Disease Markers 28 (2010) 185–193
DOI 10.3233/DMA-2010-0700
IOS Press

Functional haplotypes in the promoter region of transcription factor *Nrf2* in chronic obstructive pulmonary disease

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Abstract. Nuclear factor (erythroid-derived 2)-like 2 (Nrf2) protects against oxidative stress which is important in the pathogenesis of chronic obstructive pulmonary disease (COPD). Three single nucleotide polymorphisms and 1 triplet repeat polymorphism are found in the promoter region of the *Nrf2* gene. Molecular haplotyping of the *Nrf2* promoter region was performed using DNA obtained from the peripheral blood of 69 COPD patients. The luciferase activities of *Nrf2* promoter constructs containing all possible combinations of the 4 polymorphisms were determined and found to differ among the 16 haplotypes.The haplotypes isolated from the subjects were divided into 3 groups (L: low; M: medium; H: high) on the basis of luciferase activities. The proportions of subjects belonging to global initiative for chronic obstructive lung disease stage 3 or 4 decreased from the group with the LL haplotype to that with the HH haplotype. Presence of the LH or MM haplotype (hazard ratio, 3.36; 95% confidence interval, 1.16–9.69), gender (0.13; 0.02–0.67), and post-bronchodilator FEV_1 value of predicted (0.95; 0.91–0.99) are significant predictors of respiratory failure development.The haplotype of the *Nrf2* gene promoter affects its activity, and is associated with the severity and the development of respiratory failure in COPD.

Keywords: Respiratory failure, COPD, haplotype, Nrf2, promoter

1. Introduction

Chronic obstructive pulmonary disease (COPD) is an inflammatory lung disease characterized by systemic and chronic localized inflammation induced by oxidative stress [1]. Oxidative stress regulates both signal transduction and histone modification in lung inflammation. Reactive oxygen species (ROS) generated as a result of oxidative stress enhance inflammation by activating stress kinases and redox-sensitive transcription factors [2]. In COPD, levels of ROS increase while those of antioxidant enzymes decrease, and the imbalance between oxidative stress and endogenous antioxidant mechanisms results in chronic inflammation [1].

The expression of nearly 50 antioxidant and cytoprotective genes in the lung may be transcriptionally controlled by nuclear factor (erythroid-derived 2)-like 2 (NFE2L2 or Nrf2) [3]. Nrf2 is a "cap 'n' collar" basic leucine zipper transcription factor associated with its negative regulator Keap1 (kelch-like ECH-associated protein 1) in the cytoplasm of unstressed cells [4]. Under conditions of oxidative stress, Nrf2 is released from Keap1 and translocated to the nucleus [5]. The heterodimer formed by Nrf2 and small Maf proteins serves as a transcriptional activator that recognizes the antioxidant response element in the promoters of many phase II detoxifying enzymes [4,6,7]. The Nrf2-Keap1 system is one of the major cellular defense mechanisms

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ISSN 0278-0240/10/$27.50

against oxidative stress and contributes to protection against various pathologies, including carcinogenesis, liver toxicity, respiratory distress, and inflammation [6].

Nrf2-disrupted mice have earlier onset and more extensive cigarette smoke-induced emphysema than their wild-type littermates [3]. The Nrf2 protein levels are lower in the lung tissue of patients with emphysema [8–10]. *Nrf2* mRNA expression in pulmonary macrophages is lower in COPD patients [11]. *Nrf2* expression patterns differ among nonsmokers, healthy smokers, and COPD patients [12]. Cigarette smoke contains more than 10^{14} free radicals per puff, causes lung damage, and elevates inflammatory responses in the lung; it is also implicated in the pathogenesis of COPD [13]. 1-[2-Cyano-3-,12-dioxooleana-1,9(11)-dien-28-oyl]imidazole, a potent Nrf2 activator, protects mice from developing emphysema caused by chronic exposure to cigarette smoke [14]. Resveratrol activates Nrf2 and protects against cigarette smoke-mediated oxidative stress in human epithelial cells [14]. Under conditions of oxidative stress, Nrf2 activates the biosynthesis of glutathione, the levels of which are much lower in the epithelial lining fluid of COPD patients than in that of control subjects [15]. Nrf2 probably plays an important role in COPD pathogenesis.

