL-6 is currently unavailable for clinical practice, and the use of KL-6 as a serum biomarker for ILDs among non-Japanese populations remains limited. We recently found indications that circulating levels of KL-6 were higher in European cohorts than levels reported earlier from the Japanese population.[15,16] Furthermore, it has been reported that the serum levels of KL-6 in patients with sarcoidosis vary according to the status of rs4072037, a single nucleotide polymorphism (SNP) on exon 2 of the *MUC1* gene.[17] KL-6 levels were highest for the G/G genotype, lowest for the A/A genotype, and at intermediate levels for the A/G genotype. In addition, available data from the HapMap database indicate that the genotypes of this polymorphism are found at different frequencies in Caucasian and Japanese ethnic groups.[18]

Based on these observations, we hypothesized that there might be differences in the circulating KL-6 levels of patients with ILDs and healthy subjects between European (e.g., German) and Japanese populations. To test this hypothesis, we evaluated the serum KL-6 levels and determined the rs4072037 genotypes in *MUC1* in both German and Japanese cohorts that included patients with ILDs and healthy subjects. The cutoff values for serum KL-6 levels that discriminated patients with ILDs from healthy subjects were determined and compared between the German and Japanese cohorts, and associations between serum KL-6 levels and rs4072037 genotypes in *MUC1* were also analyzed.

Methods

Study subjects

Between February 2007 and December 2011, 152 consecutive patients with ILDs at Ruhrlandklinik, University Hospital (Essen, Germany) and 115 patients with ILDs at Hiroshima University Hospital (Hiroshima, Japan) were enrolled in this study. Seventy-six German and 110 Japanese healthy volunteers were also included as healthy control subjects. Each patient with ILD underwent a physical examination, pulmonary function tests, chest computed tomography (CT), and/or SLB. Diagnoses of IIPs were made based on the criteria of the American Thoracic Society (ATS)/European Respiratory Society (ERS) published in 2002, which included characteristic clinical and/or CT findings and/or histological findings.[7] Each healthy volunteer underwent pulmonary function tests and chest X-ray studies, and those with apparent lung disease, such as ILDs or chronic obstructive pulmonary disease (COPD), were excluded. This study was approved by the Ethics Committees of Ruhrlandklinik (IRB 06-3170) and Hiroshima University Hospital (IRB 326) and conducted in accordance with the ethical standards established in the Helsinki Declaration of 1975. All patients and healthy volunteers gave informed consents in writing and permission to use their samples.

Lung function values

Physiologic assessment included measurements of thoracic gas volume, total lung capacity, forced vital capacity (FVC), forced expiratory volume in 1 second (FEV_1), and single-breath diffusing capacity of the lung for carbon monoxide (DL_{CO}), as previously described.[16] The protocol for lung function measurements conformed to ATS recommendations.[19]

Measurement of serum KL-6 levels

Serum samples were obtained from 267 patients with ILDs and 186 healthy subjects and stored at −80 °C until analyzed. Serum KL-6 levels were measured by a sandwich-type electrochemiluminescence immunoassay (ECLIA) using a Picolumi 8220 Analyzer (EIDIA Co. Ltd. Tokyo, Japan), as previously described.[20,21]

DNA preparation and genotype analyses of *MUC1* rs4072037

We extracted DNA from peripheral whole venous blood samples using the phenol-chloroform extraction and ethanol precipitation methods, as previously described.[22] The rs4072037 genotype was determined using a real-time polymerase chain reaction (RT-PCR) method. We used a commercially available SNP genotyping assay (TaqMan SNP Genotyping Assay C 27532642-10; Life Technologies

Corp., Carlsbad, California, USA) and the Applied Biosystems 7500 Fast RT-PCR System (Life Technologies Corp.).

Statistical analysis

Individual variables for two groups were analyzed by the Mann–Whitney *U*-test or chi-square test. The significance levels for multiple pairwise comparisons were set according to Bonferroni's correction. Linear regression analysis was conducted to study the independent effect of age, gender, smoking status, ethnicity, diagnostic category, lung function values, rs4072037 genotype, and presence or absence of ILD on serum KL-6 levels. The usefulness of serum KL-6 as a diagnostic biomarker for ILDs was assessed by receiver operating characteristic (ROC) analysis, and the optimal cutoff values for discriminating the patients with ILDs from healthy subjects were determined. All statistical analyses were performed using SPSS for Windows, version 18.0 (SPSS Inc. Chicago, USA).

