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Functional SNPs of the *CCL5* gene and non-emphysematous phenotype in patients with COPD

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Abstract

We previously reported that a *gain-of-function* -28G allele at the promoter SNP (-28C>G) in the *CCL5* gene was associated with susceptibility to late-onset asthma in patients who developed asthma at age 40 or older. The clinical diagnosis of COPD includes emphysema and small airway disease, and upregulation of CCL5 has been described in the airways of patients with COPD. We hypothesized that the *CCL5* gene has a genetic impact on the variable expression of emphysema in patients with COPD.

We studied 267 patients with COPD. All patients underwent high-resolution lung CT scans, and visual scoring (CT score) was performed to determine emphysema severity. We genotyped 3 SNPs at the *CCL5* gene, including -403G>A, -28C>G, and +375T>C.

We found a significant difference in CT score according to the *CCL5* genotypes; the -28G allele was inversely associated with CT score (p=0.00038). When the analysis was confined to 180 patients with bronchial reversibility less than 15%, even stronger evidence for this association was noted (p=0.00002).

Functional SNPs in the *CCL5* gene were associated with milder emphysema. Together with our previous findings, our studies may identify the *CCL5* gene as a common pathway in the pathogenesis of late-onset asthma and COPD with milder emphysema.

Introduction

We previously found that the -28C>G promoter polymorphism in the *CCL5* gene, which has been associated with increased levels of mRNA expression and protein *in vitro* [1], was associated with susceptibility to late-onset asthma among patients who developed disease after age 40 [2]. In general, late-onset asthma is not strongly associated with specific allergen sensitization. Rather, infections, including respiratory viruses, may be more likely to be involved in the pathophysiology of late-onset asthma through host response mechanisms [3]. Viral infections are associated with most exacerbations of asthma and COPD [4-8], and the most prominent aspect of the epithelial immune response toward viral respiratory infections consists of the production and release of CCL5 [9-12]. In fact, an exacerbation in mild COPD is associated with the upregulation of CCL5 in both inflammatory and epithelial cells of the bronchial mucosa [13, 14].

The chronic airflow limitation associated with COPD is caused by a mixture of small airway disease and emphysema, the relative contributions of which vary from person to person [15]. These phenotypic variations of COPD may be influenced by several innate susceptibility factors to environmental stimuli, including tobacco smoking and viral respiratory infections. However, the relative importance of genetic factors in the pathogenesis of airway disease and emphysematous components of COPD is unknown.

Given that accumulation of inflammatory immune cells and airway wall remodeling processes are common characteristics in the small airways of patients with asthma and COPD [16], a common genetic susceptibility may be present, with latent viral infections predisposing some patients to experience increased airway inflammation. The *CCL5* gene may be involved in the pathogenesis of epithelial remodeling and chronic hyperreactivity in response to viral infections.

In the current study, using a well-characterized COPD cohort of Japanese subjects [17], we examined the specific hypothesis that functional SNPs at the regulatory region of the *CCL5* gene and their haplotypes have a genetic impact on the variable expression of the emphysematous phenotype in patients with COPD.

Methods

See online depository for additional details.

Study subjects

Among 274 patients with COPD recruited for the Hokkaido COPD cohort study [17], a total of 267 patients, whose genetic samples were available, were examined in the current study. Study approval was obtained from governing ethics committees for each study center, and all subjects provided written informed consent.

Lung computed tomography scans

Information regarding the CT scanners and parameters assessed has been described previously [17]. The severity of emphysema was visually assessed by three independent pulmonologists according to the modified Goddard scoring system [17, 18]; the pulmonologists were blinded to any clinical information regarding the patient. We analyzed 6 images in 3 slices in the lungs, including the aortic arch, the carina, and 1 to 2 cm above the highest hemi-diaphragm. Each image was scored from 0 to 4 as follows: normal (score 0), up to 5% affected (score 0.5), up to 25% affected (score 1), up to 50% affected (score 2), up to 75% affected (score 3), more than 75% affected (score 4); the average score of 6 images was considered representative of the severity of emphysema in the lungs. When the three independent pulmonologists split in their evaluation, we took used the score assessed by the majority.

