Polymorphism of Clara Cell 10-kD Protein Gene of Sarcoidosis

Takashi Ohchi, Noriharu Shijubo, Isao Kawabata, Shingo Ichimiya, Shin-ichiro Inomata, Akihiro Yamaguchi, Yoshifumi Umemori, Yoshihisa Itoh, Shosaku Abe, Yomei Hiraga, and Noriyuki Sato

Third Department of Internal Medicine; Department of Pathology, Sapporo Medical University School of Medicine; Department of Respiratory Medicine, Sapporo Hospital, Hokkaido Railway Company, Sapporo; and Department of Laboratory Medicine, Asahikawa Medical College, Asahikawa, Japan

Clara cell 10-kD protein (CC10) exhibits potent antiinflammatory properties. G38A polymorphism was found in the CC10 gene. We investigated the genetic influence of the allele on the development of sarcoidosis using case control analysis in a Japanese population (265 sarcoidosis cases and 258 control subjects). The A allele frequency in sarcoidosis cases (45.1%) was significantly higher than healthy control subjects (34.9%, $p = 0.0002$). According to outcomes, we divided 223 patients with follow-up periods of 3 years or more into two subgroups (55 progressive and 168 regressive disease). The A allele frequency in patients with progressive disease was significantly higher than control subjects (odds ratio = 4.55; 95% confidence interval, 2.97–6.97; $p < 0.0001$), whereas that of regressive disease was not. The A/A genotypes had significantly lower bronchoalveolar lavage fluid CC10 levels than the G/G (nonsmokers, $p = 0.0054$, and smokers, $p = 0.0045$) and G/A genotypes (nonsmokers, $p = 0.0022$, and smokers, $p = 0.0402$). The reporter gene assay showed significantly lower reporter activities in the presence of interferon-γ for the 38A construct than the 38G construct ($p = 0.0177$). The G38A polymorphism in the CC10 gene may influence protein expression and be associated with the development of progressive sarcoidosis.

Keywords: Clara cell 16-kD protein; Clara cell secretory protein; protein 1; secretoglobin; uteroglobin

Sarcoidosis results from marked macrophage and $CD4^+$ helper T-cell activity, immune dysregulation, and formation of noncaseating granulomas in affected organs. Although the initiating antigen(s) remains unknown, familial aggregation and ethnic predominance suggest that an inherited susceptibility to sarcoidosis exists (1, 2). Attempts to identify sarcoidosis susceptibility genes have focused on the genes residing in the major histocompatibility complex, particularly the HLA genes and immunorelevant genes (1–6). In addition, T-cell and macrophage/monocyte activations are restricted to the involved organs, despite the systemic nature of the disease. In approximately 70% of patients with sarcoidosis, spontaneous regressions are observed (7, 8). Mechanisms for downregulating inflammation should be present in sarcoidosis; however, the prerequisites or mechanisms for induction of spontaneous regression are unknown.

Clara cell 10-kD protein (CC10) is the predominant product in nonciliated bronchiolar epithelial cells (Clara cells) (9). CC10 is a homodimer of polypeptide of 70 amino acids covalently bound in an antiparallel manner (10). CC10 has been referred to as the Clara cell phospholipid binding protein, the Clara cell secretory protein, the Clara cell 16-kD protein, or the polychlorinated biphenyl–binding protein and is identical to uteroglobin (UG) and protein 1/urinary protein 1 (9). At a nomenclature meeting held during a symposium on the UG/Clara cell protein family (9), a new generic name of secretoglobin was coined (9, 10). CC10/UG is assigned as secretoglobin 1A1 in the secretoglobin family. CC10/UG possesses varied biochemical and biological properties, including phospholipase A2- (9) and phospholipase C-inhibitory activity (11). CC10 was shown to be a potent inhibitor of IFN-γ (8, 12). Inversely, IFN-γ enhanced CC10 expression in airway epithelial cells (13–15). CC10 levels have been reported to change in the lung fluid and serum of inflammatory lung diseases (16–18), including sarcoidosis (8, 19). CC10 deficiency results in increased sensitivity to hyperoxia-induced lung injury by increasing proinflammatory cytokine expression (20). The evidence has led to speculation that CC10 may function as a downregulator of inflammation in the lung.