Results

Serum KL-6 levels were significantly higher in healthy German subjects than in healthy Japanese subjects

The clinical characteristics of 267 patients with ILDs (152 Germans and 115 Japanese) and 186 healthy subjects (76 Germans and 110 Japanese) are shown in Table 1. Serum KL-6 levels in patients with ILDs and in healthy subjects are shown in Fig. 1a. The mean serum KL-6 levels in healthy German subjects (German HS), German patients with ILDs (German ILDs), healthy Japanese subjects (Japanese HS), and Japanese patients with ILDs (Japanese ILDs) were 331.5 ± 13.6 U/ml (mean ± SEM), 1831.0 ± 110.4 U/ml, 233.7 ± 8.1 U/ml, and 1519.0 ± 97.9 U/ml, respectively. In both German and Japanese cohorts, the serum KL-6 levels were significantly higher in patients with ILDs than in healthy subjects ($p < 0.001$ and $p < 0.001$, respectively). Furthermore, serum KL-6 levels in German HS were found to be significantly higher than those in Japanese HS ($p < 0.001$). The serum KL-6 levels in German ILDs tended to be higher than the levels in Japanese ILDs, although the difference was not statistically significant. We also compared serum KL-6 levels between German and Japanese patients within each of the following diagnostic categories of ILD: idiopathic pulmonary fibrosis (IPF), nonspecific interstitial pneumonia (NSIP), cryptogenic organizing pneumonia (COP), and drug-induced ILD. There were no significant differences in serum KL-6 levels according to the ethnicities of patients with each type of ILD (Fig. 1b).

The optimal cutoff value for serum KL-6 levels that discriminated patients with ILDs from healthy subjects was higher in the German cohort than in the Japanese cohort

To evaluate the ability of the serum KL-6 level for discriminating patients with ILDs from HS, ROC curves were drawn for each cohort. The area under the ROC curve (AUC) was 0.967 ($p < 0.001$) for the German cohort (Fig. 1c) and 0.987 ($p < 0.001$) for the Japanese cohort (Fig. 1d). Based on these ROC analyses, the optimal cutoff value for the serum KL-6 level showing the best sensitivity and specificity was 659 U/ml for the German cohort and 461 U/ml for the Japanese cohort (Table 2).

Table 1 The clinical characteristics of study subjects.

Patients with ILDs	German	Japanese	*p* value
Number of subjects	152	115	
Age, years	67.4 ± 0.8	67.5 ± 0.8	0.659
Gender, male/female	94/58	77/38	0.388
Smoking status, non/Ex/Cu/ unknown	68/56/15/13	44/55/14/2	0.282
VC, percent predicted	67.3 ± 1.6	72.0 ± 2.1	0.103
DL_{CO}, percent predicted	48.3 ± 1.5	47.5 ± 1.8	0.642
Diagnostic categories of ILDs			
IPF, *n*	92	61	0.320
NSIP, *n*	44	34	
COP, *n*	6	10	
Drug-induced ILD, *n*	10	10	

Healthy subjects	German	Japanese	*p* Value
Number of subjects	76	110	
Age, years	45.1 ± 1.2	45.8 ± 0.7	0.345
Gender, male/female	33/43	62/48	0.083
Smoking, non/Ex/ Cu/ unknown	36/9/19/12	66/15/29	0.876

Data are shown as mean ± SEM. Statistical significance was tested by Mann–Whitney *U* test or Chi-square test. ILD, interstitial lung disease; Non, non-smoker; Ex, ex-smoker; Cu, current smoker; VC, vital capacity; DL_{CO}, diffusing capacity of the lung for carbon monoxide; IPF, idiopathic pulmonary fibrosis; NSIP, nonspecific interstitial pneumonia; COP, cryptogenic organizing pneumonia.