To confirm the accuracy and reliability of our visual assessment, we compared the severity of emphysema assessed visually with that assessed by three-dimensional computerized analyses. The method of computerized assessment of emphysema for the whole lung was described previously and appears in detail in the online depository. A

strong correlation between the two methods of assessment was found (n=137, r=0.835, $p<0.0001$) [17].

Pulmonary function test

Spirometry was performed before and 30 minutes after a bronchodilator (salbutamol 400 μg) was given. Bronchial reversibility was expressed as the percent change in FEV_1 after salbutamol administration. The carbon monoxide diffusing capacity (DLco) test was also performed, and DLco adjusted with levels of hemoglobin over alveolar ventilation (V_A) was calculated.

Allele-Specific PCR and Detection of Fluorescence-Labeled PCR Fragments

Three SNPs (-403G>A [rs2107538], -28C>G [rs2280788], and +375T>C [rs2280789]) at the regulatory region were genotyped using an assay that combines kinetic (real-time quantitative) PCR with allele-specific amplification, as described previously [2].

Statistical analysis

The linkage disequilibrium (LD) between 3 SNPs was analyzed, and all SNPs were tested for conformation with Hardy-Weinberg expectations in patients with COPD using

Haploview software, version 3.2 [19]. We examined the genetic impact of 3 regulatory SNPs on the CT score using a multivariate stepwise linear regression model. The model included sex, age, smoking status (current or ex), pack-years of smoking, BMI, DLco/V_A, FEV_1 (% predicted), levels of total serum IgE, and peripheral blood eosinophil counts. We also examined the genetic effect of the regulatory SNPs on CT score using only 180 patients whose bronchial reversibility was less than 15% (model 2), because a possibility might remain that the presence of bronchodilator reversibility indicates the presence of coexisting asthma, although patients with physician-diagnosed asthma were carefully excluded from the study [17].

We also examined an association between the extent of emphysema as judged by a low attenuation volume (LAV) automatically assessed by 3D-CT and the 3 SNPs (model 3; n=105).

The association between common haplotypes with a frequency of >1% and CT score was tested using global and haplotype-specific statistics using the Haplo.Score program [20].

Results

The clinical characteristics of subjects are summarized in Tables 1 and 2. The genotypic distribution of all 3 SNPs were in Hardy-Weinberg equilibrium (HWE). Among COPD-related phenotypes including BMI, FEV_1 (% predicted), bronchial reversibility, and CT score, a significant difference was found in CT score according to the -28C>G SNP ($p<0.05$) and the -403G>A SNP ($p<0.05$, Table 2). In the multiple linear regression analysis using 267 patients (model 1), the -28C>G *CCL5* SNP, but neither the -403G>A nor the +375T>C, was significantly associated with CT score. The presence of the -28G allele was significantly associated with lower CT score; mean (SD) levels of CT score were 1.49 (0.93), 1.15 (0.87), and 0.93 (0.69) for the -28CC homozygotes, the -28CG heterozygotes, and the -28GG homozygotes, respectively ($p=0.00038$, Table 3 and Figure 1E in the online depository). In the subgroup analysis (model 2), the association between the -28G allele and lower CT score was the most significant ($p=0.00002$, Table 3) in 180 patients who had bronchial reversibility <15%. A linear dose-response relationship between genotype and phenotype was consistently found between the CT score and the -28C>G genotype. Three independent variables, including smoking status (current or past), baseline FEV_1 (% predicted), and $DLco/V_A$, were consistently associated with CT score in both models, and other variables were excluded from the models. The adjusted R-squared values associated with these fitting models (model 1 and model 2) were 40% and 47%, respectively. This

indicated that the models including smoking, baseline FEV_1 (% predicted), $DLco/V_A$, and the -28C>G genotype explain only 40 to 47% of the variance in CT score, and that unidentified factors other than those studied in the current study also significantly influence the extent of emphysema in patients with COPD.

When we used LAV assessed by computerized analysis as an index of the severity of emphysema, the inverse association with the -28G allele remained significant (p=0.0015, Figure 1E), even though the number of patients in this analysis was limited (n=105). Because of the low frequency of the homozygous mutant genotype, we also analyzed data by combining this group with the heterozygote. When we compared the homozygous wild-type with the combined genotype (heterozygote and homozygous mutant), a similar inverse association was found between the emphysema score and the presence of the -28G allele in model 1 (p=0.00059), model 2 (p=0.00005), and model 3 (p=0.0049).