The promoter region of the *Nrf2* gene has 3 single nucleotide polymorphisms (SNPs; -686 A/G, -684 G/A, and -650 C/A) and 1 triplet repeat polymorphism [-20 to -6 $(CCG)_{4or5}$] [16]. Using the luciferase assay, it was shown that the SNPs in the promoter region affected the basal expression level of the *Nrf2* gene and were associated with the risk of developing acute lung injury after major trauma [17]. This study examines the luciferase activities of constructs containing all possible combinations of the 4 polymorphisms in the promoter region of the *Nrf2* gene and determines the distribution of haplotypes in COPD patients. Haplotypes of the *Nrf2* gene promoter were associated with disease severity, which was assessed by the pulmonary function test and categorized according to the global initiative for chronic obstructive lung disease (GOLD) guideline [18] in COPD patients. The polymorphism effect of the *Nrf2* gene promoter on the first development of respiratory failure in COPD subjects was also investigated.

2. Patients and methods

2.1. Patients

COPD was diagnosed by irreversible airflow limitation evidenced by a post-bronchodilator forced expiratory volume in 1 s/forced vital capacity (FEV_1/FVC) $< 70\%$ in the pulmonary function test [18]. Sixty-nine COPD patients were recruited with informed consent signed for this study and received the pulmonary function test via a spirometer (ASP-pro; E. Jaeger Laboratories, Wurzburg, Germany) in accordance with the ATS guideline [19]. The severity of COPD was classified according to the FEV_1 value of the predicted following the GOLD guideline [18]. Complicated pneumoconiosis with progressive massive fibrosis was defined by the presence of one or more large opacities $\geqslant 1$ cm [20,21]. Twenty-one subjects experienced one or more episodes of respiratory failure which needed ventilatory support before August 24, 2009. The age at the first occurrence of respiratory failure was recorded. Mean follow-up time of all subjects in this institution was 7.9 ± 2.5 years. Subjects with congestive heart failure were excluded. This study was approved by the Institutional Review Board of Chang Gung Memorial Hospital. Informed written consent was obtained from each participating patient.

2.2. Determination of haplotypes

Genomic DNA was extracted from 2 ml of peripheral venous blood using a DNA extraction kit (Puregene; Gentra Systems, Minneapolis, MN, USA). An upstream primer 5′-GCGTGGTGGCTGCGCTTT-3′ (-848 to -831) and a downstream primer 5′-GGCAGCTCCAAGTCCATCATG-3′ ($+83$ to $+103$) modified from Yamamoto et al. [16] were used to amplify the -848 to $+103$ fragment of the *Nrf2* gene promoter. Polymerase chain reaction (PCR) was performed in a 25 μl reaction mixture containing 100 ng of genomic DNA, 1 U proofreading Taq DNA polymerase (Blend Taq-Plus DNA polymerase; Toyobo, Osaka, Japan), 2.5 μl of $10 \times$ PCR buffer, 2 mM of each dNTP, 1.5 M betaine, and 0.625 μg of each primer. PCR was carried out in a thermocycler (PCRSprint; ThermoHybaid, Middlesex, UK) under the following cycling conditions: 95°C for 4 min followed by 35 cycles at 94°C for 1 min, 61°C for 1 min, and 72°C for 50 s, and a final extension at 72°C for 10 min. Genotypes of the PCR products were determined by DNA sequencing (ABI 3730 XL DNA Analyzer; Applied Biosystems, Foster City, CA, USA). If the haplotypes could not be determined accurately from the genotype, the PCR product was transformed into *Escherichia coli* DH5α using a TA cloning vector (pGEM-T Easy Vector System; Promega, Madison, WI, USA), and a single colony was picked for plasmid isolation. The plasmid was isolated using a mini-prep kit (Protech, Taipei, Taiwan), and the extracted DNA was sent for sequencing.