Distribution of the rs4072037 genotypes in *MUC1* was different in the German and Japanese cohorts

DNA was extracted from blood samples in 193 out of 267 patients with ILDs (113 Germans and 80 Japanese) and 145 out of 186 HS (35 Germans and 110 Japanese), and the rs4072037 genotypes of *MUC1* were determined. The distributions of the SNPs in the German and Japanese cohorts are shown in Table 3. In both the German and Japanese cohorts, the distributions of the rs4072037 genotypes were in Hardy–Weinberg equilibrium ($p = 0.679$ and $p = 0.839$, respectively). In addition, the distributions of the rs4072037 genotypes in both HS and patients with ILDs varied with ethnicity (Table 3, $p < 0.001$ and $p < 0.001$, respectively); the frequency of the G/G genotype was

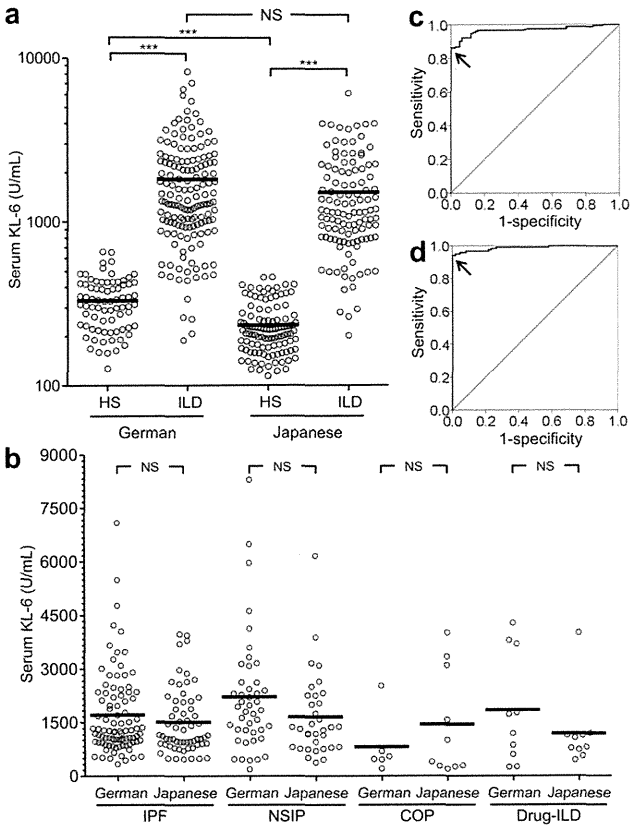

Figure 1 Serum levels of KL-6 according to ethnicity, (a) compared between healthy subjects and patients with interstitial lung diseases (ILDs), and (b) compared within each diagnostic category of ILD. Each point represents the serum KL-6 level in the studied subjects (HS, healthy subjects; ILDs, patients with interstitial lung diseases; IPF, idiopathic pulmonary fibrosis; NSIP, nonspecific interstitial pneumonia; COP, cryptogenic organizing pneumonia; drug-ILD, drug-induced ILD; NS, not significant). The horizontal bars represent the mean values. (a) The significance level was set at $\alpha = 0.0125$ (four comparisons in four groups). ***$p < 0.001$ (Mann–Whitney U test). Receiver operating characteristic (ROC) analyses were performed for patients with ILDs and healthy subjects in (c) German and (d) Japanese cohorts. Arrows at the top left corner show the points with best sensitivities and specificities.

Table 2 Optimal cutoff values of serum KL-6 according to ethnicity or rs4072037 genotype.

	Ethnicity		rs4072037 Genotype		
	German	Japanese	A/A	A/G	G/G
Cutoff value, U/ml	659	461	397	461	503
Sensitivity	0.862	0.939	0.958	0.986	1.000
Specificity	1.000	1.000	1.000	0.981	1.000
Accuracy	0. 908	0.969	0.977	0.984	1.000

The optimal cutoff values are determined as the serum levels of KL-6 which show the best sensitivity and specificity in ROC analysis. Accuracy = (true positives + true negatives)/total subjects.

significantly higher and the frequency of the A/A genotype was significantly lower in the German cohort than in the Japanese cohort. However, the distributions of the rs4072037 genotypes did not differ between HS and patients with ILDs in either the German or Japanese cohorts.

