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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

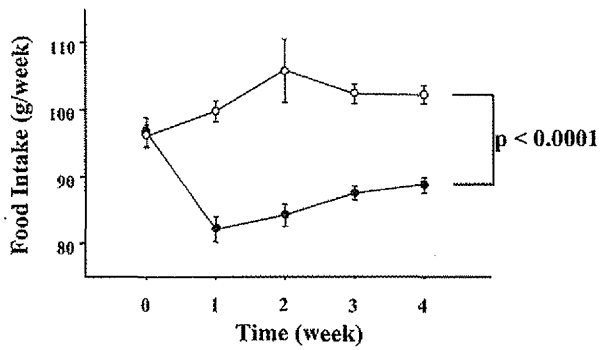


**Fig. 1.** Effects of cigarette smoke exposure on food intake in WKY rats. Circles show the mean values of the group not exposed to cigarette smoke, while solid dots are those of the smoke-exposed group. Each point indicates the mean ± S.D. of 10 animals. Data were analyzed by two-way analysis of variance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

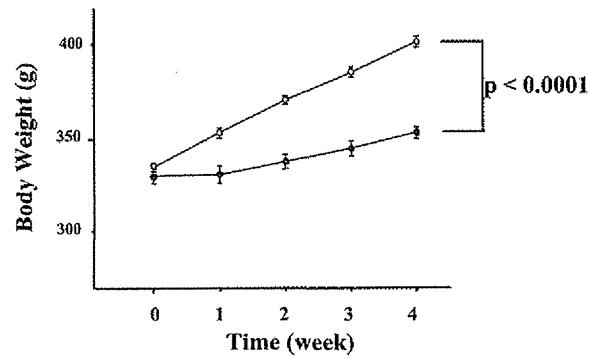


**Fig. 2.** Effects of cigarette smoke exposure on body weight in WKY rats. Circles show the mean values of the non-exposed group, while solid dots are those of the smoke-exposure group. Each point indicates the mean ± S.D. of 10 animals. Data were analyzed by two-way ANOVA. The cigarette smoke-exposure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

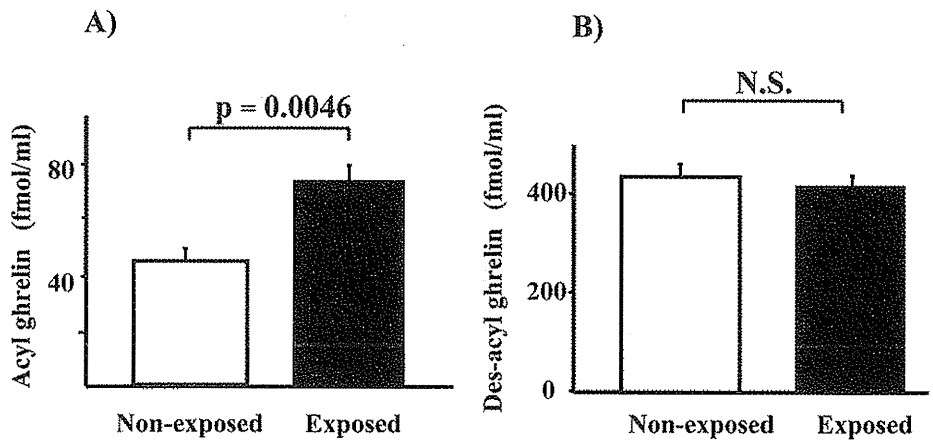


**Fig. 3.** Influences of cigarette smoke exposure on ghrelin levels in plasma. The outlined bars show the group not exposed to cigarette smoke, while the solid bars show those the smoke-exposed group. Each value indicates the mean ± S.D. of 10 animals. Data were analyzed by the Mann-Whitney U test. 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.

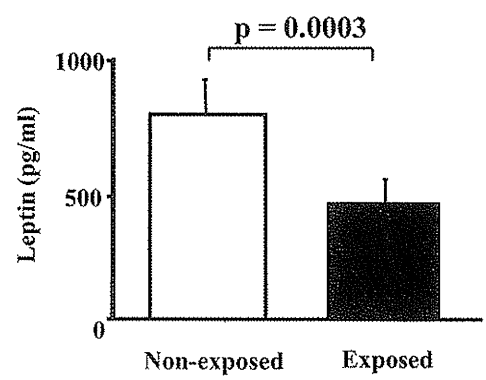


**Fig. 4.** Influences of cigarette smoke exposure on leptin level in plasma. The outlined bars show the group not exposed to cigarette smoke, while the solid bars show the smoke-exposed group. Each value indicates the mean ± S.D. of 10 animals. Data were analyzed by the Mann-Whitney U test. The cigarette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

# Association between Episodes of Upper Respiratory Infection and Exacerbations in Adult Patients with Asthma

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*Background.* Asthma has several phenotypical features, including recurrent exacerbations and recurrent episodes of upper respiratory infection (URI). *Purpose.* A retrospective study was performed to identify the characteristics of adult patients with recurrent exacerbations of asthma, especially in association with recurrent episodes of URI. *Methods.* Information was collected using a self-administered questionnaire given to 7070 patients in autumn–winter 2006, 4859 patients in spring–summer 2007, and 4452 patients in autumn–winter 2007. The patients reported the degree of symptoms and the frequency of febrile episodes of URI and exacerbations. Severe exacerbations were defined as a self-report of asthma-related hospitalization, an emergency department visit, or a requirement for systemic corticosteroids. Recurrent febrile URI and exacerbations were defined as two or more episodes within the previous 6 months. A Poisson regression model was used to identify the factors that were predictors of a risk for exacerbations. *Results.* Of the 6266 patients who completed the questionnaire, the frequencies of febrile URI and episodes of severe exacerbations were 1.54 and 0.54 per subject per year, respectively. Logistic regression analysis showed that an older age [odds ratio (OR): 1.57; 95% confidence interval (CI): 1.15–2.13], female sex (OR: 1.58; 95% CI: 1.20–2.08), recurrent febrile episodes of URI (OR: 2.68; 95% CI: 1.47–4.91), a history of previous exacerbation within 1 year (OR: 1.74; 95% CI: 1.28–2.34), disuse of inhaled corticosteroids (ICSs) (OR: 2.63; 95% CI: 1.68–4.12), and disuse of add-on leukotriene receptor antagonists (LTRAs) (OR: 1.42; 95% CI: 1.06–1.74) were independently associated with moderate to severe symptom-severity. Poisson regression analysis showed that the independent factors that contributed to the frequency of recurrent severe exacerbations were female sex (regression coefficient $\beta = 0.62$, $p < .01$), an episode of sputum with coughing ($\beta = 1.23$, $p < .01$), nocturnal awakening ($\beta = 1.22$, $p < .01$), and severe exacerbation ($\beta = 0.78$, $p < .01$) within the previous 6 months. *Conclusion.* Symptom-severity of asthma and the frequency of severe exacerbations were associated with previous exacerbations and susceptibility to URI.

