ELECTRONIC LETTER

Association of susceptibility to the development of pneumonia in the older Japanese population with haem oxygenase-1 gene promoter polymorphism

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Background: Oxidative stresses including cigarette smoking are implicated in the pathogenesis of cerebrovascular diseases, which are associated with pneumonia because of frequent aspiration. Haem oxygenase-1 (HO-1) acts in cytoprotection against oxidants, provides anti-inflammatory effects, and inhibits atherogenesis. A (GT)_n dinucleotide repeat in the human *HO-1* promoter modulates *HO-1* gene expression and shows length polymorphism, which is grouped into three classes: class S (<27 repeats), class M (≥27, <33 repeats), and class L (≥33 repeats) alleles.

Objective: To investigate the correlation between the *HO-1* gene polymorphism and development of pneumonia in elderly Japanese.

Methods: The length of the (GT)_n repeats was analysed in 200 elderly patients with pneumonia and 200 control subjects. The association of the *HO-1* gene polymorphism with risk of pneumonia was estimated by logistic regression.

Results: The proportion of allele frequencies in class L, and the proportion of genotypic frequencies in the L-allele carriers (L/L, L/M, and L/S), was significantly higher in patients with pneumonia than in controls (20% v 10% in class L, and 34% v 18% in L-allele carriers). After adjustment for potentially confounding factors, both cerebrovascular disorders and *HO-1* gene L-allele carriers were significant and independent risk factors for pneumonia. The adjusted odds ratio for L-allele carriers v non-L-allele carrier was 2.1 (95% confidence interval, 1.2 to 3.6).

Conclusions: The large size of a (GT)_n repeat in the *HO-1* gene promoter may be associated with susceptibility to pneumonia in the older Japanese population.

Pneumonia is not only a common infection in older people, it is also the most common cause of death from nosocomial infection in the Japanese population.¹ Disorders of the central nervous system are more likely to develop in the elderly, and pneumonia has been estimated to occur in about one third of patients with stroke.² Cerebrovascular disease is associated with a high incidence of pneumonia owing to frequent aspiration.³ As well as factors including diabetes mellitus, hyperlipidaemia, and hypertension, oxidative stresses such as cigarette smoking are also associated with the pathogenesis of cerebrovascular disease.⁴ Genetic factors affecting antioxidants may be involved in the susceptibility to atherosclerosis of the cerebral arteries and the subsequent development of pneumonia in the elderly. Although the antioxidant enzymes inhibit the formation of atherosclerosis,⁵ the roles of reduced expression

of these enzymes on the development of pneumonia in elderly people are still uncertain.

Haem oxygenase (HO) oxidatively degrades haem to biliverdin, which is subsequently reduced to bilirubin, an efficient scavenger of reactive oxygen species (ROS), by biliverdin reductase.⁶ HO-1, an inducible form of HO—and also a constitutive form of HO, including HO-2—provides cellular protection against haem mediated and non-haem-mediated oxidant injury.⁶ HO-1 is thought to be an essential component in protection against various ROS.

A (GT)_n repeat in the 5' flanking region of the human *HO-1* gene is polymorphic,⁷ and modulates human *HO-1* gene transcription by thermal stress⁸ and hydrogen peroxide.⁷ The size of the (GT)_n repeat in the *HO-1* gene is associated with the antiapoptotic effects of HO-1 in lymphoblastoid cell lines.⁹ We have shown that the size of the (GT)_n repeat in the *HO-1* gene is associated with susceptibility to chronic pulmonary emphysema (CPE)⁷ and lung adenocarcinoma,¹⁰ and with longevity¹¹ in Japanese populations. This *HO-1* gene polymorphism is also associated with coronary artery disease, one of vascular diseases related to ROS.¹² However, the association between the size of the (GT)_n repeat in the *HO-1* gene and the development of pneumonia in older populations is still uncertain.

In the present study, we screened allelic frequencies of the (GT)_n repeats in the *HO-1* gene promoter in elderly people with and without pneumonia, and examined the association between the risk of senile pneumonia and length of the (GT)_n repeats.