The optimal cutoff values for serum KL-6 levels that discriminated patients with ILDs from healthy subjects were different, depending on the rs4072037 genotype

To determine the effects of rs4072037 SNPs on serum KL-6 levels in healthy subjects, serum KL-6 levels were compared between the patients with rs4072037 A/A, A/G, and G/G genotypes. Because there were no significant ethnic differences between the serum KL-6 levels of patients with each genotype (data not shown), both German and Japanese subjects were included in each genotype analysis. The mean serum KL-6 levels in HS were determined to be 210.4 ± 7.6 U/ml for the A/A genotype, 286.9 ± 13.8 U/ml for the A/G genotype, and 354.5 ± 361 U/ml for the G/G genotype. As shown in Fig. 2a, the serum KL-6 levels in the HS with A/G or G/G genotypes were significantly higher than the serum KL-6 levels in the subjects with the A/A genotype ($p < 0.001$ and $p = 0.001$, respectively).

The serum KL-6 levels were then compared between the ILD patients with rs4072037 A/A, A/G, and G/G genotypes. The mean serum KL-6 levels in patients with ILDs were 1421.2 ± 99.7 U/ml for the A/A genotype, 2129.0 ± 176.9 U/ml for the A/G genotype, and 1915.6 ± 244.8 U/ml for the G/G genotype. As shown in Fig. 2a, the serum KL-6 levels were significantly higher in ILD patients with the A/G genotype than in patients with the A/A genotype ($p \leq 0.001$). We also compared the serum KL-6 levels between patients with rs4072037 A/A, A/G, and G/G genotypes within each diagnostic category of ILD (IPF, NSIP, COP, and drug-induced ILD). There were no significant differences in serum KL-6 levels according to rs4072037 genotypes of patients with each type of ILD (Fig. 2b).

To evaluate the ability of serum KL-6 levels to discriminate patients with ILDs from HS within each genotype, ROC curves were drawn for each rs4072037 genotype. The AUC values were 0.991 (95% CI, 0.000–1.000, $p < 0.001$) for the A/A genotype (Fig. 2c), 0.990 (95% CI, 0.000–1.000, $p < 0.001$) for the A/G genotype (Fig. 2d), and 1.000 (95% CI, 1.000––1.000, $p < 0.001$) for the G/G genotype (Fig. 2e). As shown in Table 2, the optimal cutoff values of serum KL-6 levels that discriminated patients with ILDs from HS were determined to be 397 U/ml for the A/A genotype, 461 U/ml for the A/G genotype, and 503 U/ml for the G/G genotype.

Serum KL-6 levels are independently correlated with the rs4072037 genotype in *MUC1*

To obtain more information on variables that might affect serum KL-6 levels, correlations between the serum level of KL-6 and clinical characteristics, including age, gender, smoking status, ethnicity, specific rs4072037 genotype, and the presence or absence of ILD, were examined using linear regression analysis. Univariate analysis confirmed that older

Table 3 Frequency of each genotype of rs4072037.

Patients with ILDs	Total	A/A	(%)	A/G	(%)	G/G	(%)
German							
Number of subjects	113	38	(33.6)	49	(43.4)	26	(23.0)
Standardized residuals		−5.3		2.4		4.3	
Japanese							
Number of subjects	80	58	(72.5)	21	(26.3)	1	(1.2)
Standardized residuals		5.3		−2.4		−4.3	
Healthy subjects	Total	A/A	(%)	A/G	(%)	G/G	(%)
German							
Number of subjects	35	9	(25.7)	17	(48.6)	9	(25.7)
Standardized residuals		−4.0		1.7		4.3	
Japanese							
Number of subjects	110	71	(64.5)	36	(32.8)	3	(2.7)
Standardized residuals		4.0		−1.7		−4.3	

$p < 0.001$, tested by chi-square test.
ILDs, interstitial lung diseases.