2.3. Luciferase assay

A *Kpn*I-upstream primer 5′-AAGGTACCGCGTGGTGGCTGCGCTTT-3′ and *Xho*I-downstream primer 5′-AACTCGAGGGCAGCTCCAAGTCCATCATG-3′ were used to obtain amplicons of the *Nrf2* gene promoters from −848 to +103 with digestion sites at each end using the PCR protocol described above. To study the effects of deleting the polymorphism sites on promoter activity, a *Kpn*I-d680 primer 5′-GGTACCTCAGGGTGACTGCGAACAC-3′ and *Kpn*I-d650 primer 5′-GGTACCGCTGTCCACATCTCCCCTAG-3′ were used to replace the *Kpn*I-upstream primer in order to amplify the segment of the *Nrf2* gene promoter from −679 to +103 and −649 to +103, respectively. The PCR products were first TA cloned, sequenced, and then digested by *Kpn*I and *Xho*I (New England Biolabs, Beverly, MA, USA). The double-digested fragment of the *Nrf2* gene promoter was fused upstream of the firefly luciferase gene in the pGL3-basic vector (Promega), and the resulting construct was then transfected into A549 cells. Luciferase activity was determined with 20 μl of lysate/sample using the Dual-Luciferase assay system (Promega) and a luminometer (TD 20/20; Turner Design, Sunnyvale, CA, USA). The haplotype AGA5 [-686(A/G)/-684(/A/G)/-650(A/C)/number of CCG triplet repeats] was chosen as the reference. All luciferase activity measurements were repeated at least 3 times.

2.4. Mutagenesis

Site-directed mutagenesis with overlap extension by PCR [22] was used to generate constructs whose haplotype was not isolated from the recruited subjects (AAC4, AAA5, AAA4, GAC4, GAC5, GAA4, GAA5, and GGA5). Primers 5′-GGGAGTTCAGAGGGGAGCGTTCAGGGTGAC-3′ and 5′-AACACGAGCTGCCGGAGCTGTCCACATCTC-3′ with their complements changed G at −684 to A and C at −650 to A, respectively, to obtain the haplotypes not isolated from subjects using the same PCR cycling conditions as described above. The PCR products were TA cloned, sequenced, and subjected to the luciferase activity measurements described above.

2.5. Statistical analyses

Relative luciferase activities of the haplotype or deleted constructs were grouped using one-way analysis of variance (ANOVA) with Student-Newman-Keuls post-hoc test. Hardy-Weinberg equilibrium was assessed using goodness of fit chi-square or Fisher's exact test for biallelic markers. Cochran Armitage test was used to test a trend in the proportion of subjects with GOLD stage 3 or 4. The chi-square analysis or Fisher's exact test was used to compare discrete variables. ANOVA and Student's t-test were used to detect differences in the pulmonary function tests among and between groups, respectively. The proportional-hazards regression model of Cox with stepwise selection [23] by Bayesian information criterion (BIC) was used to determine the significance of the following: gender; age; current smoking status; smoking index; comorbidities; and the haplotype of low, medium, or high luciferase activity (see Results) as predictors for the first occurrence of respiratory failure. A $p-$value < 0.05 was considered to be significant.

3. Results

The characteristics of the COPD subjects are listed in Table 1.

Samples of electropherogram showing the polymorphisms in the promoter region of *Nrf2* gene are presented in Fig. 1. The distributions of haplotype, genotype, and allelic frequency are listed in Table 2. All genotypes of different polymorphism sites agreed with the Hardy-Weinberg equilibrium. The allelic frequencies of the 4 polymorphisms are similar to those reported by Yamamoto et al. [16].

Relative luciferase activities of full and deleted constructs with different haplotypes are presented in Fig. 2. The relative luciferase activities of full constructs are significantly different among the 16 haplotypes. The haplotypes were subsequently grouped into 3 levels on the basis of their luciferase activities: low (L) for AGC4 and AGC5; medium (M) for AGA4, GGC4, and GGC5; and high (H) for AGA5, GGA4, and AAC5. In constructs with the −686/−684 site deleted (−679 to +103), a C, but not A, at the −650 polymorphism site interacts with CCG copy number to affect relative luciferase activity. Deleted constructs with different CCG copy number only (−649 to +103) have similar relative luciferase activities. Comparisons among full and deleted constructs grouped by the combination of −650 polymorphism and CCG copy number are shown in Fig. 3. The presence of a −650 site in the construct has less relative luciferase activity than that consisted of triplet repeat only. Inclusion of the −686/−684 sites further decreases relative luciferase activity to various