METHODS

Clinical protocol and patient characteristics

We studied 200 elderly patients with pneumonia and 200 elderly control subjects without pneumonia, attending the departments of internal medicine in six hospitals in Miyagi prefecture. The hospitals were a university hospital, a Red Cross hospital, three public general hospitals, and a municipal hospital. All participants were Japanese and aged 65 and older. To evaluate whether *HO-1* genotypes are associated with the development of pneumonia in elderly Japanese people, we selected the subjects with a performance status of 2 or better¹³ and in a stable state as potential participants, because those with too low a performance status ran a greater risk of infectious disease, which might mask the preventive effect of any genetic factors. Patients were given a score of 0 if they were fully active and asymptomatic, 1 if they were symptomatic but fully ambulatory, 2 if they were

Abbreviations: COPD, chronic obstructive pulmonary disease; CPE, chronic pulmonary emphysema; HO, haem oxygenase; HO-1, inducible haem oxygenase; ROS, reactive oxygen species; TNF, tumour necrosis factor

Table 1 Characteristics of the study subjects

Characteristics	Control subjects (n = 200)	Patients with pneumonia (n = 200)	p Value
Age (years)*	73.8 (0.7)	75.4 (1.0)	NS
Sex			
Male	99 (50%)	101 (50%)	NS
Female	101 (50%)	99 (50%)	
Performance status			
0–1	114 (57%)	108 (54%)	NS
2	86 (43%)	92 (46%)	
Smoking history (pack-year)*	18.2 (2.6)	19.3 (2.8)	NS
Cerebrovascular disease			
Yes	14 (7%)	101 (50%)	<0.0001
No	186 (93%)	99 (50%)	
COPD			
Yes	35 (18%)	38 (19%)	NS
No	165 (82%)	162 (81%)	
Congestive heart failure			
Yes	17 (9%)	28 (14%)	NS
No	183 (91%)	172 (86%)	
Hypertension			
Yes	43 (22%)	59 (30%)	NS
No	157 (78%)	141 (70%)	
Diabetes mellitus			
Yes	21 (10%)	34 (17%)	NS
No	179 (90%)	166 (83%)	
Hyperlipidaemia			
Yes	9 (5%)	10 (5%)	NS
No	191 (95%)	190 (95%)	

Values are n (%) or *mean (SD).

COPD, chronic obstructive pulmonary disease.

symptomatic and confined to bed or chair for less than 50% of their waking hour, 3 if they were symptomatic and confined to bed or chair for more than 50% of their waking hours, and 4 if they were completely bedridden. The study was approved by the Tohoku University ethics committee, and informed consent was obtained from each subject. This study was carried out between April 2002 and December 2004.

During the study period, 264 elderly patients with pneumonia were identified. Pneumonia was defined as pulmonary infiltrate on chest radiograph, cough, and a temperature higher than 38.0°C.³ All patients with pneumonia had the features of pulmonary infiltrate on chest radiographs, cough, and a temperature above 38.0°C. The patients were enrolled consecutively. Among them, we selected for the case group those with a performance status of 2 or better and in a stable state. We excluded patients who were immunocompromised—for example, those with active malignant disease, on renal dialysis, receiving corticosteroid treatment, or with HIV-1 infection. Patients were also excluded if they had obvious swallowing dysfunction, chronic sepsis in pressure sores, venous ulcers, or an indwelling urinary catheter. After these selections and exclusions were applied, 200 elderly patients with pneumonia were enrolled in the case group.

Potential control subjects were 439 elderly patients who continued attending the departments of hospitals over the study period and who had never had pneumonia at any time in their life including the study period. Control subjects were excluded if their past history relating to pneumonia were unclear. After the same selection and exclusion criteria as in the case group were applied, 383 control subjects were available for frequency matching. To carry out a case–control study, we randomly selected 200 control subjects in a frequency matched manner from the control cohort. They were frequency matched on age (± 5 years), sex, smoking history, and performance status with the patients with pneumonia. Physical characteristics, smoking history, and complications in patients with pneumonia and control subjects are shown in table 1.