age (regression coefficient [*B*] = 35.413, standard error [SE] = 4.090, $p \leq 0.001$), German ethnicity ($B = 730.926$, SE = 125.844, $p \leq 0.001$), the rs4072037 G/G genotype ($B = 333.670$, SE = 93.596, $p < 0.001$), and the presence of ILD ($B = 1489.534$, SE = 104.439, $p \leq 0.001$) were significantly correlated with higher serum KL-6 levels (Table 4a); and multivariate analysis revealed that the rs4072037 G/G genotype ($B = 213.973$, SE = 81.802, $p = 0.009$) and the presence of ILD ($B = 1533.652$, SE = 159.022, $p \leq 0.001$) were independently correlated with higher serum KL-6 levels. To further evaluate the correlation between the serum KL-6 levels and clinical characteristics, we performed subgroup analyses based on subjects with or without ILDs. For the HS, univariate analysis confirmed that older age ($B = 2.648$, SE = 0.853, $p = 0.002$), German ethnicity ($B = 63.969$, SE = 18.190, $p = 0.001$) and rs4072037 G/G genotype ($B = 69.949$, SE = 11.162, $p \leq 0.001$) were significantly correlated with higher serum KL-6 levels (Table 4b); and multivariate analysis revealed that older age (B = 3.035, SE = 0.741, $p < 0.001$) and the rs4072037 G/G genotype ($B = 64.924$, SE = 11.589, $p < 0.001$) were independently correlated with higher serum KL-6 levels. For the patients with ILDs, univariate analysis demonstrated that decreased %VC ($B = 13.082$, SE = 5.681, $p = 0.006$), decreased $\%DL_{CO}$ ($B = 12.664$, SE = 5.681, $p = 0.027$), and rs4072037 G/G genotype ($B = 358.590$, SE = 124.140, $p = 0.004$) were significantly correlated with higher serum KL-6 levels (Table 4c); and multivariate analysis revealed that only the rs4072037 G/G genotype ($B = 301.665$, SE = 130.177, $p = 0.022$) was independently correlated with higher serum KL-6 levels.

Discussion

In this study, we found in both the German and Japanese cohorts that serum KL-6 levels were significantly higher in patients with ILDs than in healthy subjects. We also found that the optimal cutoff value for the serum KL-6 level that discriminated patients with ILDs from healthy subjects was higher in the German cohort than in the Japanese cohort. The distribution of the rs4072037 genotypes in *MUC1* was shown to differ between the German and Japanese cohorts. In addition, multivariate regression analyses, which included both German and Japanese subjects, revealed that a specific rs4072037 genotype in *MUC1* and the presence of an ILD independently affected serum KL-6 levels.

Our study showed that the difference in the cutoff values for serum KL-6 levels of German and Japanese cohorts that discriminated patients with ILDs from healthy subjects was at least in part due to different distributions of the rs4072037 genotypes in *MUC1*. The G/G genotype was more common in the German cohort, and the A/A genotype was more common in the Japanese cohort. The distributions of the rs4072037 genotypes in the German and Japanese cohort were shown to be similar to the respective distributions in CEU (Utah residents with ancestry from northern and western Europe) and in JPT (Japanese in Tokyo, Japan) populations, which are reported in the HapMap database.[18]

The rs4072037 SNP is located in exon 2 of *MUC1*. Previous studies reported that this SNP regulates splicing site selection and is in strong linkage disequilibrium with variable numbers of tandem repeats (TRs) in *MUC1*. The G allele correlates with a large number of TRs and the A allele correlates with a small number of TRs.[23,24] Because the sialylated sugar chains on MUC1, which are believed to be recognized by the anti-KL-6 monoclonal antibody ,[11,12] are known to be abundant in the TR domain, larger numbers of TRs may contain more KL-6 antigen, resulting in higher serum KL-6 levels.[25–27] In agreement with these previous reports, our data showed that the serum KL-6 levels in the A/G and G/G genotype cohorts were higher than those in the A/A genotype cohort.[17] Based on these results, we hypothesize that the higher levels of serum KL-6 in the German compared with the Japanese cohort may be caused