Three regulatory SNPs (–403G/A, -28C/G, and +375C/T) were in a significant LD; 3 SNPs were shown to be part of a single haplotype with *D′* values ranging from 0.94 to 0.98. The 4 most common haplotypes constituted 95.9% of haplotypes in the 267 patients with COPD (Table 4). The haplotype comprising 3 SNPs was significantly associated with the CT score (global p-value=0.0023). The haplotype -403A/-28G/+375C was most strongly associated with lower CT scores (p=0.0010), as judged by haplotype-specific scores

on the basis of 10 000 simulations, whereas the -403G/-28C/+375T haplotype was associated with higher CT scores (p=0.014, Table 4). Confining the analysis to 180 patients without bronchial reversibility strengthened the association between the haplotype and CT score (global p-value=0.00075); the -403A/-28G/+375C haplotype was inversely associated with CT score (p=0.00015), and the -403G/-28C/+375T haplotype was associated with CT score (p=0.0011). However, the association observed in the haplotype analysis was not stronger than that observed for the single locus analysis using the -28C>G SNP.

Discussion

COPD is a heterogeneous condition including emphysema and small airway disease. In the current study, we investigated the genetic effects of functional alleles at the *CCL5* gene regulatory region in a well-characterized cohort of 267 patients, and found that the *gain-of-function* allele was inversely associated with the severity of emphysema in patients with COPD. Given that upregulation of CCL5 in the airways has been associated with an exacerbation of COPD, and after identifying a significant association between the *CCL5* -28G allele and late-onset asthma [2], we were able to test whether the allele has a genetic effect on variable COPD phenotypes in a hypothesis-driven association study. This type of study is more powerful statistically than the typical association study that tests multiple genes

with no *a priori* hypothesis. By investigating patients with asthma and COPD, a series of our studies may identify the *CCL5* gene as a shared genetic risk factor for these chronic inflammatory airway diseases.

Three common SNPs with functional relevance have been identified (*-403G>A, -28C>G,* and *+375T>C*) in the regulatory region of the *CCL5* gene; these 3 SNPs influence transcriptional activity *in vitro* and subsequent CCL5 expression in human cell lines [1, 21, 22]. These SNPs were associated with increased protein levels of CCL5 [2, 23] as well as increased blood eosinophil counts [24]. These SNPs have also been associated with several inflammatory immune diseases including asthma, allergic rhinitis, and atopic dermatitis [2, 21, 23, 25]. In the context of haplotypes involving these SNPs, the -28G/-403G haplotype was associated with near-fatal asthma in Chinese children [24]. In addition, the -403A/-28G haplotype was shown to be associated with a slower rate of $CD4^+$ T-cell depletion in HIV-1-infected Japanese subjects [1], and haplotypes that included +375C displayed a strong dominant association with rapid progression to AIDS among HIV-1-infected individuals in African-American, European-American, and combined cohorts [22]. Therefore, we believe that the genetic association observed in the present study is due to the functional consequences that these functional SNPs have on *CCL5* transcriptional activity, although we cannot exclude the possibility that they act as markers of another important genetic

abnormality without being functionally relevant themselves. It is also interesting to note that the frequency of the -28G allele differs according to ethnicity: the frequency of the -28G allele is about 15 to 20% in Asians including Japanese, Chinese and Koreans, while it is very low (up to 2%) in Caucasians and African-Americans. Therefore, the genetic impact of the -28G allele observed in our studies seems to be clinically important, especially in Asian populations.

In patients with COPD, relative contributions of small airway disease and emphysema on the degree of airflow limitation vary [15]. Indeed, we have shown that severity of emphysema is widely varied even in the same stage of COPD [17], and thus COPD patients with milder emphysema despite severe airflow limitation could be considered as having predominantly small airway disease. Within the context of our previous finding that the -28G allele was associated with late-onset asthma, the current observation of an inverse association between the allele and CT score in patients with COPD leads to a specific hypothesis that an increased severity of small airway disease caused by a gain effect of the -28G allele may underlie chronic inflammation and remodeling at the small airways of late-onset asthma and COPD with milder emphysema. Alternately, a low attenuation area may reflect hyperlucency due to air trapping rather than emphysema itself, thus confounding the assumption that the CT score purely indicates the extension of

emphysema [26]. However, we took high-resolution CT scans at full inspiration; thus this latter possibility would be less likely compared with the case where conventional CT scans were taken at expiration. In addition, if low attenuation areas reflected not only emphysema but also hyperlucency due to air trapping, one would expect a good correlation between CT scores and airflow limitation; however, this was not the case in our population [17].