Analysis of length variability of (GT)_n repeats in HO-1 gene promoter

Genomic DNAs were extracted from leucocytes in peripheral venous blood by conventional procedures. The 5′-flanking region containing a poly (GT)_n repeat of the *HO-1* gene was amplified by polymerase chain reaction (PCR)^{7–11} with a fluorescently labelled primer p1-s (5′-AGAGCCTGCAGC TTCTCAGA-3′) and an unlabeled antisense primer p1-as (5′-ACAAAGTCTGGCCATAGGAC-3′), which were designed

Table 2 Allele and genotypic frequencies of HO-1 at polymorphic locus

	Control subjects (n = 200)	Patients with pneumonia (n = 200)	OR (95% CI) v all other classes or subjects	p Value
Allele class				
L	38 (10%)	79 (20%)	2.3 (1.5 to 3.5)	<0.0001
M	189 (47%)	159 (40%)	0.7 (0.5 to 0.9)	<0.05
S	173 (43%)	162 (40%)	0.9 (0.7 to 1.2)	NS
Genotype group				
L-allele carrier	36 (18%)	68 (34%)	2.3 (1.5 to 3.7)	<0.001
Non-L-allele carrier	164 (82%)	132 (66%)		

CI, confidence interval; OR, odds ratio.

Table 3 Multivariate analysis of risk factors related to pneumonia in older adults

Variable	OR (95% CI)	p Value
Haem oxygenase-1 genotype subgroup		
L-allele carriers v non-L-allele carriers	2.1 (1.2 to 3.6)*	<0.01
Cerebrovascular disease		
Yes v no	28.0 (13.3 to 58.6)†	<0.0001

*OR was calculated with the non-L-allele carriers as the reference group, and adjusted for age, gender, performance status, smoking history, and complications.

†OR was calculated with the patients without cerebrovascular disease as the reference group, and adjusted for age, gender, performance status, smoking history, *HO-1* genotype, and complications other than cerebrovascular disease.

CI, confidence interval; OR, odds ratio.

according to the published sequence.^{7 14} The PCR was carried out over 30 cycles of 20 seconds at 94°C, 10 seconds at 60°C, and 20 seconds at 72°C. The PCR products were analysed in a DNA sequencer (ALF express II DNA sequencer version 2.2, Amersham Pharmacia Biotech, Piscataway, New Jersey, USA). Each size of (GT)_n repeat in the PCR product was calculated with ALFwin fragment analysis version 1.03 (Amersham Pharmacia Biotech) using four cloned alleles as size markers, which were already sequenced with the ABI prism dye terminator sequencing kit (Perkin-Elmer Applied Biosystems, Foster City, California, USA).⁷ The repeat numbers of these size markers were 16, 23, 29, and 38, respectively. The investigators of genetic analysis were blinded with respect to the status of the subjects.

Carboxyhaemoglobin concentrations in patients with pneumonia

Blood samples were taken from the radial artery in patients with pneumonia on the first day of hospital admission. The patients for the carboxyhaemoglobin analysis were all non-smokers and consisted of five L-allele carriers and five non-L-allele carriers (L/L genotype and S/S genotype, respectively), who showed a similar C reactive protein concentration (15.0 to 20.0 mg/dl) and white blood cell (WBC) count (9500 to 12 500 cells/μl) at the time of analysis. The carboxyhaemoglobin concentrations were measured with a spectrophotometer (ASL System, Radiometer, Copenhagen, Denmark).¹⁵

Statistical analysis

In the analysis of *HO-1* gene polymorphism in this study, the patient and control groups were frequency matched by age,

sex, performance status, and smoking history. For statistical analysis, age and smoking history (pack-year) between the two groups were compared using Student's *t* test, and sex, performance status, and the frequency of the complications between the two groups were compared using χ^2 tests (table 1), as described previously in coronary artery disease.¹² The proportion of allelic frequencies and genotypic frequencies between the two groups were also compared using the χ^2 test (table 2). Factors associated with the presence of senile pneumonia such as age, sex, performance status, smoking status, complications, and *HO-1* gene polymorphism (L-allele carrier) were examined with multivariate analysis by logistic regression analysis (table 3). Odds ratios (OR) and their 95% confidence intervals (CI) were calculated to assess the relative risk conferred by a particular genotype (L-allele carrier), and adjusted for age, sex, performance status, smoking history, and complications using logistic regression as described previously (table 3).¹² All the statistical analyses were undertaken using SYSTAT (version 10.2; SYSTAT Software, Richmond, California, USA). The values for age and smoking history (pack-year) are reported as means (SD). The *HO-1* genotype distributions were in Hardy-Weinberg equilibrium. Significance was accepted at $p < 0.05$.