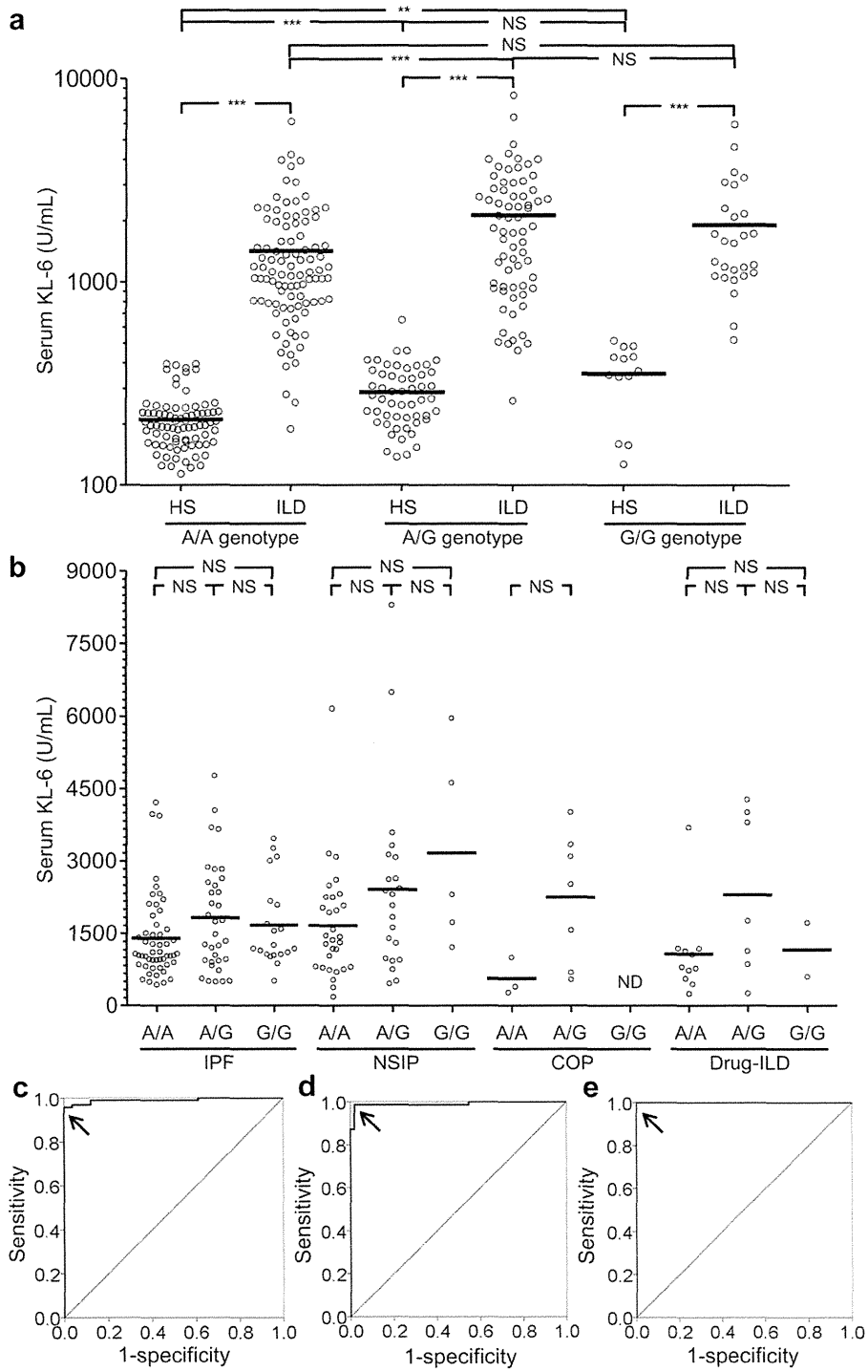

Figure 2 Serum levels of KL-6 according to rs4072037 genotype, (a) compared between healthy subjects and patients with interstitial lung diseases (ILDs), and (b) compared within each diagnostic category of ILD. Each point represents the serum KL-6 level in the studied subjects (HS, healthy subjects; ILDs, patients with interstitial lung diseases; IPF, idiopathic pulmonary fibrosis; NSIP, nonspecific interstitial pneumonia; COP, cryptogenic organizing pneumonia; drug-ILD, drug-induced ILD; NS, not significant). The horizontal bars represent the mean values. (a) The significance level was set at $\alpha = 0.0056$ (nine comparisons in six groups). $^{**}p < 0.0056$, $^{***}p < 0.001$ (Mann–Whitney U test). (b) The significance level was set at $\alpha = 0.017$ (three comparisons in three groups) for IPF, NSIP, and drug-ILD, and at $\alpha = 0.05$ (Bonferroni's correction was not adopted) for COP. Receiver operating characteristic (ROC) analyses were performed between patients with ILDs and healthy subjects in (c) A/A cohort, (d) A/G cohort, and (e) G/G cohort of rs4072037. Arrows at the top left corner show the points with best sensitivities and specificities.

Table 4 Linear regression analysis for serum KL-6.

a. Whole studied subjects	Regression coefficient	Standard error	*p* Value
Univariate analysis			
Age, years	35.413	4.090	<0.001
Gender, female vs male	135.546	133.043	0.309
Smoking, Non vs Ex vs Cu	23.001	88.610	0.795
Ethnicity, Japanese vs German	730.926	125.844	<0.001
rs4072037, A/A vs A/G vs G/G	333.670	93.596	<0.001
With or without ILDs, HS vs ILDs	1489.534	104.439	<0.001
Multivariate analysis			
Age, years	4.989	5.263	0.344
