

effect of both corticosteroid and anticoagulant. Although the number of patients and the observation period in the present study were limited, our data support a new therapeutic approach using anticoagulants in patients with IPF.

According to this study, the major cause of death in patients with IPF was acute exacerbation, in which the plasma d-dimer level was elevated. The acute exacerbation is defined clinically after excluding infection and heart failure. IV administration of dalteparin sodium significantly decreased the plasma d-dimer level and mortality during rehospitalization with exacerbation of IPF. Interestingly, the plasma d-dimer level was slightly elevated in IPF patients at entry to this study (Table 1). Since an elevated plasma d-dimer level is thought to be a marker for intravascular coagulation, these results suggest the presence of an activated coagulation system in patients with IPF and a relationship between intravascular coagulation and mortality in acute exacerbations of IPF. Panos et al<sup>9</sup> described that pulmonary embolism is an important cause of death in IPF patients. However, Kotani et al<sup>21</sup> reported that an increased procoagulant activity was observed in BAL fluid from IPF patients. Tissue factor expression and fibrin deposition were detected by specific antibodies in the alveoli from patients with IPF.<sup>22</sup> Since d-dimer is derived from fibrin, plasma d-dimer may be influenced by increased intra-alveolar fibrin deposition. Thus, careful hemostatic analysis might reveal important prognostic factors in IPF patients.

The exact mechanism of the beneficial effect of anticoagulation therapy for IPF is not known. Histologic analysis on the patients who died due to exacerbation of IPF in the nonanticoagulant group revealed the usual interstitial pneumonia with superimposed features of acute lung injury. There was a honeycomb appearance and an exudative or organizing phase of diffuse alveolar damage. These findings were compatible with previous reports<sup>2,3</sup> showing a pattern of usual interstitial pneumonia and acute lung injury with or without hyaline membranes. Although evidence of massive pulmonary embolisms was not found in the samples, a few small clots were detected in the alveolar capillary bed and there was evidence of fibrin deposition in the alveolar space. Furthermore, it is described that extravascular fibrin deposition and coagulation play an important role in the pathogenesis of acute lung injury and related fibrosis.<sup>23</sup> The extravascular function as well as the intravascular function of anticoagulant therapy might be associated with the beneficial effects evident in this study.

There was a tendency for a longer hospitalization-free state in the anticoagulant group compared to the nonanticoagulant group, but this was not statistically

significant. This suggests that anticoagulation with oral warfarin in the outpatient setting is insufficient to prevent risk of acute exacerbation of IPF. However, anticoagulant therapy following readmission to the hospital, *ie*, IV heparin administration, could reduce the mortality of patients with acute exacerbation. The reason for the discrepancy between the beneficial effect of heparin on mortality of acute exacerbations of IPF and the ineffectiveness of oral warfarin in preventing the risk of acute exacerbation is not clear. One possibility is the absence of rigid control of anticoagulation in outpatients. It is more difficult to rigidly control the intravascular coagulation by means of oral warfarin administration compared to IV heparin therapy that can be used in hospitalized patients. Another possibility is that heparin exerts additional effects compared to anticoagulation with warfarin. Several *in vitro* studies<sup>24,25</sup> indicate that heparin may directly down-regulate the expression of various factors implicated in the progression of interstitial fibrosis such as transforming growth factor- $\beta_1$ , endothelin-1, and fibroblast growth factor-2. These reports suggest the existence of important differential effects between oral warfarin and IV heparin (dalteparin sodium injection) administration.

In summary, we demonstrated the beneficial effect of combined anticoagulant and corticosteroid therapy on the survival of IPF patients. Increased plasma d-dimer level was observed in patients with IPF, suggesting the presence of an activated hemostasis system. The main effect of anticoagulant therapy is lowering the mortality rate after acute exacerbation of IPF. Anticoagulant therapy may be an additional new strategy to treat IPF patients.

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# Increased Arterial Carboxyhemoglobin Concentrations in Chronic Obstructive Pulmonary Disease

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**Rationale:** Exhaled carbon monoxide (CO) and arterial blood carboxyhemoglobin concentrations (Hb-CO) increase in inflammatory pulmonary diseases. **Objectives:** To study whether arterial Hb-CO is useful to monitor disease activity in patients with chronic obstructive pulmonary disease (COPD) who had stopped smoking. **Methods:** We measured arterial Hb-CO, arteriovenous Hb-CO differences, and FEV<sub>1</sub> in 58 patients with COPD and 61 ex-smoking control subjects. **Results:** Arterial Hb-CO concentrations in patients at stable conditions were higher than those in control subjects ( $p < 0.0001$ ). Furthermore, the Hb-CO concentrations in patients at the exacerbations ( $p < 0.0001$ ) were higher than those at the stable conditions. Arterial Hb-CO concentrations in patients at stage III were higher than those in patients at stage II, and the Hb-CO concentrations in patients at stage IV were higher than those in patients at stage III at the stable conditions and exacerbations. Arterial Hb-CO correlated with exhaled CO in patients with COPD at stage II and stage III at the exacerbations. Arterial Hb-CO inversely correlated with the arterial blood partial oxygen pressure and FEV<sub>1</sub>. Arteriovenous Hb-CO differences in patients at the exacerbations did not differ from those in patients at stable conditions and from those in control subjects. Moreover, arterial Hb-CO correlated with serum C-reactive protein values and serum lipid peroxide concentrations. **Conclusions:** These findings suggest that increased arterial Hb-CO may relate to severity in patients with COPD because of lung and systemic inflammation and production of reactive oxygen species.

**Keywords:** carbon monoxide; carboxyhemoglobin; chronic obstructive pulmonary disease; heme oxygenase; systemic inflammation

Chronic obstructive pulmonary disease (COPD) is a major global health problem that has an increasing disease burden and effect on health care spending and is characterized by airflow limitation that is not fully reversible (1). Various factors are associated with the pathogenesis of COPD, including oxidative stress (2), inflammatory cells (3, 4), such as activated neutrophils and mononuclear leukocytes (5), and mediators, including tumor necrosis factor  $\alpha$  (6, 7). Furthermore, these factors are associated with the exacerbations of COPD (7–9) caused by airway infection of viruses and bacteria (10, 11).

Many reports have suggested that airflow limitation presented by FEV<sub>1</sub> inversely correlates with several inflammatory indicators, such as nitric oxide (NO) levels in exhaled air (12, 13), 4-hydroxy-2-nonenal levels in the lung (14), and sputum interleukin-8 levels and soluble tumor necrosis factor receptor (4) in patients with COPD. NO, hydrogen peroxide, ethane, and 4-hydroxy-2-nonenal in exhaled air have been suggested to be

noninvasive markers in monitoring the production of reactive oxygen species in the lung, and inflammation in the airway and lung in patients with COPD (12–17).

On the other hand, the inducible form of heme oxygenase (HO), HO-1, is reported to be expressed in airway epithelial cells (18), endothelial cells (19), and alveolar macrophages (20). Carbon monoxide (CO) is produced endogenously by HO, and is known to be present in measurable quantities in the exhaled air of normal subjects (21, 22). Upregulation of HO-1 by oxidant stress and proinflammatory cytokines (23) in airway and lung inflammation is suggested to cause the increased levels of exhaled CO in patients with inflammatory pulmonary diseases such as bronchial asthma, acute pneumonia, silicosis, bronchiectasis, upper respiratory tract infections (URTIs), and seasonal allergic rhinitis (20, 22, 24–29). Arterial blood carboxyhemoglobin (Hb-CO) concentrations correlate to exhaled CO concentrations (21), and have also been suggested to be an inflammatory marker in inflammatory pulmonary disease, including bronchial asthma, acute pneumonia, and silicosis (27–29). Blood gas analysis is performed in patients with COPD not only at the first visit to the hospital to estimate respiratory failure but also at visits to the hospital because of exacerbations. The arterial Hb-CO concentrations can be measured at the measurement of blood gas. However, Hb-CO concentrations at stable conditions and exacerbations have not been studied in patients with COPD.

In the present study, we studied the relationship between Hb-CO concentrations and disease severity in patients with COPD. We also measured the arteriovenous (a-v) Hb-CO concentration differences in patients with COPD to study the systemic and lung inflammation (28) in these patients (30, 31). Some of the results of these studies have been previously reported in the form of an abstract (32).

## METHODS

### Subjects

We studied 61 control subjects and 58 patients with COPD (Table 1). Of patients with COPD, 28 were classified into stage II, 10 into stage III, and 20 patients into stage IV according to the criteria by the Global Initiative for Chronic Obstructive Lung Disease (33). To avoid the influence of ambient CO on the Hb-CO, we enrolled subjects living uptown in Sendai City in the Miyagi prefecture with the same environment as previously described (28). Furthermore, passive smokers in patients with COPD and control subjects were excluded.

Exacerbations of COPD were defined with the criteria previously described (11, 34). At the exacerbations, 18 of 58 patients had symptoms of URTIs defined with the methods previously described (34). Type A influenza virus was isolated from sputum from 7 of the 18 patients with URTIs, and type B influenza was isolated from three patients. In another 30 patients with COPD, 13 patients had acute bronchitis with purulent sputum, and six patients had pneumonia. The patients with exacerbated COPD had been receiving treatment as previously described (35).