**Keywords** asthma, exacerbation, upper respiratory infection

## INTRODUCTION

Asthma is a syndrome with heterogeneous phenotypes, including the recently proposed entity of "exacerbation-prone" asthma, which involves frequently occurring exacerbations (1). The characteristic features of exacerbation-prone patients include irreversible airflow limitation, a history of cigarette smoking, psychosocial dysfunction, medication noncompliance, and comorbidities including rhinosinusitis, obesity, gastroesophageal reflux, and intolerance to nonsteroidal anti-inflammatory medications (2, 3). Recent asthma exacerbations are a predictor of future exacerbations (4), but not all exacerbations are associated with severe disease.

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Periodic exacerbations are generally thought of as a worsening of asthma symptoms in response to a variety of triggers. Upper respiratory infection (URI) due to viral infection is recognized as a major trigger of acute exacerbation of asthma in children and adults and results in frequent outpatient visits and hospitalization (5–7). The frequency of URI is suspected to contribute to exacerbation-prone asthma, but the factors underlying the exacerbation-prone phenotype are incompletely understood.

Asthma guidelines recommend achieving and maintaining current control, as well as reducing the future risk of exacerbations (8, 9). We examined the prevalence of URI and exacerbations in patients with adult asthma using a self-administered questionnaire to determine the risk factors for severe exacerbations. We focused on the association between the episodes of URI and a deteriorated asthma status, such as that in patients with moderate to severe symptom-severity and severe exacerbations.

## METHODS

### *Subjects and Study Design*

The study was designed as a questionnaire-based retrospective study to capture all febrile episodes of URI, symptom-severity, and severe exacerbations that occurred in adult patients over a 6-month period. Adult patients with asthma that had been diagnosed by physicians were consecutively recruited during their regular visits to 446 centers, including the outpatient departments of 7 university hospitals, 24 general hospitals, and 315 clinics. The questionnaires were handed to the subjects in autumn–winter in 2006, spring–summer in 2007, and autumn–winter in 2007. Male and female asthma patients aged above 18 years were eligible for inclusion in the study if they had a diagnosis of moderate or severe persistent asthma confirmed by a chest physician, and the diagnosis had occurred at least 6 months before the beginning of the study. Each center's Institutional Review Board approved the protocol and all subjects gave signed informed consent.

### *Questionnaire*

Questions to each patient concerning febrile episodes of URI, symptoms associated with asthma, and exacerbations were based on symptom-severity scores (10) and were modified to fit the design of this study (see Appendix). Another questionnaire was concurrently given to the physicians regarding the events of asthma-related hospitalization and treatment, based on chart review.

### *Definition of Symptom-Severity and Severe Exacerbations*

The definition of moderate to severe symptom-severity was based on symptom-severity scores (10), as having at least one symptom of cough, sputum with coughing, and nocturnal awakening per week. Among the cold-severity indicators validated in the Wisconsin Upper Respiratory Symptom Survey (WURSS) (11), fever was one of the symptoms associated with acute exacerbations of asthma requiring hospitalization (12). To avoid inclusion of data for rhinitis and milder common colds, URIs were defined as self-reported episodes of nasal stuffiness and discharge, combined with sneezing, sore throat, or cough with fever. The American Thoracic Society/European Respiratory Society (ATS/ERS) Task Force on Asthma Control and Exacerbations defined exacerbations as events characterized by a change from the patient's previous status that prompts a need for a change in treatment (13). In this study, severe exacerbations were defined as self-reported episodes requiring systemic glucocorticosteroids, an emergency room (ER) visit, or asthma-related hospitalization. Recurrent events of exacerbations and URI were defined as two or more episodes within the previous 6 months. The primary outcomes were the rates of URI and severe exacerbations of asthma per patient per 6 months.

### *Statistical Analysis*

SPSS version 18.0 (SPSS, Chicago, IL, USA) was used for all calculations. Factors associated with poor asthma control were evaluated by logistic regression analysis. The results are expressed as odds ratios (ORs) with 95% confidence intervals (CIs). The frequency of exacerbations was analyzed using a simple model assuming a Poisson distribution. The associations between recurrent severe exacerbations and clinical variables were initially explored by univariate regression. Multivariate regression models were then used to evaluate the relationship between recurrent severe exacerbations and variables found to be significant in univariate analysis, while adjusting for potential confounding factors that were also significant in univariate analysis.

## RESULTS

### *Patient Characteristics*

The self-administered questionnaire survey was given to a total of 16,381 adult patients with asthma in three separate periods (Table 1): 7070 subjects in autumn–winter 2006, 4859 subjects in spring–summer 2006, and 4452 subjects in autumn–winter 2007. A total of 11,573 completed questionnaires (70.6%) were received from 6266 patients (some patients completed questionnaires in two or all three of the periods). Of these patients, 42.7% were male. There were no significant differences in response rates among patients of different sex or age.

### *Incidence of URI*

A total of 8876 URI episodes were documented in the 11,573 patients over 6 months, giving a rate of 1.54 episodes per patient per year. Recurrent febrile URI, which was defined as two or more episodes per 6 months, occurred in 17.4% of the patients. The incidence of febrile URI in autumn–winter was more frequent than that in spring–summer ($p < .05$) (Figure 1).