Ethnicity, Japanese vs German	117.823	120.173	0.328
rs4072037, A/A vs A/G vs G/G	213.973	81.802	0.009
With or without ILDs, HS vs ILDs	1533.652	159.022	<0.001

b. Healthy subjects	Regression coefficient	Standard error	*p* Value
Univariate analysis			
Age, years	2.648	0.853	0.002
Gender, female vs male	11.760	16.229	0.470
Smoking, Non vs Ex vs Cu	1.159	9.300	0.901
Ethnicity, Japanese vs German	63.969	18.190	0.001
rs4072037, A/A vs A/G vs G/G	69.949	11.162	<0.001
Multivariate analysis			
Age, years	3.035	0.741	<0.001
Ethnicity, Japanese vs German	28.572	17.447	0. 104
rs4072037, A/A vs A/G vs G/G	64.924	11.589	<0.001

c. Patients with ILDs	Regression coefficient	Standard error	*p* Value
Univariate analysis			
Age, years	10.616	8.997	0.239
Gender, female vs male	55.201	188.244	0.770
Smoking, Non vs Ex vs Cu	111.173	135.264	0.412
VC, percent predicted	13.082	4.722	0.006
DL_{CO}, percent predicted	12.664	5.681	0.027
Ethnicity, Japanese vs German	355.953	181.940	0.052
Diagnostic category, drug-ILD vs COP vs IPF vs NSIP	195.010	102.851	0.059
rs4072037, A/A vs A/G vs G/G	358.590	124.140	0.004
Multivariate analysis			
VC, percent predicted	2.219	5.72	0.699
DL_{CO}, percent predicted	11.682	6.366	0.069
rs4072037, A/A vs A/G or G/G	301.665	130.177	0.022

Non, non-smoker; Ex, ex-smoker; Cu, current smoker; HS, Healthy subjects; ILDs, interstitial lung diseases; VC, vital capacity; DL_{CO}, diffusing capacity of the lung for carbon monoxide; drug-ILD, drug-induced interstitial lung disease; COP, cryptogenic organizing pneumonia; IPF, idiopathic pulmonary fibrosis; NSIP, nonspecific interstitial pneumonia.

by higher frequencies of rs4072037 A/G and G/G genotypes in the German than in the Japanese cohort.