None of the control subjects were receiving long-term medication. All control subjects and all patients with COPD were ex-smokers. To

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TABLE 1. SUBJECT CHARACTERISTICS

Subject Category	Age (yr)	Male/female (no.)	CRP (mg/dl)	FEV <sub>1</sub> (%pred)	PaCO <sub>2</sub> (mm Hg)	PaO <sub>2</sub> (mm Hg)	Smoking History (pack-yr)
Control (n = 61)	69.7 ± 1.2	55/6	0.06 ± 0.01	95.5 ± 0.8	40.5 ± 0.2	90.2 ± 0.8	27 ± 2
COPD (n = 58)	70.9 ± 1.1	53/5	4.90 ± 0.62*	48.9 ± 2.2*	53.5 ± 2.1*	64.4 ± 1.7*	58 ± 2*

Definition of abbreviations: COPD = chronic obstructive pulmonary disease; CRP = C-reactive protein.

Values are mean ± SE.

\*p < 0.0001.

exclude current smokers, we measured urinary cotinine concentrations with high-performance liquid chromatography (36).

This study was approved by the Tohoku University Ethics Committee, and informed consent was obtained from each subject.

#### Measurement of Arterial and Venous Blood Hb-CO and Exhaled CO

We took blood from the radial artery and the median cubital vein in patients with COPD and in control subjects. We measured the Hb-CO concentrations with a spectrophotometer (18, 27, 37), and calculated the a-v Hb-CO concentration differences (28). We also measured exhaled CO concentrations in the control subjects and patients, as previously described (21, 22, 25, 26, 38). To avoid the influence of ambient CO on Hb-CO, Hb-CO and exhaled CO levels were measured at least 3 hours after arrival in a room in the hospital with low ambient CO.

#### Measurement of Serum Lipid Peroxide

Concentrations of lipid peroxide (LPO) in arterial blood serum were measured with methods as previously described (39, 40).

#### Study Protocol

We examined the relationships between arterial Hb-CO and either exhaled CO, PaO<sub>2</sub> and PaCO<sub>2</sub>, serum C-reactive protein (CRP), and LPO, or FEV<sub>1</sub>.

#### Statistical Analysis

The age, sex, Hb-CO concentrations, a-v Hb-CO difference, exhaled CO concentrations, arterial blood PaO<sub>2</sub> and PaCO<sub>2</sub> values, serum CRP values, serum LPO concentrations, and FEV<sub>1</sub> in each group are reported as mean ± SE. Statistical analysis of these values was performed by one-way analysis of variance and followed by the Newman-Keuls test. Linear regression analysis was performed using the method of least squares, to compare the relationship between arterial blood Hb-CO and either exhaled CO, PaCO<sub>2</sub>, PaO<sub>2</sub>, CRP value, LPO concentrations, or FEV<sub>1</sub> in patients with COPD at the exacerbations. Significance was accepted at p < 0.05.

## RESULTS

#### Subject Characteristics

The subject characteristics of patients with COPD and control subjects are shown in Table 1. There was no statistically significant difference in age and sex between normal control subjects and patients. All control subjects and patients were ex-smokers. Furthermore, urinary cotinine concentrations of all subjects were less than 30 ng of the cotinine/mg creatinine ratio, showing that all subjects in this study were not current smokers (22). The patients were treated with sustained-release theophylline and inhaled anticholinergic agents (oxitropium bromide). Furthermore, 10 of 58 patients were treated with inhaled corticosteroids (beclometasone dipropionate or fluticasone propionate).

#### Arterial Blood Hb-CO Concentrations

To examine the reproducibility of the arterial blood Hb-CO measurement in stable COPD, we measured the Hb-CO concentrations twice with a 4-week interval in patients with COPD at

stable conditions. The Hb-CO concentrations were stable and reproducible between two measurements (0.81 ± 0.02 vs. 0.82 ± 0.02%, n = 58, p > 0.50). Hb-CO concentrations in patients with COPD at stable conditions (0.81 ± 0.02%, n = 58) were significantly higher than those in control subjects (0.55 ± 0.02%, n = 61, p < 0.0001; Figure 1). Among the patients with COPD at stable conditions, Hb-CO increased in the severe stage of COPD. Hb-CO concentrations in patients at stage III (0.83 ± 0.03%, n = 10, p < 0.05) were higher than those in patients at stage II (0.70 ± 0.03%, n = 24). Hb-CO concentrations in patients at stage IV (0.95 ± 0.03, n = 20, p < 0.05) were also higher than those in patients at stage III.

The arterial blood Hb-CO concentrations in all patients with COPD at the exacerbations (1.09 ± 0.04%, n = 58) were significantly higher than those at a stable condition before the exacerbations (0.81 ± 0.02%, p < 0.0001; Figure 1). Furthermore, among the patients at the exacerbations, Hb-CO increased in the severe stage of COPD. At the exacerbations, Hb-CO concentrations in patients at stage III (1.12 ± 0.05%, n = 10, p = 0.0001) were higher than those in patients at stage II (0.85 ± 0.03%, n = 28). Hb-CO concentrations in patients at stage IV (1.41 ± 0.07%, n = 20, p = 0.01) were also higher than those in patients at stage III. Furthermore, in patients at stage IV, the Hb-CO changes between stable conditions and exacerbations (0.47 ± 0.05%) were higher than those in patients at stage III (0.29 ± 0.05%, p < 0.01), and the Hb-CO changes in patients at stage III were higher than those in patients at stage II (0.14 ± 0.02%, p < 0.05).

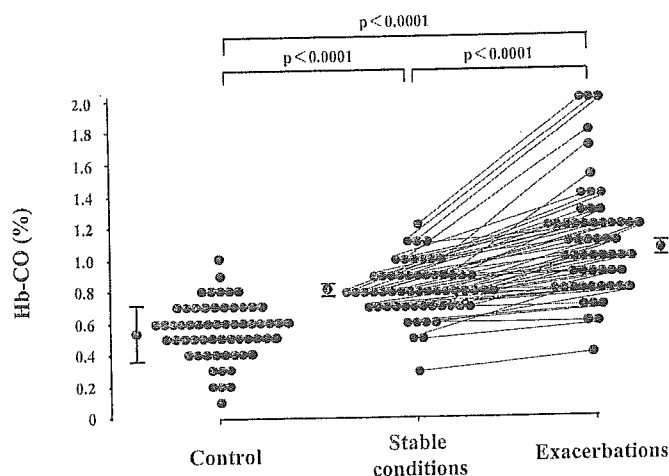


Figure 1. Arterial blood carboxyhemoglobin (Hb-CO) concentration in healthy ex-smoker control subjects (n = 61) and in patients with chronic obstructive pulmonary disease (COPD; n = 58) at the exacerbations and in patients with COPD at a stable condition before the exacerbations. The error bars indicate mean ± SEM.

Hb-CO concentrations in patients with the symptoms of URTIs ( $1.10 \pm 0.08\%$ ,  $n = 18$ ) were higher than those in control subjects ( $p < 0.0001$ ) and those in patients at the stable conditions ( $0.82 \pm 0.04\%$ ,  $p < 0.0001$ ), but did not differ from those in patients without URTIs at the exacerbations ( $1.09 \pm 0.06\%$ ,  $n = 40$ ,  $p > 0.8$ ).

Hb-CO concentrations in patients treated with inhaled corticosteroids did not differ from those in patients without inhaled corticosteroids at the stable conditions ( $0.87 \pm 0.03$  vs.  $0.80 \pm 0.03\%$ ,  $n = 10$ ,  $p > 0.2$ ) and at the exacerbations ( $1.24 \pm 0.08$  vs.  $1.06 \pm 0.05\%$ ,  $p > 0.1$ ).

In the present study, the Hb-CO concentrations in ex-smoking control subjects ( $n = 54$ ) with a smoking history of more than 60 pack-years, which was the average smoking history of patients in this study (Table 1), did not differ from the Hb-CO concentrations in the control subjects ( $n = 7$ ) with a smoking history of less than 60 pack-years (data not shown).

### Venous Blood Hb-CO Concentrations

The venous blood Hb-CO concentrations were measured in 14 patients with COPD and 16 control subjects. The venous blood Hb-CO concentrations in patients with COPD at a stable condition ( $0.71 \pm 0.04\%$ ,  $n = 14$ ) were significantly higher than that in control subjects ( $0.45 \pm 0.03\%$ ,  $n = 16$ ,  $p < 0.0001$ ). Furthermore, among the patients at the stable condition, the venous blood Hb-CO increased at the severe stage of COPD. The venous blood Hb-CO concentrations in patients at stage IV ( $0.83 \pm 0.05\%$ ,  $n = 6$ ,  $p < 0.01$ ) were higher than those in patients at stage II ( $0.58 \pm 0.05\%$ ,  $n = 4$ ). However, the venous blood Hb-CO concentrations in patients at stage III ( $0.65 \pm 0.07\%$ ,  $n = 4$ ,  $p > 0.3$ ) did not differ from those in patients at stage II. Likewise, the venous blood Hb-CO concentrations in patients at stage IV did not differ from those in patients at stage III ( $p = 0.052$ ).

The venous blood Hb-CO concentrations in all patients with COPD at the exacerbations ( $1.02 \pm 0.09\%$ ,  $n = 14$ ) were higher than those at a stable condition ( $p < 0.0001$ ). In contrast, among the patients at the exacerbations, the venous blood Hb-CO did not increase at the severe stage of COPD (data not shown).