For statistical analysis in the study on the correlation between carboxyhaemoglobin level and *HO-1* genotype in the patients with pneumonia, the mean values for age (year), smoking history (pack-year), WBC number (cells/μl), C reactive protein (mg/dl), and carboxyhaemoglobin concentration (%) between the five L-allele carriers and the five non-L-allele carriers were compared using Student's *t* test and sex using the χ^2 test (table 4).

RESULTS

Allele frequencies of *HO-1* gene in control and patients with pneumonia in older adults

There were between 16 and 39 (GT)_n repeats in the human *HO-1* gene in the study subjects (fig 1). The distribution of the number of (GT)_n repeats was trimodal, as previously reported, with two main peaks located at 23 and 30 GT repeats and another peak located at 33 GT repeats.^{7 10 11} We therefore divided the alleles into three subclasses, as previously reported⁷: class S (<27 repeats), class M (≥27 and <33 repeats), and class L (≥33 repeats) alleles.

In the control subjects, the distributions of the 400 alleles were 173 (43%) class S, 189 (45%) class M, and 38 (10%) class L (table 2); in the patients with pneumonia, the distributions were 162 (40%) class S, 159 (40%) class M, and 79 (20%) class L. The proportion of allelic frequencies in class L was significantly higher in all patients with pneumonia ($n = 79$, 20%) than that in all control subjects ($n = 38$, 10%)

Table 4 Arterial blood carboxyhaemoglobin in patients with pneumonia

Patient	HO-1 genotype	Age (years)*	Sex†	Smoking history (pack-year)*	WBC (cells/μl)*	CRP (mg/dl)*	Arterial blood Hb-CO (%)‡
L-allele carrier 1	LL	71	M	0	12 300	18.3	0.57
L-allele carrier 2	LL	65	F	0	10 500	15.2	0.20
L-allele carrier 3	LL	79	F	0	9 700	15.7	0.80
L-allele carrier 4	LL	73	F	0	9 600	19.0	0.21
L-allele carrier 5	LL	76	F	0	10 020	19.4	1.20
Non-L-allele carrier 1	SS	65	M	0	12 400	18.5	1.50
Non-L-allele carrier 2	SS	77	M	0	11 000	16.3	1.20
Non-L-allele carrier 3	SS	79	F	0	10 500	19.2	1.02
Non-L-allele carrier 4	SS	65	F	0	9 900	15.6	1.10
Non-L-allele carrier 5	SS	75	F	0	9 600	19.5	0.90

*There was no significant difference in the mean value between L-allele carrier and non-L-allele carrier ($p > 0.7$).

†There was no significant difference in the ratio between L-allele carrier and non-L-allele carrier ($p > 0.5$).

‡There was a significant difference in the mean value between L-allele carrier and non-L-allele carrier ($p < 0.04$).

CRP, C reactive protein; F, female; Hb-CO, carboxyhaemoglobin; M, male; WBC, white blood cell count.

Figure 1 Frequency distribution of the number of (GT)_n repeats in control subjects (n=400 alleles) and patients with pneumonia (n=400 alleles).

($p < 0.0001$). The odds ratio for pneumonia with L alleles ν non-L alleles (class M allele + class S allele) was 2.3 (95% CI, 1.5 to 3.5) (table 2).

Genotypic frequencies of HO-1 gene in control and patients with pneumonia

Six genotypes (L/L, L/M, L/S, M/M, M/S, and S/S) of (GT)_n 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).⁷ The proportion of genotypic frequencies in L-allele carriers was significantly higher in all patients with pneumonia (n=68, 34%) than that in all control subjects (n=36, 18%) ($p < 0.0001$). The odds ratio for patients with pneumonia with L-allele carriers ν non-L-allele carriers was 2.3 (95% CI, 1.4 to 3.7) (table 2).