There have been several reports of an association between diseases and a guanine–adenine substitution at position 38 (G38A) downstream from the transcription initiation site within the noncoding region of exon 1 of the CC10 gene (21–25). Serum CC10/UG levels were significantly decreased in the A/A genotype patients with asthma (21) and IgA nephropathy (22) compared with those in the G/G and G/A genotypes. We investigated the genetic influence of the A allele of G38A on the development of sarcoidosis and risk of its progression using a case control analysis in a Japanese population. We also compared CC10 levels among the genotypes and analyzed whether the G38A polymorphism is responsible for reduced transcriptional activity of the CC10 gene.

METHODS

Populations

The study was comprised of 265 patients with sarcoidosis and 258 healthy control subjects (Table 1). All participants (n = 523) were Japanese and were mainly from the north of Japan (Hokkaido). All of them gave written informed consent for enrollment in the study. The ethics committees of Sapporo Medical University and Sapporo Hospital, Hokkaido Railway Company approved the study.

A total of 265 patients with sarcoidosis were enrolled in this study. They had histologic findings consistent with sarcoidosis (noncaseating epithelioid cell granulomas) in the lung, scale node, and/or skin without evidence of mycobacterial, fungal, or parasitic infection. None had a history of exposure to organic or inorganic materials known to cause granulomatous lung diseases. Eighteen patients had stage 0 (clear chest radiograph); 167 had stage 1 (hilar lymphadenopathy); 61 had stage 2 (hilar lymphadenopathy and pulmonary infiltrates); 17 had stage 3 (pulmonary infiltrates without hilar lymphadenopathy); and 2 had stage 4 (pulmonary fibrosis without hilar lymphadenopathy). Seventy recent patients were enrolled at the time of diagnosis, and CC10 levels in serum and bronchoalveolar lavage (BAL) fluid were analyzed in this

(*Received in original form April 22, 2003; accepted in final form October 7, 2003*)

Supported in part by Grants-in-Aid for Scientific Research (B) (C) (project number 14370791, 13672411, 13670610) from Japan Society for the Promotion of Science.

Correspondence and requests for reprints should be addressed to Noriharu Shijubo, M.D., Department of Respiratory Medicine, Sapporo Hospital, Hokkaido Railway Company, North-3, East-1, Chuo-ku, Sapporo, 060-0033 Japan. E-mail address: shijubo@jrsapporohosp.com

Am J Respir Crit Care Med Vol 169. pp 180–186, 2004
Originally Published in Press as DOI: 10.1164/rccm.200304-559OC on October 9, 2003
Internet address: www.atsjournals.org

TABLE 1. CHARACTERISTICS OF THE STUDY POPULATION

			Sarcoidosis*	
	Healthy Control Subjects	Sarcoidosis	Regressive Disease	Progressive Disease
Number of subjects	258	265	168	55
Sex, male†	125 (48.4)	85 (32.1)¶	55 (32.7)¶	17 (30.9)¶
Nonsmoker†	149 (57.8)	151 (57.0)	95 (56.5)	30 (54.5)
Age, yr‡	48.7 (14.2)	45.6 (17.1)	45.7 (16.8)	45.4 (17.1)
Stage, 0, 1, 2, 3, +4†	—	17/167/61/19 (6/63/23/7)	14/110/35/9 (8/65/21/5)	0/30/18/7§ (0/55/33/13)
Number of affected organs‡	—	2.2 (0.9)	2.2 (0.9)	2.3 (1.0)
Serum ACE, IU/L, 37°C‡	—	25.4 (12.9), n = 211	24.6 (11.9), n = 134	26.5 (10.0), n = 44

Definition of abbreviation: ACE = angiotensin-converting enzyme.

* Disease progression in sarcoidosis was evaluated in 223 patients with sarcoidosis who had follow-up periods of 3 years or more.

† Values are number (%) or ‡ mean (SD).

¶ $p < 0.05$ vs. healthy control subjects.

§ $p < 0.05$ vs. regressive disease.