### *Independent Factors Associated with Asthma Symptom-Severity*

A nested case–control study was performed to identify the characteristics of adult patients with poor asthma control, especially in those with a previous history of severe exacerbations. The nested study included the 1012 patients who answered the questionnaire in all three periods. Of these patients, 66% showed moderate to severe symptom-severity, which was defined as having at least one symptom of cough, sputum, and nocturnal awakening per week. The results of logistic regression analysis of covariate factors for symptom-severity are shown in Table 2. An older age (OR: 3.42; 95% CI: [1.75–8.06]; $p < .01$), female sex (OR [CI] = 1.59 [1.22–2.07]; $p < .01$), ≥3 episodes of febrile URI in the previous 6 months or an episode of sputum with coughing (OR = 3.83 [2.29–6.40], $p < .01$), previous exacerbations within 1 year (OR = 4.96 [1.75–14.0], $p < .01$), disuse of inhaled corticosteroids (ICSs) (OR = 2.70 [1.82–4.00], $p < .01$), and disuse of add-on leukotriene receptor antagonists (LTRAs) (OR = 1.58 [1.21–2.07], $p < .01$) contributed significantly to symptom-severity. In contrast, disuse of add-on long-acting $\beta$-agonists (LABAs) and add-on theophylline did not contribute to symptom-severity.

TABLE 1.—Characteristics of the patients.

| | First questionnaire | Second questionnaire | Third questionnaire |
|---|---|---|---|
| Number of responses | 7084 | 4858 | 4451 |
| Responses with complete answers | 4038 (57%) | 3090 (64%) | 2798 (63%) |
| Age (year) | 54.5 (54.0–55.0) | 56.2 (55.6–56.8) | 56.8 (56.2–57.4) |
| Sex (male/female) | 1784/2254 | 1331/1759 | 1272/1526 |
| Frequency of URI per 6 months | 0.79 (0.76–0.83) | 0.80 (0.73–0.86) | 0.60 (0.56–0.64) |
| Frequency of events using SABA per 6 months | 10.0 (9.04–11.0) | 9.50 (8.33–10.7) | 11.9 (10.5–13.3) |
| Frequency of events using oral CS | 1.64 (1.38–1.91) | 1.90 (1.58–2.22) | 1.83 (1.47–2.18) |
| Frequency of events of visiting ER | 0.72 (0.56–0.90) | 0.72 (0.60–0.84) | 0.54 (0.47–0.62) |
| Coughing lasted over 1 week | 2907 (72%) | 2232 (72%) | 2159 (77%) |
| None | 0.90 (0.80–1.00) | 0.74 (0.67–0.81) | 0.65 (0.55–0.74) |
| Frequency per 6 months | | | |
| Sputum with coughing | | | |
| None | 2027 (50%) | 1621 (52%) | 1584 (57%) |
| 1–2 events per 6 months | 928 (23%) | 687 (22%) | 622 (22%) |
| 1–2 events per 1 month | 500 (12%) | 370 (12%) | 291 (10%) |
| 1–2 events per 1 week | 339 (9%) | 235 (8%) | 183 (7%) |
| Almost every day | 224 (6%) | 177 (6%) | 118 (4%) |
| Nocturnal awakening | | | |
| None | 2549 (63%) | 2135 (69%) | 1965 (70%) |
| 1–2 events per 6 months | 864 (21%) | 554 (18%) | 523 (19%) |
| 1–2 events per 1 month | 366 (9%) | 258 (8%) | 209 (7%) |
| 1–2 events per 1 week | 189 (5%) | 118 (4%) | 75 (3%) |
| Almost every day | 70 (2%) | 25 (1%) | 26 (1%) |
| Rhinitis symptoms | | | |
| None | 1671 (41%) | 1250 (40%) | 1314 (47%) |
| Nasal obstruction | 1394 (35%) | 1077 (35%) | 852 (30%) |
| Runny nose | 1581 (39%) | 1233 (40%) | 973 (35%) |
| Sneeze | 1187 (29%) | 935 (30%) | 703 (25%) |
| Treatment | | | |
| ICS[a] | 5869 (83%) | 4064 (84%) | 3655 (82%) |
| Add-on LABA | 2258 (32%) | 1789 (36%) | 1760 (38%) |
| Add-on LTRA | 3061 (43%) | 2246 (45%) | 2059 (46%) |
| Add-on theophylline | 2664 (38%) | 1804 (37%) | 1698 (38%) |

Notes: Data are expressed as number (%) or mean (95% CIs) unless otherwise indicated.
CS, corticosteroid; ICS, inhaled corticosteroid; LABA, long-acting $\beta_2$ agonist; LTRA, leukotriene receptor antagonist; SABA; short-acting $\beta_2$ agonist, URI, upper respiratory infection; CI, confidence interval.
[a]Including ICS therapy alone and additional therapy with LABAs, LTRAs, and/or theophylline.

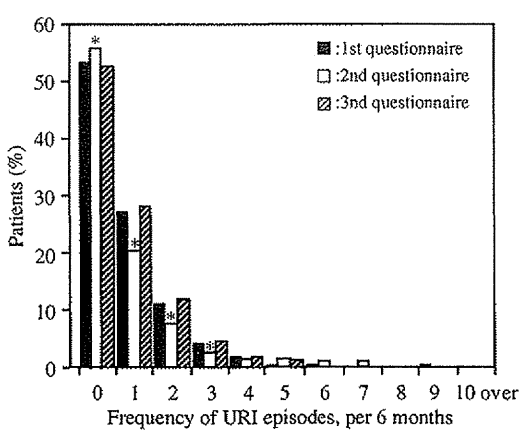


FIGURE 1.—Frequency of febrile URI episodes in adult patients with asthma in three periods. A self-administered questionnaire was given to 7070 patients in autumn–winter 2006 (1st questionnaire), 4859 patients in spring–summer 2007 (2nd questionnaire), and 4452 patients in autumn–winter 2007 (3rd questionnaire).
Note: *$p < .05$, compared with the first questionnaire.