Risk factors for pneumonia

On multivariate analysis, cerebrovascular disease ($p < 0.0001$) and *HO-1* genotype ($p < 0.01$) were significantly and independently associated with the development of pneumonia (table 3), when the variables were adjusted by age, sex, performance status, smoking history, and complications including congestive heart failure, COPD, hypertension, diabetes mellitus, and hyperlipidaemia. The adjusted odds ratio (95% CI) was 2.1 (1.2 to 3.6) for *HO-1* genotype and 28.0 (18.3 to 58.6) for cerebrovascular disease (table 3).

Carboxyhaemoglobin concentrations in patients with pneumonia

To show the correlation between *HO-1* genotype and HO-1 activity caused by the inflammation of pneumonia, we examined the carboxyhaemoglobin concentration in several patients with pneumonia on their first day of hospital admission. The subjects for carboxyhaemoglobin analysis were five L-allele carriers and five non-L-allele carriers (L/L genotype and S/S genotype, respectively). There were no significant differences in age, sex, smoking history, WBC count, and C reactive protein concentration level between these two groups. However, the patients without the L-allele showed significantly higher carboxyhaemoglobin levels than

those with the L-allele (1.14 (0.23)% ν 0.5 (0.42)%, respectively; $p < 0.04$) (table 4).

DISCUSSION

In this study we analysed *HO-1* gene polymorphism and showed that the proportion of allele frequencies in class L and the proportion of genotypic frequencies in the L-allele carriers (L/L, L/M, and L/S) were significantly higher in elderly people with pneumonia than in control subjects. The proportion of subjects with cerebrovascular disease in the pneumonia group was significantly higher than in the control group. With multivariate analysis, *HO-1* genotype and the presence of cerebrovascular disease were significant and independent risk factors for pneumonia. These findings suggest that the large size of a (GT)_n repeat in the *HO-1* gene promoter may be associated with the development of pneumonia in older Japanese people with cerebral infarction.

Disorders of the central nervous system are more likely to develop in the elderly, and pneumonia has been estimated to occur in about one third of patients with stroke.² Basal ganglia infarction is associated with a high incidence of pneumonia owing to frequent aspiration³ resulting from the reduction in the cough and swallowing reflexes.¹⁶ In fact, in the present study, half these older patients with pneumonia also had cerebrovascular disease.

Oxidative stress such as cigarette smoking⁴ is one of the important risk factors for cerebrovascular diseases, including basal ganglia infarction. Various ROS including superoxide and hydrogen peroxide induce lipid peroxide formation, which is a key process in atherosclerotic plaques in hypercholesterolaemia.¹⁷ ROS are also involved in the brain tissue damage in stroke.¹⁸ On the other hand, antioxidant systems such as glutathione, superoxide dismutase, and HO are suggested to protect the vascular disease caused by ROS.¹⁹ The initial degradation of haem by microsomal HO involves the liberation of iron and CO and the formation of biliverdin, which is subsequently reduced to bilirubin by cytosolic biliverdin reductase.⁶ Higher intracellular HO-1 activity may increase the content of bilirubin, which is an efficient scavenger of ROS,⁶ and a natural inhibitor of intimal hyperplasia after balloon injury.²⁰ In fact, Ishikawa *et al.*

reported inhibitory effects of HO-1 on the atherogenesis in hyperlipidaemic rabbits.²¹ Enhanced endothelial cell injury caused by oxidative stress was observed in a human case of *HO-1* deficiency.²² Reduced expression of HO-1 might be partly associated with the development of stroke and subsequent pneumonia.

A (GT)_n dinucleotide repeat in the 5'-flanking region of human *HO-1* gene shows length polymorphism.⁷ We previously reported the influence of the number of the (GT)_n repeats on the inducibility of the *HO-1* gene promoter under oxidative stimulus by transient transfection assay in human cell lines. The promoter activity of *HO-1* is modulated by the length variability of the (GT)_n repeats, and large (GT)_n repeats have a potent inhibitory activity on H₂O₂ induced gene expression of HO-1.⁷ Furthermore, Epstein-Barr virus transformed lymphoblastoid cell lines were established from smokers with class L alleles (L/L) and with class S (S/S). When treated with H₂O₂, lymphoblastoid cells with the L/L genotype showed lower viability than those with the S/S genotype.⁹ The GT dinucleotide repeat polymorphism has emerged as a potent genetic risk factor in various diseases, including vascular diseases such as coronary arteriosclerosis¹² and restenosis after balloon angioplasty.²³ These findings are consistent with the view that tissues of the non-L allele carrier could employ the antioxidant activity of HO-1 to a greater extent than that of the L-allele carrier when exposed to reactive oxygen species.¹⁰ Large (GT)_n repeats may affect the protective function against oxidant induced vascular endothelial injury and arteriosclerosis through the inhibition of HO-1 expression.