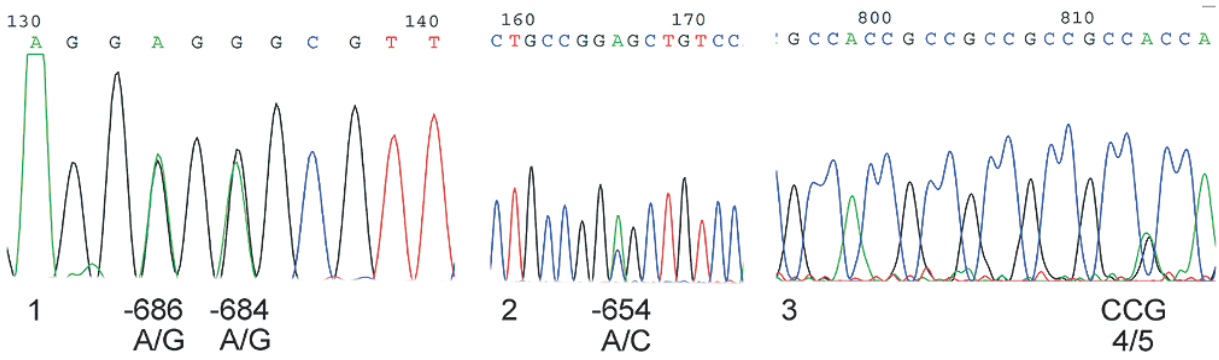

Fig. 1. Electropherograms of the 4 polymorphism sites of *Nrf2* promoter: (1) A/G at both the −686 and the 684 sites; (2) A/C at the −650 site; and (3) CCG_4/CCG_5 of triplet repeat polymorphism (−20 to −6).