### A-v Blood Hb-CO Concentration Differences

To examine the site of endogenous production of CO, we measured the a-v blood Hb-CO concentration differences in patients with COPD. In these patients, the a-v Hb-CO differences at a stable condition ( $0.14 \pm 0.02\%$ ,  $n = 14$ ) did not differ from those in control subjects ( $0.13 \pm 0.03\%$ ,  $n = 16$ ,  $p > 0.7$ ). Furthermore, among the patients at a stable condition, the a-v Hb-CO differences did not increase at the severe stage of COPD (data not shown).

The a-v Hb-CO differences in all patients with COPD at the exacerbations ( $0.16 \pm 0.03\%$ ,  $n = 14$ ) did not differ from those in control subjects ( $p > 0.5$ ; Figure 2). Furthermore, among the patients at the exacerbations, the a-v Hb-CO differences did not increase with severity of COPD (data not shown).

The a-v Hb-CO differences in patients with COPD who had URTIs also did not differ from those in patients without URTIs at the exacerbations (data not shown).

### Exhaled CO Concentrations

Exhaled CO concentrations in patients with COPD at the stable conditions ( $3.5 \pm 0.1$  ppm,  $n = 58$ ) were significantly higher than those in control subjects ( $1.8 \pm 0.1$  ppm,  $n = 61$ ,  $p < 0.0001$ ). In the patients at stage II and stage III, exhaled CO concentrations ( $3.8 \pm 0.2$  ppm,  $n = 38$ ) at the exacerbations were higher than those at the stable conditions ( $p < 0.001$ ). In

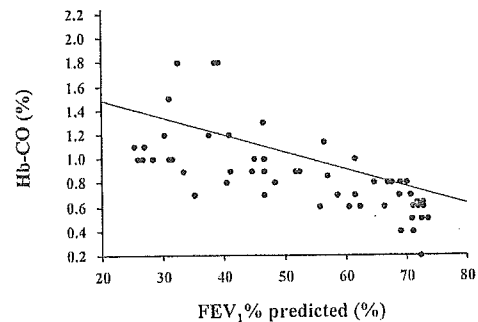


Figure 2. Relationship between the arterial blood Hb-CO concentrations and FEV<sub>1</sub> in patients with COPD ( $n = 54$ ) at the exacerbations ( $p < 0.0001$ ,  $r = -0.70$ ).

contrast, in the patients at stage IV, exhaled CO concentrations at the exacerbations did not differ from those at the stable conditions (data not shown). Exhaled CO concentrations in patients with URTIs ( $4.0 \pm 0.2$  ppm,  $n = 18$ ) were also higher than those in control subjects ( $p < 0.0001$ ) and those in the patients at stable conditions ( $3.2 \pm 0.2$  ppm,  $p < 0.05$ ). Exhaled CO correlated with the Hb-CO in patients at stage II and stage III at the exacerbations ( $n = 38$ ,  $r = 0.530$ ,  $p < 0.001$ ), but did not correlate with the Hb-CO in patients at stage IV at the exacerbations (data not shown).

In patients with COPD at stage III, the exhaled CO concentrations in patients treated with inhaled corticosteroids ( $3.8 \pm 0.5$  ppm,  $n = 4$ ) did not differ from those in patients without inhalation of corticosteroids at the exacerbations ( $4.4 \pm 0.3$  ppm,  $n = 6$ ,  $p > 0.3$ ). Likewise, in patients at stage IV, the exhaled CO concentrations in patients treated with inhaled corticosteroids ( $3.1 \pm 0.8$  ppm,  $n = 5$ ) did not differ from those in patients without inhaled corticosteroids at the exacerbations ( $3.3 \pm 0.6$  ppm,  $n = 12$ ,  $p > 0.8$ ).

### Factors Associated with Arterial Blood Hb-CO Concentrations

In patients with COPD at the exacerbations, arterial Hb-CO concentrations inversely correlated with arterial blood PaO<sub>2</sub> values ( $63.3 \pm 1.4$  mm Hg,  $n = 58$ ;  $r = -0.70$ ,  $p < 0.0001$ ), and correlated with arterial blood PaCO<sub>2</sub> values ( $53.5 \pm 2.1$  mm Hg,  $n = 58$ ;  $r = 0.52$ ,  $p < 0.0001$ ). Likewise, in the patients at the exacerbations, arterial blood Hb-CO concentrations correlated with serum CRP values ( $4.9 \pm 0.6$  mg/dl,  $n = 58$ ;  $r = 0.56$ ,  $p < 0.0001$ ). Arterial Hb-CO concentrations also correlated with serum LPO concentrations ( $5.63 \pm 0.39$  nmol/ml,  $n = 58$ ;  $r = 0.56$ ,  $p < 0.0001$ ). Furthermore, in patients at the exacerbations, the Hb-CO inversely correlated with FEV<sub>1</sub> ( $n = 54$ ,  $r = -0.70$ ,  $p < 0.0001$ ; Figure 2). On the other hand, in all four patients with an Hb-CO of more than 1.4% at the exacerbations, FEV<sub>1</sub> was less than 40%, and in all eight patients with the Hb-CO of more than 1.2% at the exacerbations, FEV<sub>1</sub> was less than 50% (Figure 2).

### DISCUSSION

The present study demonstrated that, at stable conditions, Hb-CO concentrations in patients with COPD were higher than those of control subjects. At stable conditions and exacerbations, the Hb-CO concentrations in patients at stage III were higher than those in patients at stage II, and the Hb-CO concentrations in patients at stage IV were also higher than those in patients at stage III. In patients at stage IV, the Hb-CO changes between stable conditions and exacerbations were higher than those in patients at stage III, and the Hb-CO changes in patients at stage III

were higher than those in patients at stage II. In patients with COPD at the exacerbations, Hb-CO concentrations also inversely correlated with FEV<sub>1</sub> and arterial blood Pa<sub>O<sub>2</sub></sub>. At the exacerbations, FEV<sub>1</sub> was less than 40% in all four patients with the Hb-CO concentrations of more than 1.4%, and FEV<sub>1</sub> was less than 50% in all eight patients with Hb-CO concentrations of more than 1.2%. These findings suggest that measurement of Hb-CO may be a useful marker of severity, especially to define the severe exacerbations of COPD, although there is an overlap of the Hb-CO concentrations between control subjects and patients with COPD and between patients with COPD at stable conditions and exacerbations. The Hb-CO concentrations also correlated with serum values of CRP and LPO, suggesting that the Hb-CO concentrations might be also associated with the lung and airway inflammation and production of reactive oxygen species in the patients.

Smoking history in control subjects differed from that in patients with COPD in the present study, although all control subjects and all patients with COPD were ex-smokers. To avoid the effects of ambient CO from smoking on Hb-CO and exhaled CO levels, we excluded current smokers by measuring urinary cotinine concentrations (41), and we confirmed that all subjects had not smoked for at least 3 months before the samplings. Furthermore, none of the control subjects and patients with COPD was a passive smoker. On the other hand, the Hb-CO concentrations in control subjects with a smoking history of more than 60 pack-years, which was the average smoking history of patients with COPD in this study, did not differ from the Hb-CO concentrations in the control subjects with a smoking history of less than 60 pack-years. These findings suggest that a different smoking history did not influence the Hb-CO in the control subjects.

Increased Hb-CO in patients with COPD in this study is consistent with that observed in previous studies in patients with bronchial asthma, pneumonia, and interstitial lung disease (27, 28), in which increased Hb-CO might relate to the inflammation in airway and/or lung parenchyma. At stable conditions in this study, airway inflammation (5) and production of reactive oxygen species (15) might be associated with increased Hb-CO (23) in patients with COPD. Airway inflammation caused by infection of bacteria and viruses (10, 11, 25) might further increase endogenous CO production in patients with COPD at exacerbations.

In patients with COPD at the exacerbations caused by bacterial and viral infections, Pa<sub>O<sub>2</sub></sub> and FEV<sub>1</sub> decrease and Pa<sub>CO<sub>2</sub></sub> concentrations increase (10, 11). At the exacerbations in this study, 18 of 55 patients (31%) represented the symptoms of URTI, 13 patients (24%) had acute bronchitis with purulent sputum, and six patients (11%) had pneumonia. The Hb-CO concentrations might be increased through the production of proinflammatory cytokines and NO (9, 23, 42–44) in airway virus infection, and through reactive oxygen species (15, 23) from neutrophils (8) in bacterial infection. These factors might also relate to the increased CRP and LPO levels (45, 46).

Systemic inflammation and production of reactive oxygen species in the organs, including muscles, have been reported in patients with COPD (30, 31). To examine the site of endogenous production of CO, we measured the a-v Hb-CO difference, which is a better way to define the site of inflammation, in the lung or other organs, in patients with bronchial asthma and pneumonia (28). In patients with COPD, the a-v Hb-CO differences at stable conditions and exacerbations did not differ from those in control subjects. Furthermore, among the patients with COPD at stable conditions, the a-v Hb-CO differences in patients were not increased in the severe stage of COPD. The loss of a-v Hb-CO differences in the patients in the present study suggests that CO might be also produced in organs other than the lung, such as

muscles (30). On the other hand, Hb-CO inversely correlated with FEV<sub>1</sub> in the patients at the exacerbations, suggesting that lung and airway might be also the site of CO production.