Multivariate logistic regression analysis showed that recurrent febrile episodes of URI (OR = 2.68 [1.47–4.91]), disuse of ICS (OR = 2.63 [1.68–4.12]), a previous exacerbation within 1 year (OR = 1.74 [1.28–2.34]), female sex (OR = 1.58 [1.20–2.08]), older age (OR = 1.57 [1.15–2.13]), and disuse of add-on LTRAs (OR = 1.42 [1.06–1.74]) were independently associated with symptom-severity.

*Independent Factors Associated with Frequency of Severe Exacerbations*

To analyze previous episodes of severe exacerbations, we examined the responses of the 1012 patients who answered the questionnaire 3 times. In these patients, the frequency of severe exacerbations was 0.27 per subject per 6 months and 5.5% had ≥2 severe exacerbations. The results of univariate Poisson regression analysis are shown in Table 3. In a multivariate Poisson regression model, the independent factors that contributed to the frequency of severe exacerbations were a history of previous exacerbations within 1 year ($\beta = 0.99$, $p < .01$), an episode of sputum with coughing ($\beta = 0.97$, $p < .01$), an episode of nocturnal awakening ($\beta = 0.72$, $p < .01$), and female sex ($\beta = 0.59$, $p < .01$) (Table 4). The contributions of age ($\beta = 0.34$, $p = .26$), episodes of rhinitis symptoms ($\beta = -0.52$, $p = .88$), and any treatment were not significant. The

TABLE 2.—Univariate and multivariate logistic analysis of factors associated with moderate to severe symptom-severity.

| | Univariate analysis OR (95% CI) | Multivariate analysis OR (95% CI) |
|---|---|---|
| Age | | |
| < 65 years old | 1 | 1 |
| ≥ 65 years old | 3.42* (1.75–8.06) | 1.57* (1.15–2.13) |
| Sex | | |
| Male | 1 | 1 |
| Female | 1.59* (1.22–2.07) | 1.58* (1.20–2.08) |
| URI | | |
| < 3 episodes in the past 6 months | 1 | 1 |
| ≥ 3 episodes in the past 6 months | 3.83* (2.29–6.40) | 2.68* (1.47–4.91) |
| Previous exacerbation | | |
| None | 1 | 1 |
| ≥ One episode in the past year | 4.96* (1.75–14.04) | 1.74* (1.28–2.34) |
| ICS[a] | | |
| Use | 1 | 1 |
| Disuse | 2.70* (1.82–4.02) | 2.63* (1.68–4.12) |
| Add-on LABA | | |
| Use | 1 | 1 |
| Disuse | 0.78 (0.60–1.02) | 0.98 (0.73–1.30) |
| Add-on LTRA | | |
| Use | 1 | 1 |
| Disuse | 1.58* (1.21–2.07) | 1.42* (1.06–1.74) |
| Add-on theophylline | | |
| Use | 1 | 1 |
| Disuse | 1.25 (0.96–1.62) | 1.24 (0.82–1.90) |

Notes: Poor asthma control is defined as having one or more symptom of cough, sputum with coughing and nocturnal wakening per week. ORs and 95% CIs of analyses using logistic regression models for associations between older age, female sex, recurrent URI, and a previous exacerbation are shown.
OR, odds ratio; CI, confidence interval; ICS, inhaled corticosteroid; LABA, long-acting $\beta_2$ agonist; LTRA, leukotriene receptor antagonist; URI, upper respiratory infection.
[a]ICS therapy alone and additional therapy with LABA and/or LTRA and/or theophylline were included.
*$p < .01$.

TABLE 3.—Univariate Poisson analysis of factors associated with the frequency of severe exacerbations.

| | $\beta$ (95% CI) | |
|---|---|---|
| Age | | $p = .26$ |
| <65 years old | | |
| ≥65 years old | 0.34 (−0.26 to 0.94) | |
| Sex | | $p < .01$ |
| Male | | |
| Female | 0.62 (0.17–1.07) | |
| Episodes of URI | | $p = .06$ |
| <3 episodes in the past 6 months | −0.43 (−0.88 to 0.02) | |
| ≥3 episodes in the past 6 months | | |
| Episodes of rhinitis symptom | | $p = .88$ |
| None | −0.52 (−0.74 to 0.64) | |
| ≥One episode in the past 6 months | | |
| Episodes of sputum with coughing | | $p < .01$ |
| None | 1.23 (0.77–1.68) | |
| ≥One episode in the past 6 months | | |
| Episodes of nocturnal awakening | | $p < .01$ |
| None | 1.22 (0.61–1.82) | |
| ≥One episode in the past 6 months | | |
| Previous exacerbation | | $p < .01$ |
| none | 0.78 (0.6–1.02) | |
| ≥one episode in the past 6 months | | |
| ICS[a] | | $p = .08$ |
| Disuse | 0.16 (−0.08 to 0.52) | |
| Use | | |
| Add-on LABA | | $p = .12$ |
| Disuse | 0.24 (−0.14 to 0.52) | |
| Use | | |
| Add-on LTRA | | $p = .23$ |
| Disuse | 0.21 (−0.11 to 0.46) | |
| Use | | |
| Add-on theophylline | | $p = .66$ |
| Disuse | −0.32 (−0.72 to 0.08) | |
| Use | | |

Notes: Severe exacerbations are defined as a self-report of an asthma-related hospitalization, an emergency department visit, or a requirement for systemic corticosteroids.
CI, confidence interval; ICS, inhaled corticosteroid; LABA, long-acting $\beta_2$ agonist; LTRA, leukotriene receptor antagonist; URI, upper respiratory infection.
[a]Including ICS therapy alone and additional therapy with LABAs, LTRAs, and/or theophylline.
$\beta$ = regression coefficient.

Poisson regression model predicting the frequency of severe exacerbations from the four independent significant factors was statistically significant with a chi-square likelihood ratio = 171.503, df = 3 ($p < .0001$).