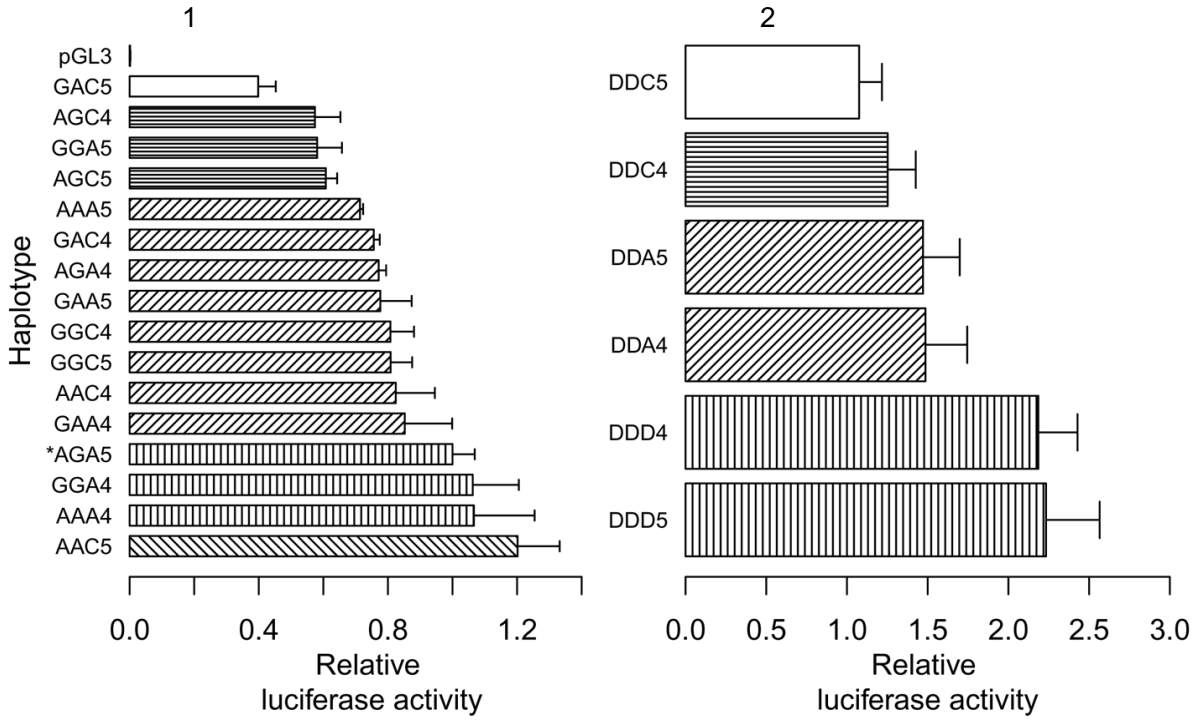

Fig. 2. Homogeneous subsets of different haplotypes (1) or deleted constructs (2) based on relative luciferase activities. The haplotype is expressed as the allele at positions −686/−684/−650/ plus the number of CCG repeats at −20 to −6, and "D" represents a deleted construct without that position. The relative luciferase activity of AGA5 (*) was used as the reference.

extents depending on allelic combination of the 2 other polymorphisms.

Pulmonary function tests of COPD patients are shown in Table 3. Despite the lack of significance, the FEV_1 of predicted increased and the proportion of subjects with GOLD stage 3 or 4 decreased from the LL to the HH haplotype. Due to the close similarity in the proportion of subjects with GOLD stage 3 or 4, LH and MM haplotypes were grouped together, and the Cochran Armitage test showed a significant trend ($p = 0.0496$) of decrease in the proportion from the LL (low-low) to HH (high-high) haplotype. Cox regression with stepwise selection by BIC obtained a subset of predictors for the first respiratory failure: presence of LH (low-high) or MM (moderate-moderate) haplotype (hazard ratio, 3.36; 95% confidence interval, 1.16–9.69); post-bronchodilator FEV_1 value of predicted (0.95; 0.91–0.99); gender (0.13; 0.02–0.67).