The estimation of Hb-CO from exhaled CO measurements is suggested to be inaccurate in patients with severe airflow obstruction (47), and Hb-CO might be increased in patients with severe airflow obstruction because of reabsorption of CO (47). In fact, in patients with COPD at stage IV, exhaled CO concentrations at the exacerbations were lower than those at stable conditions. Therefore, we performed direct measurement of Hb-CO concentrations in the present study. Furthermore, increased exhaled CO concentrations were associated with increased Hb-CO concentrations in patients at the exacerbations in patients at stage II and stage III. These findings suggest that increased Hb-CO concentrations might represent the elevated endogenous CO production, although the influence of reabsorption of CO on Hb-CO was not excluded.

We previously reported that arterial Hb-CO is correlated with FEV<sub>1</sub> in asthma exacerbations (28). This study also demonstrated that the Hb-CO concentrations inversely correlated with FEV<sub>1</sub> in patients with COPD. These findings suggest that airway narrowing might relate to the increased Hb-CO concentrations. However, many mechanisms have been reported to be associated with COPD exacerbations, including mucus hypersecretion, airway edema, and bronchoconstriction (48), all of which cause airway narrowing. On the other hand, CRP values in patients with COPD at exacerbations in this study were higher than those in patients with bronchial asthma in the previous study (27). The stronger inflammation in patients with COPD might be associated with the greater Hb-CO concentrations in patients with COPD compared with those in patients with bronchial asthma (11). However, we could not estimate the effect of bronchoconstriction on Hb-CO in this study, because we did not measure the changes in FEV<sub>1</sub> after inhalation of anticholinergic drugs or  $\beta$ -adrenergic stimulants.

In the present study, Hb-CO concentrations in patients with COPD receiving inhaled corticosteroids did not differ from those in patients with COPD without inhalation of corticosteroids. Inhaled corticosteroids might fail to inhibit airway inflammation in these patients with severe COPD with frequent exacerbations, as demonstrated in patients with severe asthma (49).

Exhaled CO levels are reported to be low in patients deficient in  $\alpha$ 1-antitrypsin (41). However, we did not examine the relationship between  $\alpha$ 1-antitrypsin levels and Hb-CO levels, because  $\alpha$ 1-antitrypsin deficiency in patients with COPD is very rare in the Japanese population (50).

In summary, we have demonstrated that arterial Hb-CO concentrations increased in patients with COPD at a stable condition compared with those in normal control subjects. The Hb-CO concentrations at the exacerbations were significantly higher than those at stable conditions. The patients needed to stop breathing for 20 seconds to measure the exhaled CO concentrations. This procedure was difficult to repeat for the patients with COPD with severe dyspnea. The Hb-CO concentrations can be measured at the same time as the blood gas analysis, and should be measured first at a medical examination. The measurement of arterial Hb-CO concentration may be a simple and valuable marker to monitor the severity of systemic and lung inflammation and disease activities in patients with COPD.

**Conflict of Interest Statement:** H.Y. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript; M.Y. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript; K.N. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript; S.E. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript; T.S. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript; S.O. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

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## 4. 高齢者呼吸器感染症予防の新戦略—ワクチン療法を中心として

大類 孝 中山 勝敏 福島 健泰 千葉 大 佐々木英忠

〈要約〉 呼吸器感染症の中で肺炎は、抗菌剤の開発がめざましい今日においても、日本での疾患別死亡率の第4位を占めている。また、肺炎による死亡者を年齢別に見ると、65歳以上の高齢者が全体に占める割合は約9割と極めて高い。近年、高齢者の肺炎は複雑化し難治例が増加しつつあるといわれる。その背景には高齢化社会を迎え、さまざまな基礎疾患を抱えた易感染状態の患者が増加している点や、加齢に伴う免疫能の低下によって弱毒性の病原微生物によっても肺炎を発症しうる点などが挙げられる。よって、高齢者特に寝たきり高齢者の免疫状態を把握し、ワクチン等を用いて免疫を賦活化させ感染防御能を高める方法は、高齢者の感染症、特に肺炎の予防に有効ではないかと考えられる。本シンポジウムで、私は、初めに寝たきり高齢者の免疫能について言及し、さらにこれらの対象者におけるインフルエンザワクチン、肺炎球菌ワクチンおよびBCGワクチンの効果について解説する。

Key words：寝たきり高齢者、ツベルクリン反応、肺炎球菌ワクチン、BCGワクチン、インフルエンザワクチン  
(日老医誌 2005；42：34—36)

## 寝たきり高齢者における細胞性免疫と液性免疫

一般に免疫能はTh1リンパ球によりマクロファージ等が活性化される細胞性免疫と、Th2リンパ球により抗体産生が賦活化される液性免疫に分けて解析されることが多い<sup>2)</sup>。日常診療のなかで、とくに重要な抗体産生能（液性免疫の指標）としてインフルエンザワクチンに対する抗体産生能があげられる。また高齢者診療のなかで、簡便に評価できる細胞性免疫の指標としてツベルクリン皮内反応があげられる。そこでインフルエンザワクチンに対する抗体産生能、ツベルクリン皮内反応、CD4陽性リンパ球サブセット分析を用いて、ADLの違いによる比較検討を行った。その結果、寝たきり高齢者では、インフルエンザワクチンに対する抗体価上昇は若年者および健康高齢者と比較して差を認めず、液性免疫は維持されていることが確認された（図1）<sup>2)</sup>。一方、ツベルクリン皮内反応はADL低下群で有意に発赤最大径が短く、細胞性免疫の低下が示唆された。実際に末梢血中のTh1、Th2リンパ球のサブセット数を調べてみると、Th1リンパ球数においてはADL低下群が有意な低下を示したが、Th2リンパ球数はADLにより有意な差はみられなかった（図2）<sup>3)</sup>。

インフルエンザワクチン接種による  
症状軽減効果

以上のことから、ADLの低下している高齢者でも、インフルエンザワクチンはインフルエンザの罹患率あるいはその感染による症状を軽減しうる事が期待される。そこで、ADLの低下した高齢者を2群に分け、一方に対してインフルエンザワクチンを接種し、他方には接種せず、一冬でのインフルエンザ症状の発生状況（症状および期間）を比較してみた（図3）<sup>4)</sup>。その結果、発熱・呼吸器症状においてワクチン施行群で有意な減少を示した。以上のように、ADL低下高齢者においても、インフルエンザワクチン接種は症状軽減の点において有効であることが示された。

## ツベルクリン皮内反応による肺炎リスク評価

前述のように、ADLと液性・細胞性免疫能の解析において、ADLの低下によっても液性免疫能は大きく低下しないが、細胞性免疫能が低下することが示された。さらに、これまでの報告によると、ADLが低下している高齢者で肺炎による死亡率が高いことが知られている。以上を考え合わせると、ADL低下高齢者においては細胞性免疫の低下が肺炎の危険因子になっている可能性がある。そこで、ADL低下高齢者にツベルクリン皮内反応検査を行い、陽性の群と陰性の群における肺炎の罹患率をみてみた。その結果、ツベルクリン皮内反応陰性者のほうが陽性者よりも肺炎に罹患する率が高かった（図4）<sup>5)</sup>。このことから、ADL低下高齢者においてはツ

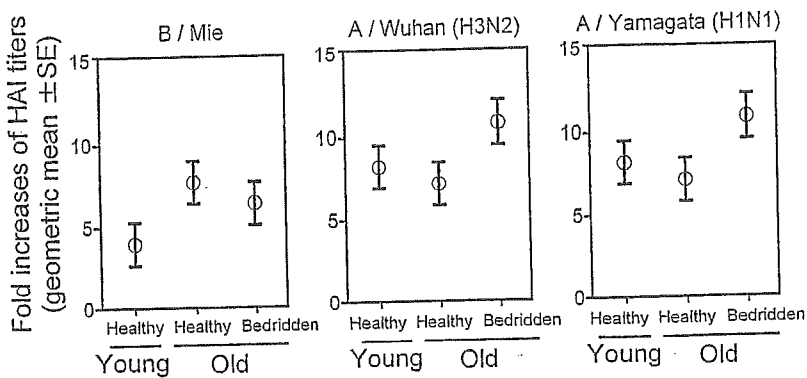


図1 インフルエンザワクチンに対する抗体価上昇率  
寝たきり高齢者におけるインフルエンザワクチンに対する抗体価上昇率は、若年者および健康高齢者と比較して差を認めず、液性免疫は維持されている (文献2より引用)。

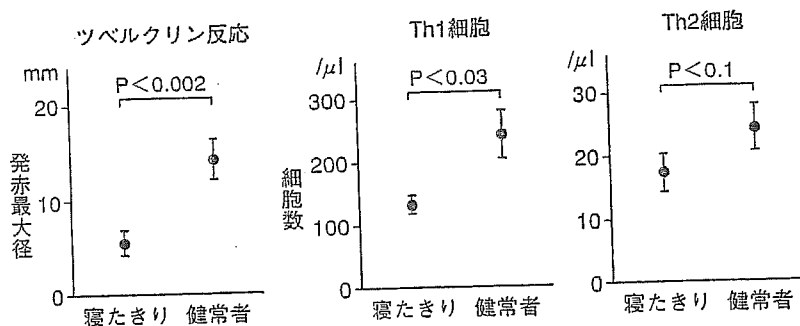


図2 末梢血中の Th1, Th2 リンパ球のサブセット数  
Th1 リンパ球数は ADL 低下群が有意な減少を示したが、Th2 リンパ球数は ADL により有意な差はみられなかった (文献3より引用)。