Increased prevalence of viral infections, as well as the persistence of cells expressing viral proteins in patients with asthma [4, 5] or COPD [6-8], have been reported, suggesting that viral infections such as human rhinovirus (HRV) and respiratory syncytial virus (RSV) may play a critical role in the pathogenesis of airway inflammation and subsequent deterioration in lung function in patients with asthma and COPD. Studies of respiratory secretions from individuals with RSV bronchiolitis showed that CCL5 is highly expressed [27], suggesting a special role for this chemokine in antiviral defense. Interestingly, a genetic variant of key receptor for CCL5, CC chemokine receptor 5, has been associated with the severity of bronchiolitis caused by RSV [28]. Viral infections are the most likely cause of CCL5 upregulation, and the epithelium at the small airways is a considerable source of CCL5. The presence of the *gain-of-function* allele as a common susceptibility factor to asthma and COPD with milder emphysema predisposes patients to greater expression of CCL5 in

response to prolonged and repeated exogenous stimuli, including viral antigens, leading to amplified inflammation at the small airways.

In a mouse model with targeted disruption of the *CCL5* gene, immune-regulatory and anti-apoptotic effects of CCL5 have been suggested [29], which are distinct from ones that have been previously identified in the setting of infection, such as initiating antiviral responses and airway inflammation by enhancing inflammatory cell recruitment. The functional properties of CCL5, if any, in humans may give an alternative explanation for our findings, such as that the presence of the -28G allele predisposes an individual to some type of protection against the development of emphysema. However, we believe that this possibility is less likely given that the -28G allele has also been associated with late-onset asthma and that increased small airway pathology is a common cardinal feature of asthma and COPD.

Although it is difficult to discriminate asthma from COPD in some older patients, we believe that the findings in the current study were not due to untoward inclusion of patients with late-onset asthma carrying the -28G allele. Among 267 patients with COPD, no correlation was found between CT score and levels of bronchial reversibility, levels of total serum IgE, peripheral blood eosinophil counts, or the frequency of atopy, which makes it unlikely that inclusion of asthmatic patients occurred more often in a group with milder

emphysema. In addition, the genetic association between the -28G allele and CT score became even stronger when we limited the analysis to patients who had bronchial reversibility less than 15% in order to lower the risk of unknowingly including patients with asthma.

Although DLco appears to be the best single physiologic measurement of emphysema severity, we found that the -28C>G SNP was significantly associated only with visually assessed CT score and not with DL/V_A. We noted wide variations in CT scores, even in patients who had the same levels of DL/V_A (Figure 2E in the online depository), despite the finding that DL/V_A was significantly associated with CT score ($p<0.0001$). Levels of DLco are usually influenced by parenchymal destruction involving respiratory bronchioles, alveoli, and the pulmonary capillary system, and they are reduced in patients with emphysema because of the loss of alveolar-to-capillary surface. However, DLco may be relatively insensitive to the loss of surface area for gas exchange when ventilation and perfusion in the lung remain well matched. Levels of DLco may also be influenced by several other factors, including pathology of alveolar septa, inequality of blood/gas distribution in the lung, and lung volume at the time of measurement, even if correction is made by alveolar volume (V_A). Therefore, we believe that the CT score indicates the emphysema severity more specifically than DL/V_A in this study.

We used a visual scoring system to assess the extent of emphysema according to the modified Goddard scoring system [17, 18]. Given that automatically calculated parameters such as LAV may be a more sensitive technique for detecting and quantifying pulmonary emphysema *in vivo*, visual assessment of emphysema is a limitation of the current study that could bias the results. However, when we compared the severity of emphysema assessed by visual score with that assessed by computerized analysis, we found a strong correlation between these two approaches [Figure 3E in the online depository, 17]. In addition, a sub-analysis using automatically calculated emphysema scores confirmed the inverse association between the functional -28G allele and the severity of emphysema. In addition, computerized analysis may not be easy to obtain in many centers, and therefore, it may not be suitable for genetic studies of complex diseases such as COPD, especially as these studies usually require a large number of subjects to identify rather small effects of a gene or genes and because they also require replication studies from independent centers.