The results of our study suggest that the *HO-1* genotype is associated with susceptibility to pneumonia independently of cerebrovascular disease. Senile pneumonia is characterised by a high likelihood of aspiration pneumonia.¹⁶ The severity of aspiration pneumonia is associated with the lung inflammation mediated by cytokines such as tumour necrosis factor α (TNF α).²⁴ On the other hand, it was reported that overexpression of the *HO-1* gene attenuated inflammation and decreased apoptosis of bronchial epithelial cells in a murine model of lung inflammation induced by *Pseudomonas aeruginosa*.²⁵ Furthermore, overexpression of the *HO-1* gene could reduce TNF α mediated apoptotic cell death in human endothelial cells.²⁶ These findings suggest that *HO-1* gene expression could be associated with the progress of aspiration pneumonia, and that reduced expression of the *HO-1* gene in elderly L-allele carriers might allow the development of pneumonia independently of cerebrovascular disease.

To examine the association between *HO-1* genotype and HO-1 activity in the pneumonia, we evaluated the carboxyhaemoglobin level in L-allele carriers and non-L-allele carriers with pneumonia. As a result, even after adjustment for the peripheral WBC count and C reactive protein level, patients without the L-allele showed higher carboxyhaemoglobin levels than those with the L-allele. Carbon monoxide (CO) is produced endogenously by HO and combines haemoglobin to form carboxyhaemoglobin complex. Therefore, the carboxyhaemoglobin concentration in the subject is a good marker of endogenous HO activity.²⁷ Furthermore, it has been reported that HO-1 is strongly induced in patients with bacterial infection.²⁸ We have already shown that arterial carboxyhaemoglobin increases at the onset of pneumonia in untreated patients returns to baseline on recovery after treatments.¹⁵ We also showed that an increase in arterial carboxyhaemoglobin in pneumonia would be caused by carbon monoxide production in pulmonary inflammation, and that the arterial carboxyhaemoglobin is significantly correlated with disease severity in patients with bacterial pneumonia.²⁹ A study of lymphoblastoid cell lines by Hirai *et al* showed that mRNA level and

activity of HO-1 were significantly higher in lymphoblastoid cells with the S/S genotype than in those with the L/L genotype after oxidant stimulation.⁹ Therefore, analysis of the carboxyhaemoglobin level in pneumonia according to *HO-1* genotype would clarify the association between the *HO-1* genotype and HO-1 activity—that is, the HO-1 protein level, resulting from pneumonia. These findings suggest that HO-1 induction might be associated with the *HO-1* genotype (S>M>L).

In contrast to arterial blood carboxyhaemoglobin concentrations, we did not measure HO-1 activity in patients with pneumonia at the onset. However, we obtained new blood samples from eight people in the control group and seven in the pneumonia group after recovery from pneumonia, and analysed the serum HO-1 protein levels using enzyme linked immunosorbent assay methods as previously described.³⁰ There was no significant difference between these two groups when they were in good physical condition (2.6 (1.2) v 2.4 (1.0) ng/ml, *p*>0.2). These values were compatible with the results from a previous report.³⁰ Because the *HO-1* gene is inducible by inflammation or oxidative stress, the baseline expression of the this gene should be low regardless of the *HO-1* genotype, which was demonstrated in lymphoblastoid cell by Hirai *et al*.⁹ Further studies are needed to clarify the relation between HO-1 activity and the *HO-1* genotype at the onset of pneumonia.

Conclusions

This is the first study to show that the 5'-flanking polymorphism in the *HO-1* gene is associated with the development of pneumonia in an older Japanese population with basal ganglia infarction. Increased susceptibility to developing pneumonia may be associated with sclerosis in the cerebral arteries.

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