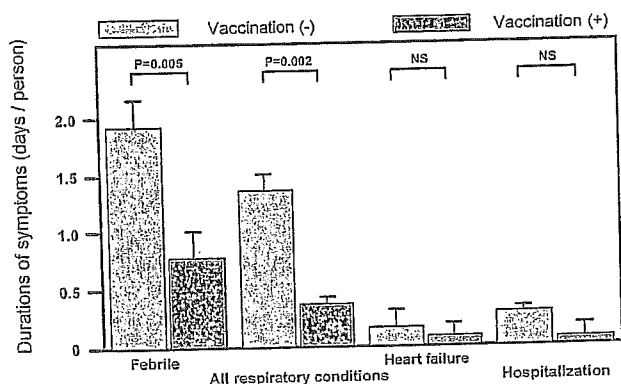


図3 インフルエンザ症状に対するインフルエンザワクチンの効果  
発熱・呼吸器症状において、ワクチン投与群で有意な減少を示した (文献4より引用)。

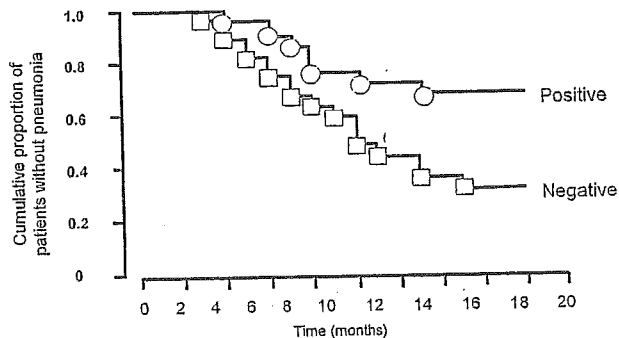


図4 ツベルクリン反応陽性者および陰性者における肺炎罹患率  
ツベルクリン皮内反応陰性者のほうが陽性者よりも肺炎に罹患する率が高かった (文献5より引用)。

ベルクリン皮内反応という簡単な検査によって、肺炎のリスクが評価できる可能性が示された。

### 肺炎球菌ワクチン投与による高齢者肺炎の予防

近年、欧米での大規模臨床研究により、高齢者に対す

る肺炎球菌ワクチンの有用性が明らかにされつつあるが、わが国における研究成果は皆無に等しい。我々は、無作為、ランダム化、前向き研究により ADL の低下した高齢者に対する肺炎球菌ワクチンの有用性について明らかにした<sup>6)</sup>。対象は、高齢者介護施設に入所中の寝たきり高齢者 294 名 (平均年齢 81 歳、男性 70 人) で、本

人および家族の同意を得た上で、無作為にワクチン投与群および非投与群に分割した。ワクチン投与群には、肺炎球菌ワクチン（ニューモバックス）0.5mLを皮下注射し、その後、両群間で1年間における発熱状況、他病院への入退院の有無、生命予後につき比較検討をした。その結果、ワクチン投与群では非投与群に比して総発熱日数の有意な減少（平均±標準誤差：3.7±0.5日/人/年 vs 6.6±0.8日/人/年， $p=0.002$ ），および肺炎による入院回数の有意な減少（0.23±0.04回/人/年 vs 0.46±0.06回/人/年， $p=0.0006$ ）を認めた。しかし、両群間で、肺炎および敗血症による死亡率には有意差を認めなかった<sup>6)</sup>。結論として、寝たきり高齢者における肺炎球菌ワクチンの投与は有効で、今後、これらの方々にはワクチン投与を積極的に推奨すべきと考えられた。

### BCG ワクチン投与による高齢者肺炎の予防

前述のように、ADL低下高齢者においては細胞性免疫の低下が肺炎の危険因子になっている可能性がある。そこで、我々は細胞性免疫賦活化作用を有するBCGワクチン接種が、寝たきり高齢者における肺炎の発症を予防し得るか否かについて検討を行った。方法は、高齢者介護施設に入所中のADLの低下した155名の高齢者を対象とし、ツ反を施行し陽性群及び陰性群に分け、さらに陰性群を無作為にBCG接種群及び非接種群に割り付けをした。そして、BCG接種4週間後に再びツベルクリン反応を施行し、陽性者を陽転群とし、その後2年間にわたり各群における肺炎の発症率を前向きに追跡調査した。その結果、ツ反陰性群では44名中19名（42%）に、陽転群では41名中6名（15%）に、ツ反陽性群では67名中9名（13%）に新たな肺炎の発症が確認され、ツ反陽転群では陰性群に比して肺炎の発症率が有意に抑制された（ $p=0.03$ ）<sup>7)</sup>。以上の結果より、BCG接種は細胞性免疫の低下した寝たきり高齢者において、肺炎発症

の予防効果を有する事が明らかにされた。

### おわりに

高齢者の肺炎は、要介護老人が死亡する直前に罹患するもので、それ以外の人には無縁と思われてきた。しかし、近年、MRIによる脳ドック検診の普及に伴い、65歳以上の健常人の約半数に、大脳基底核のロイコアライオーシスなどの脳虚血所見が認められると報告されており、このような人では大脳基底核のドーパミンの減少があり、肺炎発症の可能性が高いと考えられる<sup>8)</sup>。高齢者肺炎は、日本のような高齢化社会ではより身近な疾患であり、医療従事者の努力によってかなりの程度予防が可能である。

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### Abstract

#### Prevention of elderly pneumonia by pneumococcal, influenza and BCG vaccinations

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Pneumonia is a major cause of morbidity and mortality in elderly people, especially in those with chronic medical conditions such as chronic heart and lung diseases. We prospectively examined the effect of influenza and pneumococcal vaccinations on the rate of hospitalization for and complications of pneumonia, all respiratory tract conditions and mortality in elderly bedridden patients and found that both febrile days and pneumonia cases decreased. Thus, these results show that it is valuable to vaccinate for influenza elderly people even if they are confined to bed. Furthermore, the tuberculin skin test is an easy method to check the cell-mediated immunity in the elderly people. In the tuberculin skin test, all Japanese over 65 years old should have positive status. A negative result indicates depressed cell-mediated immunity. We undertook a trial to vaccinate tuberculin negative elderly people with BCG vaccine and found that the risk of pneumonia is decreased to a similar degree to that in subjects with positive tuberculin test results. We conclude that vaccination might be an effective strategy for the prevention of pneumonia in elderly people with limited activities of daily living.

**Key words:** Elderly pneumonia, Influenza vaccine, Pneumococcal vaccine, BCG vaccine  
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## Association of susceptibility to the development of lung adenocarcinoma with the heme oxygenase-1 gene promoter polymorphism

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**Abstract** Heme oxygenase-1 (HO-1) acts in cytoprotection against oxidants and aromatic hydrocarbons in cigarette smoke. A (GT)<sub>n</sub> dinucleotide repeat in the 5'-flanking region of the human *HO-1* gene (alias *HMOX1*) reduces HO-1 inducibility and shows length polymorphism, which is grouped into three classes: class S (<27 GT), class M (27–32 GT), and class L (≥33 GT) alleles. To investigate the correlation between the *HO-1* gene polymorphism and the development of lung adenocarcinoma, we screened 151 Japanese patients with lung adenocarcinoma and 153 control subjects. Patients and control subjects were frequency-matched by age, gender, smoking history and proportion of chronic pulmonary emphysema. The proportion of class L allele frequencies, as well as that of genotypic frequencies in L allele carriers (LL, LM, and LS), were significantly higher in patients with lung adenocarcinoma than those of control subjects. The adjusted odds ratio (OR) for lung adenocarcinoma with class L allele vs non-L allele (M + S) was 1.6 [95% confidence interval (CI) 1.0–2.5, *P*=0.03] and that with L allele carriers vs. non-L allele carriers was

1.8 (95% CI 1.1–3.0, *P*=0.02). Furthermore, the risk of lung adenocarcinoma for L allele carriers versus non-L allele carriers was much increased in the group of male smokers (OR=3.3, 95% CI 1.5–7.4, *P*=0.004). However, in the female non-smokers, the proportion of L allele carriers did not differ between patients and control subjects (OR=0.93, 95% CI 0.4–2.0, *P*=0.85). These findings suggest that the large size of a (GT)<sub>n</sub> repeat in the *HO-1* gene promoter may be associated with the development of lung adenocarcinoma in Japanese male smokers.

### Introduction

Development of lung cancer is associated with exposure to cigarette smoke, and the risk increases with the amount smoked (Loeb et al. 1984; Doll et al. 1994). Cigarette smoke contains both a gas phase and particulate matter, including polycyclic aromatic hydrocarbons and nitrosamines, which are cytotoxic, genotoxic and carcinogenic for lung cancer, including squamous cell lung carcinoma and adenocarcinoma (Loeb et al. 1984; Osann 1998). Both the gas phase and particulate matter also contain high concentrations of oxidants and free radicals, which induce DNA damage and are suggested to play an important role in the development of lung cancer (Yoshie et al. 1997; Feig et al. 1994). Although lung adenocarcinoma has been the predominant type in women and non-smoking men (Charloux et al. 1997), it is increasingly associated with cigarette smoking (Franceschi and Bidoli 1999). Activation of carcinogens is suggested in smokers who lack the glutathione S-transferase (GST) isozyme *GSTM1* gene (Nakajima et al. 1995). Although the relation between the gene polymorphism of anti-oxidant enzymes and lung cancer has been studied, including polymorphisms

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in *GST* genes (Harrison et al. 1997), the roles of reduced expression of anti-oxidant enzymes on the development of lung cancer are still uncertain.