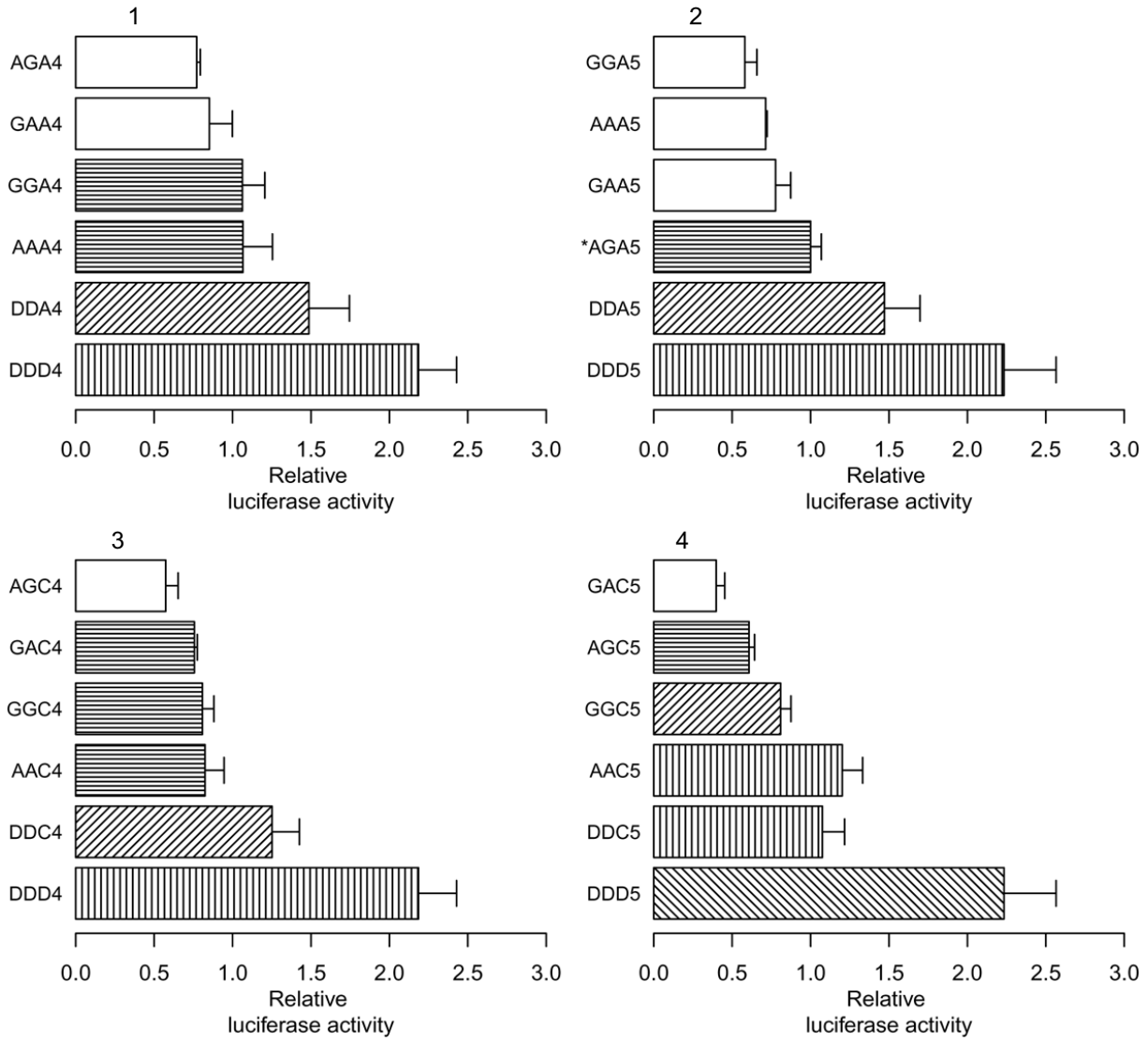

Fig. 3. Homogeneous subsets of the haplotypes and related deleted constructs based on relative luciferase activities grouped by the allele at the -650 site and the number of CCG repeats: (1) A at the -650 site and CCG_4; (2) A at the -650 site and CCG_5; (3) C at the -650 site and CCG_4; and (4) C at the -650 site and CCG_5. See Fig. 2 for the haplotype expression. The relative luciferase activity of AGA5 (*) was used as the reference.

4. Discussion

The haplotype constituted by the 3 SNPs and 1 triplet repeat polymorphism affects the luciferase activity of the *Nrf2* gene promoter. The severity of COPD patient is related to the luciferase activity of the *Nrf2* gene promoter. The risk of developing respiratory failure increases in the group with the LH or MM haplotype.

Redox balance is very important in the airways because they are the first point of contact with airborne oxidants and are exposed to a higher oxidant burden than other tissues [24]. ROS and lipid peroxidation produced by inhaled oxidants and inflammatory cells activate nuclear factor-κB and activator protein-1 with subsequent transcription of pro-inflammatory cytokines and chemokines in the alveolar space and blood of COPD patients [2]. The nuclear translocation of nuclear factor-κB and activator protein-1 caused by an increase in oxidant levels can be blocked by antioxidants such as reduced glutathione (GSH) and thioredoxin [25]. Transcription of genes of inflammatory cytokines is increased by histone acetyltransferase activ-

Table 1
Characteristics of 69 COPD subjects

Age (years)		72.4 ± 10.5
Gender (male/female)		62/7
Never smoked		8
Ex-smoker		38
Current smoker		23
Packyears		37.2 ± 33.3
Occurrence of respiratory failure		21
	Age at the first episode of respiratory failure	70.7 ± 14.1
Hypertension		33
Coronary artery disease		9
Diabetes mellitus		9
Pneumoconiosis		21
	Simple	13
	Complicated	8
Arrhythmia		7
	Atrial fibrillation or flutter	6
	Paroxysmal supraventricular tachycardia	1
Brain		7
	Stroke	5
	Other[a]	5
Liver cirrhosis		2
Bronchiectasis		2
History of tuberculous infection		10
Cancer[b]		7

[a]Three with Parkinson's disease and 2 with epilepsy.
[b]Origin of cancer: 2 with cancer of the lung; 1 each with cancer of the prostate, stomach, liver, larynx, and oral cavity.