In conclusion, together with our previous finding of an association between the -28G allele and the development of late-onset asthma, the current study indicates that the *CCL5* gene may be involved in the common pathogenesis underlying late-onset asthma and COPD with milder emphysema. Our findings lead to speculation that specific components of the innate immune system may manifest an aberrant antiviral response as a basis for

chronic inflammatory lung diseases such as asthma and COPD. Further studies of the disease phenotype presented by the current study are needed to improve our understanding of the underlying pathophysiology and to elucidate potential treatment modalities of the complex disease labeled COPD.

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Table 1 Baseline characteristics of study subjects

	Patients with COPD all patients	Patients with COPD bronchial reversibility <15%
N	267	180
Age (mean, SD)	69.6 (8.1)	68.9 (8.3)
Sex (M/F)	251/16	168/12
BMI (SD)	22.3 (3.2)	22.1 (3.1)
Current smoker (%)	27.3	30.6
Pack-year (median, range)	56.0 (12, 220)	54.5 (12, 220)
FEV1/FVC (%)	50.2 (12.1)	53.3 (11.5)
FEV1(%predicted)(SD)	63.4 (21.7)	68.7 (22.4)
*Bronchial reversibility (%)	13.3 (13.3)	5.87 (4.85)
CT score (SD)	1.40 (0.92)	1.41 (0.93)
DLco/V_A (SD)	63.2 (24.2)	62.3 (24.0)
Atopy (%)	24.1	24.9
Total serum IgE (logIU/ml)	1.78 (0.69)	1.81 (0.71)
Log[eosinophil count/mm3] (SD)	2.20 (0.33)	2.18 (0.32)
-403G>A		
GG	106	69
GA	122	82
AA	36	28
-28C>G		
CC	196	133
CG	61	41
GG	6	3
+375T>C		
TT	117	75
TC	116	79
CC	34	26

*Bronchial reversibility = [post bronchodilator FEV1 - prebronchodilator FEV1]/pre bronchodilator FEV1. We failed to obtain genotypes of the -28C>G SNP for 4 patients with COPD and failed to obtain genotypes of the -403 G>A SNP for 3 patients with COPD.

Table 2 Patient characteristics according to the 3 CCL5 polymorphisms

	-403G>A (N=264)			-28C>G (N=263)			+375T>C (N=267)		
	GG	GA	AA	CC	CG	GG	TT	TC	CC
Age (mean, SD)	69.5 (8.09)	69.9 (8.08)	68.2 (8.4)	69.4 (7.96)	70.7 (8.6)	66.0 (7.52)	69.8 (7.95)	69.7 (8.23)	68.6 (8.36)
Sex (M/F)	104/2	113/9	31/5*	184/12	58/3	5/1	115/2	106/10	30/4*
BMI (SD)	22.4 (3.35)	22.4 (3.24)	21.7 (2.71)	22.5 (3.24)	21.8 (3.18)	21.5 (3.31)	22.3 (3.30)	22.5 (3.27)	21.6 (2.72)
Current smoker (%)	26.4%	26.2%	33.3%	27.0%	26.2%	66.7%	27.4%	25.9%	32.4%
Pack-year	57	55	56	57	50	76.5	57	55	59.9
(median, range)	(12.5, 160)	(12, 220)	(19-132)	(12.5-220)	(12-174)	(38.3-105)	(12.5-57)	(12, 220)	(19,132)
FEV1(%predicted) (SD)	54.6 (19.6)	58.4 (25.5)	63.3 (19.1)	57.5 (23.3)	58.0 (21.0)	61.0 (76.9)	55.1 (19.0)	58.5 (26.2)	62.1 (18.9)
Bronchial reversibility (%)	10.53%	10.22%	6.09%	9.52%	9.27%	11.21%	10.78%	9.71%	6.59%
median (range)	-10.1, 69.2	-2.99, 60.0	-5.19, 52.7	-10.1, 69.2	-5.19, 60.0	-0.64, 25.9	-10.1, 69.2	-2.99, 60.0	-5.19, 52.7
DLco/VA	61.9 (24.2)	64.2 (25.1)	64.1 (22.4)	65.0 (24.3)	58.8 (23.2)	61.0 (24.6)	61.2 (24.1)	65.3 (24.7)	63.1 (22.5)
CT score (SD)*	1.53 (0.94)	1.38 (0.89)	1.17 (0.90)*	1.46 (0.93)	1.26 (0.87)	0.88 (0.69)*	1.51 (0.92)	1.36 (0.92)	1.2 (0.90)
Atopy (%)	25	22.1	30.6	26	18	33.3	25.2	21.6	29.4
Total serum IgE (logIU/ml)	1.82 (0.68)	1.75 (0.70)	1.80 (0.72)	1.78 (0.70)	1.81 (0.65)	1.67 (0.87)	1.83 (0.68)	1.75 (0.71)	1.74 (0.68)
Log[eosinophil count] (SD)	2.20 (0.32)	2.23 (0.32)	2.13 (0.36)	2.19 (0.34)	2.24 (0.29)	2.19 (0.20)	2.20 (0.32)	2.24 (0.32)	2.12 (0.36)
COPD stage									
I	18	33	10	47	14	0	20	32	9
II	53	49	19	86	30	6	62	44	18
III	31	33	6	52	16	0	31	33	6
IV	4	7	1	11	1	0	4	7	1