To evaluate the ability of serum KL-6 levels based on ethnicity and genotype for discriminating patients with ILDs from healthy subjects, we performed ROC analysis for each rs4072037 genotype. Importantly, the AUC value for each rs4072037 genotype (Fig. 2c–e; 0.991 for A/A, 0.990 for A/G, and 1.000 for G/G, respectively) was higher than the AUC value for each ethnicity (Fig. 1c, d; 0.967 for German and 0.987 for Japanese cohort, respectively). In addition, the sensitivity, specificity, and accuracy tended to be higher in genotype-based analysis than in ethnicity-based analysis (Table 2). These data suggest that the cutoff values of serum KL-6 levels based on rs4072037 genotypes show better abilities of discriminating patients with ILDs from healthy subjects than the cutoff values based on ethnicity. Furthermore, the rs4072037 G/G genotype was independently correlated with serum KL-6 levels, whereas ethnicity was not. Based on these results, we can conclude that the different cutoff values for serum KL-6 levels in the German and Japanese cohorts that discriminated patients with ILDs from healthy subjects were at least partially a result of the different distributions of rs4072037 genotypes in these cohorts; and, ideally, a cutoff value for the serum KL-6 levels that discriminates patients with ILDs from healthy subjects should be based on the rs4072037 genotype. However, primarily because of the expense, it may not be clinically feasible to determine the rs4072037 genotype of every patient with ILD. Therefore, we believe that cutoff values of serum KL-6 levels that discriminate patients with ILDs from healthy individuals should be determined on the basis of ethnicity.

Another interesting finding of this study was that the serum KL-6 levels in patients with ILDs were inversely correlated with the values for %DLco and %VC (Table 4c). This finding is consistent with the results of previous studies demonstrating the correlation between serum KL-6 and parameters of lung function in patients with various types

of ILDs.[28–31] Interestingly, previous studies from our laboratory demonstrated that KL-6 itself has chemotactic and antiapoptotic effects on fibroblasts, and additive effects on the proliferative and antiapoptotic activity of transforming growth factor-β toward fibroblasts.[11,32] These observations suggest that KL-6 may be involved in the fibrotic processes in the lung that lead to decreased values of %DLco and %VC.

On the other hand, there were no significant differences in the distributions of rs4072037 genotypes between healthy subjects and patients with ILDs. This finding indicates that there is no correlation between rs4072037 genotype and susceptibility to ILDs, although correlations between rs4072037 genotypes and susceptibility to dry-eye syndrome and gastric cancer have been reported.[33,34]

There are a number of limitations to this study. First, the sizes of the cohorts were relatively small, particularly with regard to the number of subjects available for analysis of rs4072037 genotypes. In addition, the patients with ILDs were older than the healthy subjects. However, multivariate analysis of all the subjects demonstrated that serum KL-6 levels did not correlate with age. Second, the relationship between radiographic manifestations of chest CT images and KL-6 levels was not analyzed. This was because we were unable to assess the extent of fibrosis using standardized criteria for both German and Japanese patients with ILDs, since different CT scanners with different slice thicknesses were used at the Ruhrlandklinik in Germany and the Hiroshima University Hospital in Japan. Third, only German and Japanese populations were studied. It remains to be seen whether the findings of the present study can be applied to other ethnic groups such as African Americans. Fourth, there were no follow-up data available for assessing prognostic values. A prospective follow-up study to investigate the association between serum KL-6 levels and prognosis is now being performed for both the German and Japanese cohorts.

Conclusions

In conclusion, we were able to demonstrate that the serum KL6 levels were significantly higher in patients with ILDs than in healthy subjects in both the German and Japanese cohorts. Because of differences in the distribution of rs4072037 genotypes in the German and Japanese cohorts, the cutoff value of KL-6 that discriminated patients with ILDs from healthy subjects was significantly higher in the German cohort than in the Japanese cohort. Although promising data were obtained in the present study, further investigations are needed to determine whether KL-6 can be used as a diagnostic biomarker in ethnic groups other than the Japanese.

Author contributions

YH drafted and finalized the manuscript, and performed some of the serum measurements, genotyping, and statistical analyses. NH, NI, AY, NK, and UC conceived the study, participated in its design and coordination, and helped to draft and finalize the manuscript. SK performed part of the statistical analysis. ST and KY performed some of the serum measurements and genotyping. FB, JG, and SO recruited the study subjects and determined their diagnoses.

Conflict of interest statement

Nobuoki Kohno holds a patent on KL-6. The remaining authors have no conflicts of interest.

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