Analysis of the expected frequency of severe exacerbations showed that one or more previous exacerbations within 6 months had an expected log count of 0.99, indicating about 0.20 more events of severe exacerbations in patients with previous exacerbations, compared to those without exacerbations, with other factors held constant (Table 4).

## DISCUSSION

To our knowledge, this study is the largest retroprospective study of URI and exacerbations in adult patients with asthma performed to date. The results indicate that the "exacerbation-prone" and "URI-prone" asthma phenotypes are common, with rates of 5.5% and 17.4%, respectively, and frequencies of severe exacerbations and febrile URI of 1.54 and 0.54 per subject per year, respectively. Recurrent URI and frequency of URI were associated with moderate to severe symptom-severity and severe exacerbation, respectively, and therefore our results support the hypothesis of an association between episodes of URI and a deteriorated asthma status.

The overall incidence of URI per subject per year of follow-up was 1.54, which is similar to the results of previous prospective reports (range 1.2–6.7) (14–19). To avoid overreporting of URI episodes, the subjects were specifically instructed not to report a URI if they were experiencing URI without fever. Severe exacerbations were defined as those requiring hospitalization or a course of antibiotics or oral corticosteroids, and this definition gave an overall rate of 0.54 exacerbations per patient per year. This frequency is in agreement with the previous reports showing that asthma leads to severe exacerbations with a frequency of 0.12–0.77 per patient per year (20–22).

Exacerbations are defined as events characterized by a change from the patient's previous status that prompts a need for a change in treatment (13). The ATS/ERS Task Force considered that episodes of "mild" exacerbations were only just outside the normal range of variation for

TABLE 4.—Multivariate Poisson analysis of factors associated with the frequency of severe exacerbations.

| | $\beta$ (95% CI) | Adjusted mean (95%CI) |
|---|---|---|
| Sex | | |
| Male | | 0.11 (0.08–0.13) |
| Female | 0.59* (0.16–1.01) | 0.19 (0.13–0.26) |
| Episodes of sputum with coughing | | |
| None | | 0.10 (0.08–0.12) |
| ≥One episode in the past 6 months | 0.97* (0.48–1.47) | 0.26 (0.15–0.38) |
| Episodes of nocturnal awakening | | |
| None | | 0.09 (0.05–0.12) |
| ≥One episode in the past 6 months | 0.72* (0.26–1.19) | 0.18 (0.13–0.23) |
| Previous exacerbation | | |
| None | | 0.12 (0.08–0.16) |
| ≥One episode in the past 6 months | 0.99* (0.34–1.65) | 0.32 (0.20–0.42) |

Notes: Severe exacerbations are defined as a self-report of an asthma-related hospitalization, an emergency department visit, or a requirement for systemic corticosteroids. Results are shown for an analysis using a Poisson regression model adjusted with covariates that were significant factors in univariate analysis. The adjusted mean is estimated as the arithmetic mean of the sample and the confidence interval is estimated using the relationship between the chi-square and Poisson distributions.
CI, confidence interval.
$\beta$ = regression coefficient; *$p < .01$.

an individual patient and that current methods of analysis could not distinguish such exacerbations from a transient loss of asthma control. In the current study, we analyzed patients' experiences of a deteriorated status of asthma to identify "moderate to severe symptom-severity" and "severe exacerbations". The prevalence of moderate to severe symptom-severity is in concordance with that in a report by Soriano et al. (23), in which it was shown that 65% of adult asthma patients had moderate to severe symptom-severity. We assessed factors that contributed to poor asthma control, which was considered as a "mild" exacerbation by the ATS/ERS Task Force (10). Our findings were an agreement with those in Temprano et al. (24) showing that women were more likely to have poor asthma control, despite higher rates of ICS use, compared with men. Previous severe exacerbations and recurrent episodes of URI were also covariates for moderate to severe symptom-severity. Hermosa et al. (25) also found that the number of exacerbations during the previous year is the variable with the greatest effect on control in patients with severe asthma. Symptom-severity is also significantly affected by adherence to treatment, patient knowledge of the disease, body mass index (BMI), and the number of visits to a physician in the previous 6 months (25). Viral infection also contributes to deteriorated asthma control, and cold-severity within the first 2 days can be used to predict subsequent changes in asthma control in adult patients (11).

Many studies have demonstrated that URI is a trigger of acute exacerbations of asthma (5–7), but there is little information on the association of the frequency of exacerbations and the episodes of URI. The hypothesis of our study was that recurrent episodes of URI might contribute to loss of control, but not to severe exacerbations. The rates of severe exacerbations were analyzed using a Poisson regression model. In agreement with a previous study (26), our results showed that asthma exacerbations are more common in females than in males. Exacerbations also occurred most frequently in individuals with severe disease (27). Previous reports have also identified other risk factors, including a history of a recent exacerbation, comorbidities such as an increased BMI and psychological problems, as well as current smoking and lower socioeconomic status. A recent exacerbation within the last 3 months is associated with a markedly increased risk of a future exacerbation (2). The host response to viral infection is also likely to influence susceptibility to asthma exacerbation, and the association of a previous exacerbation with a risk for recurrent exacerbations suggests the presence of an "exacerbation-prone" subset of patients with asthma (1).

In the current study, we showed that drug intervention with ICSs and add-on LTRAs might improve symptoms, but severe exacerbations still occurred in some patients despite the use of these drugs. Many studies have described pharmacologic interventions in asthma exacerbations (28–30), and the exacerbations have been associated with poor asthma control (31). The Formoterol and Corticosteroids Establishing Therapy (FACET) study (32) revealed that a higher ICS dose had a marked beneficial effect on exacerbation frequency, but relatively less effect on symptoms and peak expiratory flow, whereas the opposite was true with the addition of LABAs (32, 33). Rosi et al. (34) also found that the asthma exacerbation frequency is not closely related to symptoms and measures of disrupted airway function. These reports and our results demonstrate that different strategies might be needed to reduce asthma exacerbations, as well as to optimize asthma control.