Table 2
Distributions of genotypes, haplotypes[a], and allele frequencies

Genotype	N	%	Allele frequency	%
−686				
A/A	18	30	A	48
A/G	30	43	G	52
G/G	21	26		
−684				
A/G	3	4	A	2
G/G	66	96	G	98
−650				
A/A	4	6	A	27
A/C	29	42	C	73
C/C	36	52		
CCG_n[b]				
4/4	19	28	4	53
4/5	35	51	5	47
5/5	15	22		
Haplotype	N	%		
AAC5	3	2		
AGA4	6	4		
AGA5	29	21		
AGC4	9	7		
AGC5	19	14		
GGC4	56	41		
GGC5	14	10		
GGA4	2	1		

[a]The haplotype is expressed as −686(A/G)/−684 (/A/G)/−650(A/C)/number of CCG triplet repeats at −20 to −6.
[b]Copy number of CCG at −20 to −6.

ity and is repressed by histone deacetylase. Oxidative stress is one of the mechanisms by which the activity of histone deacetylase can be reduced; this activity is markedly reduced in the lung parenchyma of COPD patients and is correlated with disease severity [26,27]. Oxidative stress is important in COPD pathogenesis.

The glutathione disulfide (GSSG)/2GSH ratio is a good indicator of the cellular redox state and plays a role in maintaining most cellular molecules in a reduced state [28,29]. Increased generation of ROS in the airways of COPD patients elevates the GSSG/2GSH ratio [2,30]. Enzymes that maintain the GSSG/2GSH balance, such as glutathione peroxidase and glutathione reductase, together with proteins involved in redox signaling, such as glutathione S-transferase, peroxiredoxin, thioredoxin reductase, and tyrosine phosphatase, are regulated by Nrf2 [3,7,29]. Nrf2 is a key regulator of phase II detoxifying enzyme genes and antioxidant-responsive genes [6]. Its role in COPD pathogenesis is supported by many studies on humans and animals [3, 8,9,11,12,31–33].

Yamamoto et al. have shown that the promoter of the human *Nrf2* gene has 3 SNPs and 1 triplet repeat polymorphism [16]. Marzec et al. found that the 3 SNPs affect the basal expression level of *Nrf2* and are present in the enhancer region [17]. However, Marzec et al. did not mention the triple repeat polymorphism in their constructs and had only 4 polymorphic variants of 3 SNP sites [17]. Small simple tandem repeats in the noncoding region can have significant effects on the modulation of transcription [34]. Differences in the luciferase activities of the 16 constructs containing all possible combinations of the 4 polymorphisms suggest that the triplet repeat polymorphism also affects the basal expression level of *Nrf2*. The expression of any gene is controlled by the cooperative binding of multiple transcription factors to different *cis*-regulatory sequences that are often found close to one another [35]. Combinatorial control of *Nrf2* gene expression by multiple transcription factors is affected by all 4 polymorphisms in the promoter region. Further studies investigating the regulatory mechanism of *Nrf2* gene expression on these 4 polymorphism sites are needed.

Table 3
Pulmonary function test and GOLD stage by haplotype, genotype, and allele