Heme oxygenase (HO) oxidatively degrades heme to biliverdin, which is subsequently reduced to bilirubin, an efficient scavenger of reactive oxygen species (Stocker et al. 1987), by biliverdin reductase (Maines 1997; Shibahara 2003). HO-1, an inducible form of HO, provides cellular protection against heme- and non-heme-mediated oxidant injury (Maines 1997; Choi and Alam 1996; Yamada et al. 1999; Shibahara 2003). Likewise, an exogenous administration of HO-1 in the rat lung by gene transfer was shown to protect against injury caused by hyperoxia (Otterbein et al. 1999). These lines of evidence support that HO-1 is an essential component for the lung to protect against reactive oxygen species. Furthermore, HO-1 is suggested to act in cytoprotection against benzo(a)pyrene (Li et al. 2000), a potent carcinogen (Loeb et al. 1984).

A (GT)<sub>n</sub> repeat is the most frequent of the simple repeats scattered throughout human (Kimpapa et al. 1997) and animal genomes (Naylor and Clark 1990), and many of these exhibit length polymorphism (Kimpapa et al. 1997). This purine-pyrimidine alternating sequence negatively affects transcriptional activity in rat prolactin genes (Naylor and Clark 1990). A (GT)<sub>n</sub> repeat lying in the 5'-flanking region of the human *HO-1* gene (also known as *HMOX1*) also modulates gene transcription induced by hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) (Yamada et al. 2000, 2001). Thermal stress is also suggested to modulate HO-1 inducibility (Okinaga et al. 1996). We have recently demonstrated that the size of (GT)<sub>n</sub> repeat in the *HO-1* gene (X14782) is associated with susceptibility to chronic pulmonary emphysema (CPE), one of the oxidative stress-inducing diseases, in Japanese populations. Furthermore, the expression of the *HO-1* gene alters according to the number of (GT)<sub>n</sub> repeats in A549

transformed human lung epithelial cells and the Hep3B human hepatoma cell line (Yamada et al. 2000, 2001).

In the present study, we examined the contribution of 5'-flanking polymorphism in the *HO-1* gene in the development of lung adenocarcinoma by reason that there is an association between reactive oxygen species and the pathogenesis of lung cancer, including lung adenocarcinoma (Yoshie and Ohshima 1997; Feig et al. 1994; Franceschi and Bidoli 1999). We screened allelic frequencies of the (GT)<sub>n</sub> repeats in the *HO-1* gene promoter from subjects with and without lung adenocarcinoma and examined the association between the development of lung adenocarcinoma and length of the (GT)<sub>n</sub> repeats. We also studied the association between the development of lung squamous cell carcinoma and length of the (GT)<sub>n</sub> repeats.

## Subjects and methods

### Clinical protocol and patients characteristics

We studied 151 patients with lung adenocarcinoma and 153 control subjects without lung cancer attending the Tohoku University Hospital and Sendai Kousei Hospital. The patients were enrolled consecutively. Physical characteristics, smoking history, complications in patients with lung adenocarcinoma and control subjects are shown in Table 1. The number of lung adenocarcinoma patients in each stage was 104 in stage I, 12 in stage II, 20 in stage III, and 15 in stage IV. One hundred and thirty-nine of 151 patients with lung adenocarcinoma had received surgical operation. Smoking histories were recorded in pack years of exposure, and the median pack years for the smoking control subjects and for the smoking lung cancer subjects were 41.2 and 43.6. CPE was defined as a physical examination that demonstrated

**Table 1** Characteristics of the subjects

Details	Control subjects (n = 153)	Lung adenocarcinoma patients (n = 151)	P
Age (years) <sup>a</sup>	62.9 (9.9)	62.8 (9.8)	0.95 <sup>b</sup>
Gender (%)			
Male	82 (53.6)	80 (53.0)	0.91 <sup>c</sup>
Female	71 (46.4)	71 (47.0)	
Smoking history (%)			
Current smoker	62 (40.5)	63 (41.7)	0.69 <sup>c</sup>
Ex-smoker	7 (4.6)	10 (6.6)	
Non-smoker	84 (54.9)	78 (51.7)	
Disorders (%)			
Pulmonary emphysema	13 (8.5)	13 (8.6)	0.97 <sup>c</sup>
Bronchial asthma	10 (6.5)	4 (2.6)	0.11 <sup>c</sup>
Lung fibrosis	4 (2.6)	4 (2.6)	0.98 <sup>c</sup>
Hypertension	39 (25.5)	58 (38)	0.02 <sup>c</sup>
Diabetes mellitus	19 (12.4)	18 (12)	0.89 <sup>c</sup>
Hyperlipidemia	28 (18.3)	13 (8.6)	0.01 <sup>c</sup>
Cardiovascular	30 (19.6)	14 (9.3)	0.01 <sup>c</sup>
Cerebrovascular	5 (3.3)	13 (8.6)	0.05 <sup>c</sup>
Others	30 (19.6)	22 (15)	0.24 <sup>c</sup>

<sup>a</sup>Mean (SD)

<sup>b</sup>Unpaired *t*-test

<sup>c</sup>Chi-square test

hyperresonant chest and flattened hemidiaphragms; a chest roentgenogram that demonstrated hyperinflation, flattened diaphragms, and marked loss of vascularity; a computed-tomography scan that demonstrated areas of low attenuation; and pulmonary-function testing that demonstrated decreased FEV<sub>1</sub>:FVC ratios and impaired diffusion capacity (Celli et al. 1995).

We recruited 512 healthy subjects at a health screening before the selection of control subjects. In order to recruit healthy subjects, all the subjects were interviewed on past history and present illness, and their daily life style was evaluated. Before the matching of age, gender, smoking history and the proportion of CPE, the subjects who had obvious symptoms or disabilities were excluded. Subjects with good control in response to treatment for chronic diseases such as hypertension, diabetes mellitus, and hyperlipidemia were included. As a result, we recruited 512 healthy subjects before the selection of control subjects. To perform a case-control study, we randomly selected the 153 control subjects in a frequency-matched manner from the 512 healthy individuals. They were frequency-matched on age ( $\pm 5$  years), gender, smoking history, and the frequency of CPE in control subjects to those of patients with lung adenocarcinoma (Table 1).

All the subjects with and without lung cancer were Japanese. The study was approved by the Tohoku University Ethics Committee and Sendai Kousei Hospital Ethics Committee, and informed consent was obtained from each subject. This study was done between June 2000 and March 2003.

#### Analysis of length variability of (GT)<sub>n</sub> repeats in the *HO-1* gene promoter

Genomic DNAs were extracted from leukocytes in peripheral venous blood by conventional procedures. The 5'-flanking region containing a poly(GT)<sub>n</sub> repeat of the *HO-1* gene was amplified by polymerase chain reaction (PCR) (Kimpura et al. 1997; Okinaga et al. 1996) with a fluorescently labeled primer p1-s (5'-AGAGCCTGCAGCTTCTCAGA-3') and an unlabeled anti-sense primer p1-as (5'-ACAAAGTCTGGCCATAGGAC-3'), which were designed according to the published sequence (Shibahara et al. 1989; Yamada et al.

2000, 2001). The PCR was performed over 30 cycles of 20 s at 94°C, 10 s at 60°C, and 20 s at 72°C. The sizes of the PCR products were analyzed using cloned alleles with repeat numbers sequenced with the ABI prism dye terminator sequencing kit (Perkin-Elmer Applied Biosystems, Foster City, Calif., USA) (Yamada et al. 2001). In usual cases, a blood sample has two different sizes of (GT)<sub>n</sub> repeats from the two different alleles. Each repeat number was calculated with ALFwin Fragment Analyser version 1.03 (Amersham Pharmacia Biotech, Piscataway, N.J., USA) with four cloned alleles as size markers in the DNA sequencer (ALF express II DNA Sequencer version 2.2, Amersham Pharmacia Biotech). The repeat numbers of these cloned alleles used as size markers were 16, 23, 29, and 38, respectively. The investigators of genetic analysis were blinded with respect to the status of the subjects.

#### Statistical analysis

In the present study, the patient group and the control group were frequency-matched by age, gender, smoking history and proportion of CPE. The number of patients differed from that of control subjects (Table 1). Therefore, the matched pair design was not followed in this analysis. We used  $\chi^2$  statistics to examine the differences in gender, smoking history and the frequency of the complications including CPE between cases and controls (Table 1) as described previously in coronary artery disease by Chen et al. (2002), and in lung cancer by Wang et al. (2003). Odds ratios (ORs) and their 95% confidence intervals (CIs) were calculated to assess the relative risk conferred by particular allele and genotype, and adjusted for age, sex, and smoking history, and CPE by means of unconditional logistic regression as described previously (Wang et al. 2003) (Table 2). We also used unconditional logistic regression analysis to examine the risk of lung adenocarcinoma for L-allele carriers in male smokers. All of the statistical analyses were performed using SYSTAT (version 10.2; SYSTAT Software, Richmond, Calif., USA). The values for age are reported as mean  $\pm$  SD. Statistical analysis of age was performed by the unpaired *t*-test (Table 1). The *HO-1* genotype distributions were in Hardy-Weinberg equilibrium. Significance was accepted at  $P < 0.05$ .