Leukotrienes produced in the airway in response to infection play important roles in the mechanism of exacerbation of asthma following viral infection (35). In a few studies, LTRAs have been shown to reduce asthma symptoms or exacerbations in children with colds (36–38). However, it has also been reported that LTRAs did not improve asthma control or cold symptom scores caused by experimental rhinovirus infection (39). Our results showing that add-on LTRAs with ICSs might lessen the symptoms of asthma in adults are consistent with the previous reports that add-on LTRAs are equivalent to add-on LABAs for control of asthma symptoms (40, 41). In the current study, discontinuing add-on LABAs was not associated with developing asthma symptoms, which might be due to low drug adherence.

The limitations of the study include the selection criteria, study design, and availability of data. Because we did not require evidence of bronchodilator reversibility, the enrolled patients may have had a combination of chronic obstructive pulmonary disease (COPD) and asthma. Therefore, the results cannot be generalized to all patients with asthma with the conditions described above. The questionnaire was incomplete due to missing data and distribution of the questionnaire to some patients after cold episodes. Second, microbiological specimens were not collected during the cold episodes and this prevented

determination of the role of viral or bacterial respiratory tract infections. However, because URI is self-limiting, a viral diagnosis is not clinically indicated. In children, viral URI has been associated with >80% of asthma exacerbations (42), but a recent study showed that the virus detection rate was only 36.7% in all unscheduled hospital visits in children (43). Third, we did not assess comorbidities and airway obstruction. Several comorbidities including severe nasal sinus disease, gastroesophageal reflex, recurrent respiratory infection, and obstructive sleep apnea (44), as well as current smokers (45) and increased BMI (2, 46), are associated with an increased risk for future exacerbations. Airway obstruction is also an important risk factor for multiple asthma exacerbations requiring hospital care or systemic corticosteroids (3, 47). Finally, the survey in the study was based on recall, and recall bias can distort the description of events. However, often a person recalls positive events more than negative events, and therefore it seems reasonable to assume that this recall bias did not skew our results.

In conclusion, our data suggest an association between episodes of URI and a deteriorated asthma status. These findings require confirmation in further studies in a larger number of affected individuals.

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## DECLARATION OF INTEREST

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this article.

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## APPENDIX

### *Questionnaire*

Answer these questions and select the first answer that you think of.

1. During the past 6 months, have you had a bout of coughing that lasted over 1 week? If yes, how many events?
   ☐ No ☐ Yes ( event(s))
2. During the past 6 months, have you had any sputum with coughing? If yes, how many events?
   ☐ No ☐ 1–2 events/6 months ☐ 1–2 events/a month
   ☐ 1–2 events/a week ☐ Almost every day
3. During the past 6 months, have you been woken up at night by an attack of asthma? If yes, how many events?
   ☐ No ☐ 1–2 events/6 months ☐ 1–2 events/a month
   ☐ 1–2 events/a week ☐ Almost every day
4. During the past 6 months, have you had any of these rhinitis symptoms?
   ☐ None ☐ Nasal obstruction ☐ Runny nose ☐ Sneeze
   If yes, what was the grade of the symptoms?
   ☐ Mild ☐ Intermittent ☐ Severe ☐ Extreme
5. During the past 6 months, have you had any episodes of common cold with fever (temperature >37.0°C)? If yes, how many episodes?
   ☐ No ☐ Yes ( event(s))
6. During the past 6 months, how many times have you experienced the following events during an asthma attack?
   During these episodes, how many times did you use a reliever inhaler?
   ( event(s))
   During these episodes, how many times did you use an oral steroid?
   ( event(s))
   During these episodes, how many times did you visit an emergency room?
   ( event(s))

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# Changes of Tumor Size and Tumor Contrast Enhancement during Radiotherapy for Non-small-cell Lung Cancer May Be Suggestive of Treatment Response

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# Changes of Tumor Size and Tumor Contrast Enhancement during Radiotherapy for Non-small-cell Lung Cancer May Be Suggestive of Treatment Response

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**Non-small-cell lung cancer/Radiotherapy/Tumor enhancement/Tumor regression/Contrast-enhanced CT.**

We evaluated sequential dynamic contrast-enhanced CT (DCE-CT) scans to assess the possibility of early prediction of treatment responses by quantifying the tumor size reduction and the change in tumor enhancement during and after a course of radiotherapy (RT). Thirty-nine patients with non-small-cell lung cancer were treated with RT for initial treatment. DCE-CT scan was performed within one week before the beginning of treatment, after 17 or 18 fractions (34 or 36 Gy), and 1 week and 1 month after the end of RT. The correlation between the relative decrease in tumor diameter and that in the attenuation value was evaluated. Nineteen patients were evaluated in this study. The median tumor size was 39.5 mm at the start of treatment, 30.8 mm at 34–36 Gy, and 16.1 mm 1 month after the end of RT. The relative decrease in tumor diameter at 34–36 Gy well correlated with that 1 month after treatment ($r = 0.85$, r: Pearson's correlation coefficient, $p < 0.001$). Relative change in the attenuation value at the rim of the tumor at 34–36 Gy did not significantly correlate with the change in tumor diameter 1 month after the completion of RT, but in the center of the tumor, the change of the attenuation value in the delayed phase correlated with the change in tumor diameter. The decrease of tumor diameter during RT may be predictive of treatment response. The relative change of tumor enhancement in the center of the tumor in the delayed phase correlated with tumor shrinkage 1 month after the completion of RT.

## INTRODUCTION

Lung cancer is the most common cause of cancer mortality. Surgery is the first choice for locally resectable non-small-cell lung cancer (NSCLC), but about 80% of lung cancer patients are inoperable due to locoregional tumor extension, metastasis to other organs or poor physical condition. Radiotherapy (RT) is the definitive local treatment modality for unresectable locally advanced lung cancer patients and is often combined with chemotherapy. The current prognosis for NSCLC treated with RT is still poor, and treatment failure will occur in a significant number of patients.