		N	FVC%	FEV_1%	FEV_1/FVC (%)	GOLD stage 3 or 4
Haplotype[a]						
	LL	1	72.1	32.4	33.8	1 (100%)
	LM	15	67.7 ± 12.8	47.9 ± 12.4	54.3 ± 10.5	9 (60%)
	LH	11	67.9 ± 20.1	47.7 ± 21.0	52.0 ± 10.4	6 (54.5%)
	MM	24	73.5 ± 15.9	49.5 ± 14.9	52.1 ± 9.30	13 (54.2%)
	MH	13	71.3 ± 18.8	51.3 ± 21.1	53.7 ± 12.8	5 (38.5%)
	HH	5	79.0 ± 24.7	61.6 ± 22.0	59.3 ± 8.10	1 (20.0%)
Genotype						
−686	A/A	18	72.4 ± 20.8	52.0 ± 21.2	54.0 ± 10.9	8 (44.4%)
	A/G	30	71.0 ± 15.1	50.2 ± 16.8	53.5 ± 11.6	15 (50%)
	G/G	21	70.9 ± 16.6	47.4 ± 14.1	51.8 ± 8.8	12 (57.1%)
−684	A/G	3	71.9 ± 9.7	53.4 ± 7.0	58.0 ± 12.1	1 (33.3%)
	G/G	66	71.3 ± 17.2	49.7 ± 17.5	52.9 ± 10.5	34 (51.5%)
−650	A/A	4	83.6 ± 26	63.6 ± 24.8	56.6 ± 6.4	1 (25.0%)
	A/C	29	70.5 ± 19.3	50.4 ± 20.7	53.8 ± 11.8	13 (44.8%)
	C/C	36	70.6 ± 13.6	47.8 ± 12.3	52.2 ± 9.8	21 (58.3%)
CCG_n^b	4/4	19	74.3 ± 15.1	51.5 ± 12.5	54.3 ± 8.1	8 (42.1%)
	4/5	35	68.8 ± 15.6	47.0 ± 17.5	51.5 ± 12.1	21 (60.0%)
	5/5	15	73.3 ± 22	54.2 ± 21	55.4 ± 9.1	6 (40.0%)
Allele						
−686	A	48	71.5 ± 17.3	50.9 ± 18.4	53.7 ± 11.2	23 (47.9%)
	G	51	71.0 ± 15.6	49.0 ± 15.7	52.8 ± 10.5	27 (52.9%)
−684	A	3	72.0 ± 9.6	71.9 ± 9.7	53.4 ± 7.0	1 (33.3%)
	G	69	72.4 ± 10.5	71.3 ± 16.9	49.8 ± 17.2	35 (50.7%)
−650	A	33	72.1 ± 20.2	52.0 ± 21.3	54.2 ± 11.3	14 (42.4%)
	C	65	70.6 ± 16.2	49.0 ± 16.5	52.9 ± 10.7	34 (52.3%)
CCG_n^b	4	54	70.8 ± 15.5	48.6 ± 16.0	52.5 ± 10.9	29 (53.7%)
	5	50	70.2 ± 17.6	49.2 ± 18.7	52.7 ± 11.3	27 (54.0%)

FEV_1%: forced expiratory volume in 1 s of the predicted; FVC%: forced vital capacity of the predicted; GOLD: global initiative for chronic obstructive lung disease. FVC%, FEV_1%, and FEV_1/FVC (%) data are presented as mean ± standard deviation.
[a]Relative luciferase activity: L, low; M, medium; and H, high.
[b]Copy number of CCG at −20 to −6.

There is evidence to suggest that Nrf2 plays an important role in COPD pathogenesis [3,8–12]. Nrf2 activation has been advocated as a possible treatment for COPD [36]. The haplotypes of the *Nrf2* promoter have different luciferase activities and associated with the severity of COPD. Five models have been proposed to account for Nrf2 nuclear accumulation and the accompanying gene induction that occurs upon redox stress: Nrf2 release from cytoplasmic anchoring of Keap1; Nrf2 release from nuclear sequestration to ARE (antioxidant response element) enhancer; Nrf2 protein stabilization; antagonism of Nrf2 nuclear-cytoplasmic shuttling; Nrf2 gene induction via ARE; and xenobiotic response element on its promoter [37]. The association between luciferase activity of the *Nrf2* gene promoter and severity of COPD suggests that the last model may be operative in COPD pathogenesis.

Airway inflammation is prevalent in stable COPD and is contributed to by many inflammatory cells and related cytokines. Airway inflammation is amplified during exacerbation [38]. During acute exacerbation, the augmented airway inflammation is associated with neutrophil influx and increased oxidative stress [39]. Cellular compositions and the extent of oxidative stress in the airways of COPD subjects differ between the stable state and acute exacerbation. Gene transcription is affected by many factors, such as cytokines and signal transduction molecules, and it differs from cell type to cell type [40]. Compared to mRNA levels in non-emphysematous subjects, emphysema patients have the same *Nrf2* mRNA levels in whole lung tissue [9] but lower levels in alveolar macrophages [11]. Airway inflammation in COPD may stimulate cells to produce various profiles of *Nrf2* mRNA expression, which changes further in acute exacerbation. The result that the presence of LH or MM haplotypes is related to the development of respiratory failure is interesting, but its clarification requires further study.

Haplotypes of the *Nrf2* gene promoter are associated with differences in luciferase activity and are related to COPD severity. The presence of certain haplotypes, specifically LH or MM, is associated with the development of respiratory failure.

Acknowledgements

This study was supported by grants from Chang Gung Memorial Hospital at Keelung (CMRPG240121 and CMRPG240151).

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