**Table 2** Allele and genotype frequencies at the polymorphic locus in patients with lung adenocarcinoma and control subjects

	Control subjects ( <i>n</i> = 153)	Lung adenocarcinoma patients ( <i>n</i> = 151)	Odds ratio (95% CI)	<i>P</i>
Allele class	<i>n</i> = 306	<i>n</i> = 302		
L	39 (13%)	58 (19%)	1.6 (1.0–2.5) <sup>a</sup>	0.03
M + S	267 (87%)	244 (81%)	1.0	
Genotype subgroup	<i>n</i> = 153	<i>n</i> = 151		
L-allele carriers	36 (24%)	54 (36%)	1.8 (1.1–3.0) <sup>b</sup>	0.02
Non-L-allele carriers	117 (76%)	97 (64%)	1.0	

<sup>a</sup>Odds ratios were calculated with the non-L class (M + S) as the reference group and adjusted for age, sex, smoking history, and CPE

<sup>b</sup>Odds ratios were calculated with the non-L allele carriers as the reference group and adjusted for age, sex, smoking history, and CPE

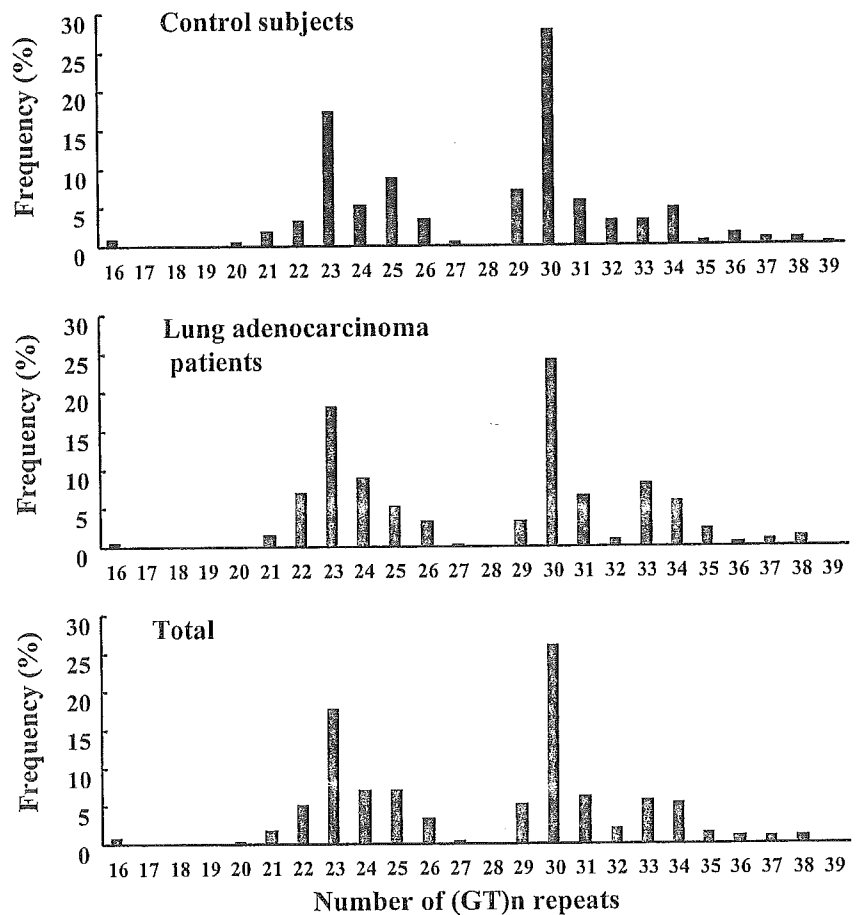
## Results

### Allele frequencies in control and patients with lung adenocarcinoma

The numbers of (GT)<sub>n</sub> repeats in the human *HO-1* gene were distributed between 16 and 39 in the subjects studied (Fig. 1). The distribution of the numbers of (GT)<sub>n</sub> repeats was trimodal, as previously reported (Yamada et al. 2000, 2001; Yamaya et al. 2003), with two main peaks located at 23 and 30 GT repeats and another peak located at 33 GT repeats. Therefore, we divided the alleles into three subclasses, as previously reported (Yamada et al. 2000, 2001): class S (< 27 repeats), class M (27–33 repeats), and class L (≥33 repeats) alleles.

In the control subjects, distribution of 306 alleles were 130 (42%) for class S, 137 (45%) for class M and 39 (13%) for class L, respectively. Likewise, in patients with lung adenocarcinoma, distributions of 302 alleles were 136 (45%) for class S, 108 (36%) for class M and 58 (19%) for class L, respectively. The proportion of allelic frequencies in class L was significantly higher in all patients with lung adenocarcinoma ( $n=58$ , 19%) than that in class L in all control subjects ( $n=39$ , 13%) ( $P=0.03$ ). The adjusted OR for lung adenocarcinoma with L allele was 1.6 (95% CI 1.0–2.5) (Table 2), compared with non-L allele (class M allele + class S allele).

**Fig. 1** Frequency distribution of the numbers of (GT)<sub>n</sub> repeats in control subjects (Control subjects,  $n=153$ ), patients with lung adenocarcinoma (Lung adenocarcinoma patients,  $n=151$ ), and total subjects including control subjects and patients (Total,  $n=304$ )



### Genotypic frequencies in control and patients with lung adenocarcinoma

Six genotypes (L/L, L/M, L/S, M/M, M/S and S/S) of (GT)<sub>n</sub> repeats in the human *HO-1* gene promoter were divided into two subgroups according to allelic subclasses: L allele carriers with a class L allele (L/L, L/M, L/S) and non-L allele carriers without a class L allele (M/M, M/S and S/S) (Yamada et al. 2000, 2001; Yamaya et al. 2003).

The proportion of genotypic frequencies in L allele carriers was significantly higher in patients with lung adenocarcinoma ( $n=54$ , 36%) than that in control subjects ( $n=36$ , 24%) ( $P=0.02$ ). The adjusted OR for lung adenocarcinoma with L-allele carriers was 1.8 (95% CI 1.1–3.0) (Table 2) compared with non-L allele carriers.

### Factors associated with genotypic frequencies in patients with lung adenocarcinoma

The *HO-1* gene is inducible by stimulation with reactive oxygen species such as H<sub>2</sub>O<sub>2</sub>, and cigarette smoke contains many reactive oxygen species. On the other hand, L-allele carriers regarding the *HO-1* gene showed susceptibility to reduced longevity and CPE in Japanese

male people (Yamaya et al. 2003; Yamada et al. 2000, 2001). Therefore, the analysis of each risk of lung adenocarcinoma for L-allele carriers in male smokers, male non-smokers, female smokers, or female non-smokers, is important. However, the numbers of the subgroup were small to be tested separately. Especially, the numbers of the subjects in male non-smokers and female smokers were far too small (34 subjects and 14 subjects, respectively). Therefore, we analyzed the adjusted risks of lung adenocarcinoma for L allele carriers in male smokers and female non-smokers. In the 128 male smokers, the proportion of genotypic frequencies in L allele carriers was significantly higher in patients with lung adenocarcinoma ( $n=28$ , 43%) than that in control subjects ( $n=12$ , 19%) ( $P=0.004$ ). The adjusted OR for lung adenocarcinoma with L allele carriers vs non-L allele carrier was 3.3 (95% CI 1.5–7.4). On the other hand, in the 128 female non-smokers, the proportion of genotypic frequencies in L-allele carriers did not differ between patients with lung adenocarcinoma and control subjects ( $n=19$ , 30% vs  $n=21$ , 32%;  $P=0.85$ ).

#### *HO-1* gene polymorphism in lung squamous cell carcinoma

We also studied the risk of lung squamous cell carcinoma for L allele carriers of the *HO-1* gene. One hundred and eight patients with lung squamous cell carcinoma, and 100 control subjects attending the Tohoku University Hospital and Sendai Kousei Hospital were enrolled to this study. The control subjects were selected in a frequency-matched fashion by age, gender, smoking history, and the frequency of CPE from 512 healthy subjects, like the study in patients with lung adenocarcinoma. The number of patients with lung squamous cell carcinoma in each stage was six in stage 0, 42 in stage I, 18 in stage II, 36 in stage III, and six in stage IV. Eighty-seven of 108 patients with lung squamous cell carcinoma had received surgical operation. In patients with lung squamous cell carcinoma, distributions of 216 alleles were 94 (44%) for class S, 90 (41%) for class M and 32 (15%) for class L, respectively. In the control subjects, distributions of 200 alleles were 82 (41%) for class S, 90 (45%) for class M and 28 (14%) for class L, respectively. In contrast to patients with lung adenocarcinoma, the proportion of allelic frequencies in class L in patients with lung squamous cell carcinoma ( $n=32$ , 15%) did not differ from that in class L in all control subjects ( $n=28$ , 14%) ( $P=1.0$ ). The adjusted OR for lung squamous cell carcinoma with L allele vs non-L allele (class M + class S) was 1.1 (95% CI 0.6–1.9). Furthermore, in patients with lung squamous cell carcinoma, the proportion of genotypic frequencies in L allele carriers ( $n=30$ , 28%) did not differ from that in control subjects ( $n=26$ , 26%) ( $P=0.8$ ). The adjusted OR for lung squamous cell carcinoma with L-allele carriers vs non-L-allele carrier was 1.1 (95% CI 0.6–2.0).