*$p<0.05$

One-way analysis of variance or chi-square test was used when appropriate.

Table 3 Linear regression analyses of the functional *CCL5* polymorphisms with CT score

(A) All patients with COPD (model 1)

	CT score		N	p-value*
	mean	SD		
-403GG	1.48	0.94	105	0.063
-403GA	1.39	0.89	121	
-403AA	1.24	0.90	36	
-28CC	1.49	0.93	196	0.00038
-28CG	1.15	0.87	61	
-28GG	0.93	0.69	6	
+375TT	1.44	0.92	116	0.12
+375TC	1.40	0.90	115	
+375CC	1.24	0.92	34	

(B) Patients with a bronchial reversibility < 15% (model 2)

	CT score		N	p-value*
	mean	SD		
-403GG	1.57	0.94	69	0.006
-403GA	1.35	0.89	82	
-403AA	1.15	0.90	28	
-28CC	1.53	0.93	133	0.00002
-28CG	1.05	0.87	41	
-28GG	0.70	0.69	3	
+375TT	1.54	0.92	75	0.009
+375TC	1.36	0.90	79	
+375CC	1.14	0.92	26	

*Linear regression models were applied to test the association between 3 polymorphisms and the CT score.

Smoking status, DLco, and prebronchodilator FEV1 (% predicted) were consistently associated with CT score in models 1 and 2.

Table 4 Haplotypes comprised of 3 *CCL5* SNPs and CT score

(A) All patients with COPD (N=267)

	-403	-28	375	frequency	haplotype-specific score	empirical-*p*
[1]	A	G	C	0.132	-3.218	0.00101
[2]	A	C	T	0.017	-0.344	0.72
[3]	A	C	C	0.210	1.021	0.31
[4]	G	C	T	0.609	2.450	0.0143

Global-*p* = 0.0023

(B) Patients with bronchial reversibility < 15% (N=180)

	-403	-28	375	frequency	haplotype-specific score	empirical-*p*
[1]	A	G	C	0.125	-3.746	0.00015
[2]	A	C	T	0.017	-0.017	0.99
[3]	A	C	C	0.235	-0.084	0.93
[4]	G	C	T	0.594	3.234	0.0011

Global-*p* = 0.00075

Frequencies of haplotypes comprised of the 3 CCL5 polymorphisms were significantly associated with CT score based on p-values from 10000 simulations of global score tests, as implemented in Haplo.score (20).

The analyses were adjusted for smoking status, DLco, and prebronchodilator FEV1 (% predicted).

Note that global score test does not give effect estimates, whereas negative Haplo-specific scores are associated with a protective effects and positive haplotype-specific scores are associated with an increased risk.