The cutoff value of angiotensin-converting enzyme is 21.4 IU/L. Chi-square contingency table analysis or analysis of variance was used where appropriate.

population. Two hundred twenty-three patients who had follow-up periods of 3 years or more (range of 44 to 430 months, median of 112 months) were divided into progressive (n = 55) and regressive disease groups (n = 168). Thirty-six patients (stage 1, n = 18; stage 2, n = 12; stage 3, n = 5; and stage 4, n = 1 at the time of diagnosis) had progressive pulmonary lesions according to the evaluation of chest X-ray films and computed tomography scans and respiratory function test results, and 16 of these 36 patients received long-term corticosteroid therapy. Cardiac sarcoidosis developed in eight patients (stage 1, n = 4; stage 2, n = 3; and stage 3, n = 1), and all needed long-term corticosteroid therapy. Nine patients (stage 1, n = 5; stage 2, n = 3; and stage 3, n = 1) had persistent muscular skin and/or bone lesions without improvement of intrathoracic lesions, and seven of nine patients received long-term corticosteroid therapy. For deterioration of ocular sarcoid lesions, seven patients (stage 1, n = 5 and stage 2, n = 2) needed a long period of corticosteroid therapy. Five of the 60 previously mentioned patients had two major progressive lesions. Thus, the 55 patients were classified into the progressive disease group. Thirty-eight patients received short-term corticosteroid therapy, and after the therapy, they had regression of disease. The remaining 130 patients had spontaneous regression of disease. Therefore, 168 patients were classified into the regressive disease group. BAL analyses at the time of diagnosis were done in 180 of the 265 patients. However, 18 patients who had received corticosteroid therapy were excluded. Therefore, 162 samples (nonsmokers, n = 91, and smokers, n = 71) were analyzed according to the genotypes.

Quantitation of CC10

CC10 concentrations were measured with ELISA employing monoclonal antibody TY-1 and TY-2 to human CC10/protein 1, as described previously (8, 9, 26). This ELISA had 10 times better sensitivity than ELISA using monoclonal antibody 6D4 to CC10 and polyclonal antibody to CC10 (9, 26). All assays were performed in duplicate.

Extraction of Genomic DNA and Genotype Determination

Genomic DNA was extracted from peripheral leukocytes isolated from ethylenediaminetetraacetic acid–anticoagulated blood through use of a commercially available DNA isolation kit (DnaQuick; Dainippon Pharmaceutical, Tokyo, Japan). An allele-specific polymerase chain reaction (PCR) was developed to detect CC10 G38A single nucleotide polymorphism (SNP). Primer sequences used for evaluating CC10 G38A SNP were ccaccagactcagagacggaaccagagaga for CC10 38-FA, cagagacggaaccagacacg for CC10 38-FG, and agcactcaccggagctgca for CC10 38-R. PCR products were generated in 10-μL volumes containing 0.25 U Taq DNA Polymerase (Amersham Pharmacia Biotech, Piscataway, NJ), 50-mM KCl, 1.5-mM $MgCl_2$, 0.2-mM dNTP (Amersham Pharmacia Biotech), primers (5 pmol/μl for CC10 38-FG, 20 pmol/μl for CC10 38-FA, and 5 pmol/μl for CC10 38-R), and 10–20 ng of genomic DNA. Amplification was performed in Gene Amp PCR System 9,700 (Biosystems, Foster City, CA). The PCR conditions were as follows: After an initial denaturation at 95° for 5 minutes, the reactions were cycled 30 times through a temperature profile of 95° for 30 seconds, 64° for 30 seconds, and 72° for 30 seconds. Then a final extension was performed at 72° for 7 minutes.

Gel electrophoretic analysis was performed on Mupid minigel at 100 V for 40 minutes. The PCR products were examined by agarose gel electrophoresis in a 3% agarose gel containing ethidium bromide (1.5 μg/ml). The bands were visualized on an ultraviolet transilluminator at 312 nm and photographed with a Polaroid camera. Genotypes of individual PCR products were confirmed using an ABI automatic sequencer (Perkin Elmer, Wellesley, MA).

Transfection and Reporter-Gene Assays

A 1,015-base pair (bp) fragment (from −960 to 55) of the human CC10 gene promoter was prepared by PCR using two forms (38G/G and 38A/A) of genomic DNA as a template, each of which was separately subcloned into an Nhe I-Hind III site of the pGL3-basic luciferase vector to generate the pGL3 38G and pGL3 38A plasmids. The human adenocarcinoma cell line NCI-H441 was maintained in RPMI-1640 containing 10% fetal calf serum. Cells were incubated in 12-well plates at 50–70% confluence. After changing culture supernatant to RPMI-1640 containing 2% or 10% fetal calf serum, cells were transfected using lipofectamin transfection reagent (Invitrogen Corp., Carlsbad, CA) with 25 ng of reporter plasmid and 25 ng of pCH110 (Amersham Pharmacia Biotech, Uppsala, Sweden) as an internal control. At 12 hours after transfection, the cells were incubated in the presence of IFN-γ (0–1,000 U). After 48 hours, the cells were harvested in Reporter Lysis Buffer (Promega, Tokyo, Japan), and the lysates were assayed for β-galactosidase and luciferase activities using the High-Sensitive β-Galactosidase Assay Kit (Stratagene, La Jolla, CA) and the Luciferase Assay System (Promega Corp.), respectively. To correct for transfection efficiency, luciferase activity was normalized to β-galactosidase activity. Relative luciferase activity of the CC10 promoter constructs was expressed based on the activity of pGL3-basic in the presence of the same *trans*-activating plasmid as 1. Five different assays were performed.