Early prediction of treatment response may allow therapy modification, such as increase of the total radiation dose and intensity of chemotherapy, and better local control. Recent data have often suggested that higher doses of radiation can improve local control and overall survival,[1–3] however, delivering doses more than 70 Gy to traditionally defined target volumes is often impossible because of normal tissue damage, especially the risk of pneumonitis and lung fibrosis. If the radiation field is small, dose escalation may be possible and the toxicity of radiotherapy can be reduced. Many investigators have observed tumor volume shrinkage to varying degrees during the course of fractionated radiation therapy,[4–8] and tumor volume shrinkage in a shorter period, specifically during the course of RT, is meaningful in clinical situations. Replanning for a shrinking field size to adapt to gross tumor volume (GTV) change will lead to greater normal tissue sparing without detrimental effects on the planned target volume (PTV) dose coverage. A recent study of head and neck cancer suggested that local blood supply increase, potentially a source of increased oxygenation, may be a positive indicator

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of therapeutic response,[9] however, a significant correlation between changes in local blood supply and therapeutic responses to RT for NSCLC has not been established yet.

Thus, we conducted a study of dynamic contrast-enhanced CT before, during, and after RT to assess the possibility of early prediction of treatment responses by quantifying the tumor size reduction and the change in tumor enhancement during and after a course of RT.

## MATERIALS AND METHODS

### *Patients*

Thirty-nine Consecutive patients with NSCLC treated with RT as initial treatment for definitive therapy under the following protocol from January 2009 to April 2010 were involved. Each patient gave written informed consent, including information on radiation exposure from both CT examinations and RT and on the adverse effects of the treatment. The ECOG Performance Status Scale of all patients ranged from 0 to 2.This study was approved by the institutional ethics committee of Nara Medical University.

All patients were proven to have NSCLC histologically (median size, 39.5 mm; range, 11.0 to 72.9 mm) and all tumors were unresectable. All patients were treated with a course of RT delivering 60–70 Gy in 30–35 fractions with or without chemotherapy. Dynamic contrast-enhanced CT (DCE-CT) scan was performed within one week before beginning the treatment, after 17 or 18 fractions (34 or 36 Gy) of RT, and 1 week and 1 month after the end of RT.

### *Dynamic contrast-enhanced CT*

Patients were scanned using a dual-source CT scanner (Somatom Definition; Siemens Medical Solutions, Forchheim, Germany). An initial non-contrast breath-hold scan encompassing the whole lung was performed. Using a dual-headed pump injector, 80 mL contrast media (300 mgI/mL (B.W. < 65 kg) or 370 mgI/mL (B.W. ≧ 65 kg)), was administered at 4 mL/s followed by 60 mL contrast media diluted 33% with saline. A contrast breath-hold scan was performed in dual mode (64 × 0.6 mm, 0.33 s rotation speed, 2 mm slice thickness, D30f kernel, 160 mAs at 140 kV, 532 mAs at 80 kV). A contrast medium bolus tracking scan was not used. Image acquisition was started 20 s after the beginning of bolus injection for the early phase, and 90 s after for the delayed phase.

The maximum tumor diameter was measured at the axial section in every series of serial DCE-CT study.

Additional analysis was carried out to assess the enhancement of the tumor center and tumor rim. The attenuation value of the tumor was measured by placing a separate ROI within the tumor rim and center at each point, respectively. All measurements in Hounsfield units were obtained from mediastinal window images to ensure that partial volume averaging was minimized. The tumor center was defined as the area more than 30% of the diameter from the edge, and the tumor rim was defined as the area less than 20% of the diameter from the tumor edge. Because of the difficulty in defining the rim and center, analysis was carried out at the maximum diameter of the axial section.

### *Statistical analysis*

The correlation between the relative decrease in tumor diameter 1 month after the end of RT and that in the attenuation value during RT was evaluated. We did not analyze the relative change of tumor enhancement 1 week after the end of RT because, in this study, we evaluated the possibility of early prediction and modification of therapy during the course of RT. Standard statistical methods were used to assess correlations in univariate analysis. Pearson's $\chi^2$ test was used for qualitative data. The correlation between the relative decrease in tumor diameter during and after the course of RT was also evaluated. All statistical analyses were performed using StatMate version 4.0 (ATMS, Tokyo, Japan).

## RESULTS

Of the 39 patients treated in this period, 2 died during the course of RT, and 2 aborted treatment because of adverse effects. In 4 patients, contrast-enhanced CT scans were not obtained because of renal dysfunction or poor general condition. In 1 patient with a 10 mm tumor, the attenuation value could not be measured in two regions. In 2 patients, CT scans were not obtained because of technical issues. In 8 patients, CT scans were not performed under this protocol. Two patients

**Table 1.** Patient characteristics

| Characteristics (n = 19) | Data |
|---|---|
| Sex | |
| Male | 18 |
| Female | 1 |
| Median age (range) (y) | 70 (41–79) |
| Histology, n (%) | |
| Squamous cell carcinoma | 12 (63.2) |
| Adenocarcinoma | 5 (26.3) |
| Mucoepidermoid carcinoma | 1 (5.3) |
| Others | 1 (5.3) |
| Stage, n (%) | |
| IIb | 3 (15.8) |
| IIIa | 9 (47.4) |
| IIIb | 6 (31.6) |
| IV | 1 (5.3) |
| Chemotherapy | |
| Carboplatin + Paclitaxel | 9 |
| Cisplatin + Docetaxel | 7 |
| Total tumor dose [median (range)] (Gy) | 66 (60–70) |

were lost to follow-up. The other 19 patients were evaluated in this study. Characteristics of patients are shown in Table 1. The median age was 70 years (range: 41–79) and 1 patient was female. The median radiation dose was 66 Gy (range: 60–70). Chemotherapy was given to 16 patients. The chemotherapy regimens included carboplatin/paclitaxel (9 patients) and cisplatin/docetaxel (7 patients). The clinical stage was IIB in 3 patients, IIIA in 9 patients, IIIB in 6 patients and IV in 1 patient. Histology of tumors was 12 squamous cell carcinomas, 5 adenocarcinomas, 1 mucoepidermoid carcinoma, and 1 NSCLC could not be classified. The individual tumor size ranged from 11.0 to 72.9 mm at the start of treatment.