#### Discussion

We show in the present study that the proportion of both allelic frequencies and the genotypic frequencies regarding large (GT)<sub>n</sub> repeats are higher in patients with lung adenocarcinoma compared with those in control subjects. Furthermore, in the male smokers, the proportion of L allele carriers was significantly higher in patients than that in control subjects. However, in the female non-smokers, the proportion of L allele carriers did not differ between patients and control subjects. These findings suggest that the microsatellite polymorphism in the *HO-1* gene promoter may be associated with the development of lung adenocarcinoma in male Japanese smokers. Because it has been reported that the class L allele is associated with the susceptibility to reduced longevity and CPE in Japanese males (Yamaya et al. 2003; Yamada et al. 2000, 2001) and coronary artery disease in diabetic patients (Chen et al. 2002), we analyzed the risk of lung adenocarcinoma for L allele in the *HO-1* gene.

A (GT)<sub>n</sub> dinucleotide repeats in the 5'-flanking region of human *HO-1* gene shows length polymorphism (Kimpara et al. 1997; Yamada et al. 2000, 2001). This purine-pyrimidine alternating sequence, possessing Z-conformation potential, negatively affects transcriptional activity in the rat prolactin gene (Naylor et al. 1990). The promoter activity of *HO-1* is modulated by the length variability of the (GT)<sub>n</sub> repeats, and large (GT)<sub>n</sub> repeats have a potent inhibitory activity on the *HO-1* gene expression by reactive oxygen species (Yamada et al. 2000, 2001). We have also previously reported the influence of the numbers of the (GT)<sub>n</sub> repeats on the inducibility of the *HO-1* gene promoter under oxidative stimulus by transient transfection assay in the A549 human type II lung cell line and the Hep3B human hepatoma cell line (Yamada et al. 2000, 2001). Significant induction of the *HO-1* promoter/*luciferase* fusion gene by H<sub>2</sub>O<sub>2</sub> was observed only when the fusion gene carried the class S allele, whereas the expression of the fusion gene carrying the class L allele remained at a control level during H<sub>2</sub>O<sub>2</sub> exposure. Furthermore, basal expression of the fusion gene with the class S allele was higher than that of the gene with the class L allele in Hep3B cells (Yamada et al. 2000, 2001). Epstein-Barr virus-transformed lymphoblastoid cell lines (LCLs) established from a patient with *HO-1* deficiency (Yachie et al. 1999) and cultured embryonic fibroblasts from the *HO-1*-targeted mice (Poss and Tonegawa 1997) showed hypersensitivity to cytotoxicity caused by H<sub>2</sub>O<sub>2</sub>. Viability of LCL with class L alleles (L/L) established from smokers without lung carcinoma was also lower than that with class S (S/S) after treatment with H<sub>2</sub>O<sub>2</sub> (Hirai et al. 2003). The GT dinucleotide repeat polymorphism has emerged as a potent genetic risk factor in various diseases, including vascular diseases as coronary arteriosclerosis (Chen et al. 2002) and restenosis after balloon angioplasty (Exner et al. 2001). These findings were



consistent with the idea that the lung of the genotype without the class L allele (alleles M/M, M/S, S/S) is capable of utilizing more of the antioxidant activity of HO-1 than that of the genotype containing the class L allele (alleles L/L, L/M, L/S) when it is exposed to reactive oxygen species in cigarette smoke. Large (GT)<sub>n</sub> repeats may affect the protective function against oxidant-induced lung cell injury through the inhibition of *HO-1* expression (Yamada et al. 2000, 2001).

Cigarette smoke, which contains reactive oxygen species and aromatic hydrocarbons, is especially the most important risk factor for lung cancer, and lung adenocarcinoma is also increasingly associated with tobacco smoking (Franceschi and Bidoli 1999). Polycyclic aromatic hydrocarbons, nitrosamines, and ROS in cigarette smoke all induce DNA damage (Loeb et al. 1984; Yoshie and Ohshima 1997; Feig et al. 1994). DNA damage leads to a deregulated cell-cycle with abnormal cellular proliferation in the subjects with loss of tumor suppressor genes such as p53, thereby resulting in the development of lung cancer (Rom et al. 2000), although we did not examine the expression of p53 in this study. Antioxidant activity of HO-1 covers a wide variety of stimulating stress (Maines 1997; Choi et al. 1996), and HO-1 acts in cytoprotection against oxidants and carcinogens, benzo(a)pyrene (Loeb et al. 1984; Li et al. 2000). Therefore, higher intracellular HO-1 activity results in an increased content of bilirubin (Tenhunen et al. 1969), which may act not only in cytoprotection against oxidants (Otterbein et al. 1999) and benzo(a)pyrene (Li et al. 2000) but also in suppression of DNA damage of the lung epithelial cells in response to cigarette smoke. Furthermore, HO-1 may inhibit the carcinogenesis through the inhibition of apoptosis (Lee et al. 1996), as shown in the effects of tumor suppressor genes (Sherr 1996).

We show in the present study that microsatellite polymorphism in the *HO-1* gene promoter may be associated with the development of lung adenocarcinoma in Japanese. However, we found no association in the microsatellite polymorphism in the *HO-1* gene promoter in patients with lung squamous cell carcinoma, as previously shown in genetic polymorphism of cytochrome P450 2A13 in lung cancer (Wang et al. 2003). Although lung squamous cell carcinoma is suggested to be induced by polycyclic aromatic hydrocarbons such as benzo(a)pyrene (Deutsch-Wenzel et al. 1983, Hecht 1999), polycyclic aromatic hydrocarbons are also known to be activated by CYP1A1 (Kandlubar and Hammons 1987). Therefore, risk of lung squamous cell carcinoma might be influenced by genetic polymorphism of enzymes other than HO-1, such as CYP1A1 (Nakachi et al. 1991).

Lung adenocarcinoma is a multifactorial disorder as previously reported (Osann 1998; Harris et al. 1993). The risk factors for lung adenocarcinoma other than cigarette smoking were also reported, including exposure to resident radon, occupational exposures, diet, and family history (Osann 1998). Women are suggested to be

at a higher risk than men for a given level of smoking (Harris et al. 1993). In the present study, more than 60% of female non-smoking patients with lung adenocarcinoma were suggested to be passive smokers. In addition to an increased risk of lung cancer in non-smokers whose spouses smoked (Hirayama 1981; Trichopoulos et al. 1981), sensitivity to these risk factors other than cigarette smoking in women may also be associated with the development of lung adenocarcinoma.

In the present study, many patients (116 of 151, 77% in lung adenocarcinoma, and 66 of 108, 61% in lung squamous cell carcinoma) were in early stage (stage 0, stage I or stage II), and most patients (139 of 151, 92% in lung adenocarcinoma, and 87 of 108, 81% in lung squamous cell carcinoma) had received surgical operation. Therefore, it should be noted that there are limitations in the present study, including a potential selection bias due to referral patterns at the hospital. Although, we demonstrated that, in the 128 male smokers, the proportion of genotypic frequencies in L allele carriers was significantly higher in patients with lung adenocarcinoma than that in control subjects, the number of subjects was still small. Further studies are needed to confirm the interaction of gender and smoking with the relationship between the 5'-flanking polymorphism in the *HO-1* gene and the development of lung adenocarcinoma.

In summary, the proportion of class L allele frequencies, as well as the proportion of genotypic frequencies in L allele carriers, was significantly higher in patients with lung adenocarcinoma compared with those of control subjects. Furthermore, the risk of lung adenocarcinoma for L allele carriers vs non-L allele carriers was much higher in the group of male smokers. However, in the female non-smoker, the proportion of L allele carriers did not differ between patients and control subjects. This is the first study to demonstrate that the 5'-flanking polymorphism in the *HO-1* gene is associated with the development of lung adenocarcinoma in Japanese smokers.

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--Letter to the Editor--

## **Acupuncture for Dysphagia in Poststroke Patients**

### **: A Videofluoroscopic Study**

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Severe dysphagia predisposes to medical complications such as aspiration pneumonia in poststroke patients.<sup>1</sup> Existing modalities for treating dysphagia are generally ineffective.<sup>2</sup> We recently reported that acupuncture on two acupoints (Zusanli ST36 and Taixi K3) restored the swallowing reflex<sup>3</sup> and gait disorders<sup>4</sup> in poststroke patients. In this study, we performed a videofluoroscopic study (VFSS) to determine if acupuncture also improves dysphagia and aspiration.

According to Magnetic Resonance Imaging findings, 32 poststroke patients ( $84 \pm 9$  (mean  $\pm$  SD) years, Female/Male 20/12) with episodes of choking while taking food or drinking liquid were selected randomly at an elderly care facility and participated in this study under written informed consent. Participants were randomly assigned into two groups. We practiced acupuncture on the intervention group (18 patients;  $77 \pm 9$  (mean  $\pm$  SD) years, F/M 10/8) three times a week for four weeks with usual care. Four disposable stainless steel fine needles (diameter 0.16 mm, length 40 mm; SEIRIN, Shizuoka, Japan) were inserted in two acupoints (ST36 and K3) bilaterally and kept at a 10 mm depth for 15 minutes without any extra stimulation such as electrical or manual. Fourteen other patients were assigned to the control group ( $79 \pm 5$  (mean  $\pm$  SD) years, F/M 10/4) and