Tumor diameters and their percentage changes from before to after therapy are detailed in Fig. 1a and 1b. The median tumor size was 39.5 mm before treatment, 30.8 mm (mean regression rate (MRR): 0.74) at 34–36 Gy, 22.8 mm (MRR: 0.56) 1 week after the end of RT, and 16.1 mm (MRR: 0.5) 1 month after the end of RT, respectively. All but 3 tumors regressed at 34–36 Gy (more than 8%) and 1 month after the end of RT by more than 30%. Three tumors regressed less than 8% at 34–36 Gy. The relative decrease in tumor diameter at 34–36 Gy well correlated with that 1 month after treatment ($r = 0.85$, r: Pearson's correlation coefficient, $p < 0.001$) (Fig. 2). Relative decrease of tumor diameter during and after the course of radiotherapy was not different between squamous cell carcinoma and adenocarcinoma in this study (data not shown). Relative change in the attenuation value in the center of the tumors at 34–36 Gy did not correlate with the change in tumor diameter 1 month after the completion of RT in the early phase ($r = -0.13$, $p = 0.60$), but in the delayed phase, it correlated with the change in tumor diameter ($r = -0.69$, $p < 0.01$) (Fig. 3). Relative change in the attenuation value at the rim of the tumors at 34–36 Gy did not correlate with the change in tumor diam-

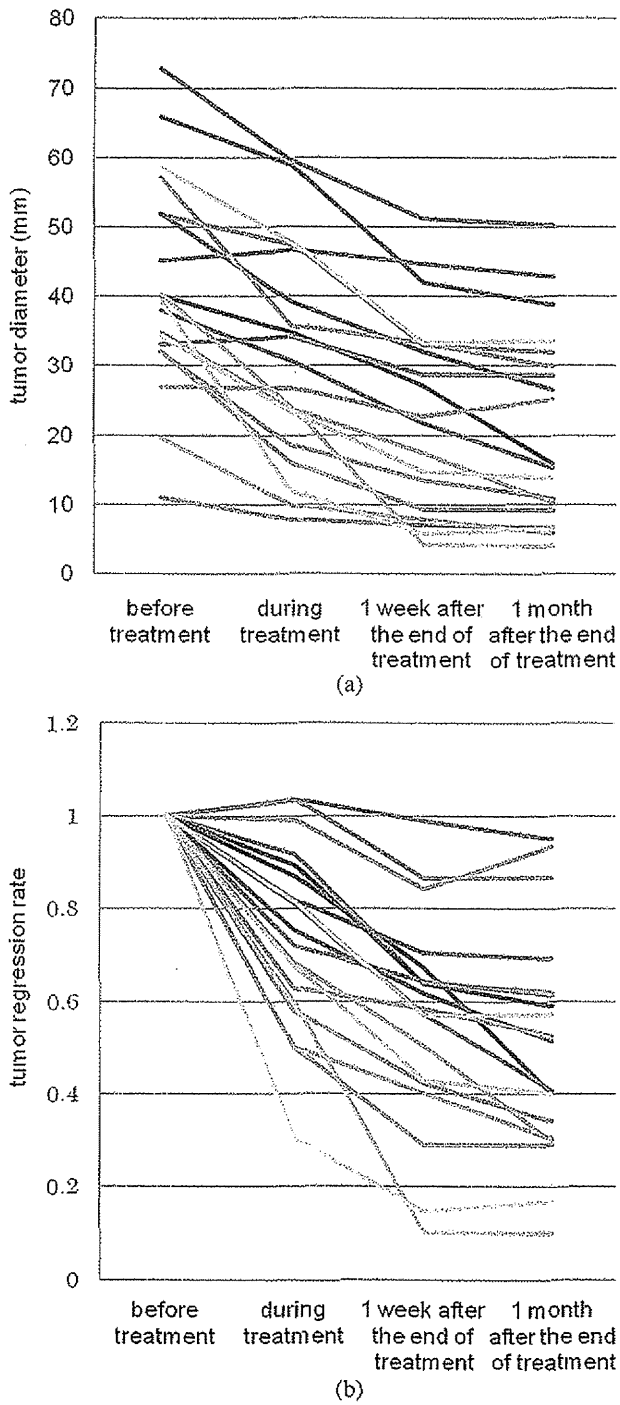


**Fig. 1.** Tumor diameters (1a) and tumor regression rate (1b) in all 19 patients included in this study from before to after therapy.

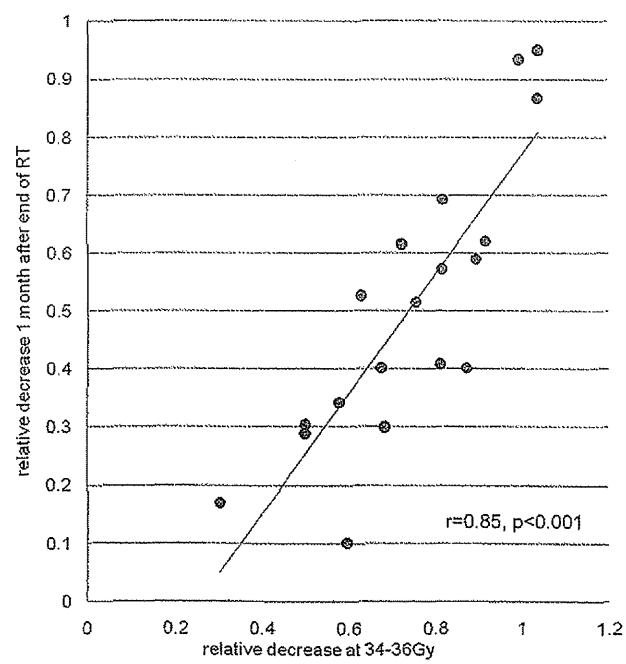


**Fig. 2.** Correlation between the relative decrease in tumor diameter at 34–36 Gy and that 1 month after the end of RT.