Statistical Analysis

Statistical analyses were performed using Statview software (SAS Institute, Inc., Cary, NC). Data were expressed as mean ± SD. The Mann-Whitney *U* test or Student's *t* test was used to compare two unpaired or paired samples. Contingency tables were analyzed for trends with the chi-square test. A p value of less than 0.05 was considered to be statistically significant.

TABLE 2. ASSOCIATION OF CLARA CELL 10-kD PROTEIN G38A POLYMORPHISM WITH SARCOIDOSIS

	CC10 G38A Genotype					
	G/G	G/A	A/A	OR	95% CI	p Value
Healthy subjects, n = 258	110 (42.6)	116 (45.0)	32 (12.4)			
Sarcoidosis, n = 265	80 (30.1)	131 (49.5)	54 (20.3)	1.60	1.25–2.05	0.0002

Definition of abbreviations: CC10 = Clara cell 10-kD protein; CI = confidence interval; OR = odds ratio.

Values are number (%). Odds ratio (95% confidence interval) was calculated for the presence of 38A allele, using chi-square contingency table analysis.

RESULTS

CC10 G38A SNP

G38A SNP was evaluated in 265 sarcoidosis patients and 258 healthy subjects by allele-specific PCR (Table 2 and Figure 1). Allele-specific PCR is a single-tube PCR-based technique using allele-specific primers that differ in length by 10 bp. Each allele-specific primer was designed to have base mismatches in the 3′ primer sequence to minimize cross-reactions of the PCR products in subsequent cycles. The PCR products are easily examined by electrophoresis on high-resolution gels. G/G and A/A genotypes showed a single band of 120 and 110 bp, respectively, and G/A genotype showed two bands of 110 and 120 bp (Figure 1). The genotypes of CC10 G38A were also confirmed by nucleotide sequencing analysis.

Table 2 lists genotype distributions of CC10 G38A SNP in patients with sarcoidosis and control subjects. G38A SNP fulfilled Hardy-Weinberg expectations for the control subjects. We found the A allele frequency to be significantly higher than the control subjects (odds ratio = 1.60; 95% confidence interval, 1.25–2.05; p = 0.0002).

G38A Genotypes and the Relationship of their Clinical Parameters

The characteristics of the patients were compared according to the genotypes of G38A SNP. There were no significant differences in sex, ages of onset, distributions of radiographic stages, number of affected organs, or angiotensin-converting enzyme activities among the genotypes (Table 3). When data of BAL examinations in nonsmoker and smoker subgroups were analyzed, there were no significant differences in any parameters among the genotypes (Table 4).

The association of G38A polymorphism with risk of disease progression was analyzed in the 223 patients who had follow-up periods of 3 years or more (Table 5). The 38A allele frequency in patients with progressive disease was significantly higher than that in control subjects (odds ratio = 4.55; 95% confidence interval, 2.97–6.97; p < 0.0001). In contrast, there was no significant difference in the A allele frequency between healthy control subjects and patients with regressive disease.

CC10 Levels According to the Genotypes

CC10 levels in the serum and BAL fluid at the time of diagnosis were compared according to the CC10 G38A genotypes. Because it has been reported that smoking habits influence CC10 levels (8, 15, 27), we evaluated CC10 levels in the nonsmoker and smoker subgroups (Figure 2). The A/A genotypes had significantly lower BAL fluid CC10 levels than the G/G (nonsmokers, p = 0.0054, and smokers, p = 0.0045) and G/A genotypes (nonsmokers, p = 0.0025, and smokers, p = 0.0402), and the G/A genotypes showed significantly lower BAL fluid CC10 levels than the G/G genotypes (nonsmokers, p = 0.0022, and smokers, p = 0.0054). Serum CC10 levels were significantly decreased in the A/A genotypes compared with in the G/G genotypes (nonsmokers, p = 0.0088, and smokers, p = 0.0452). Although

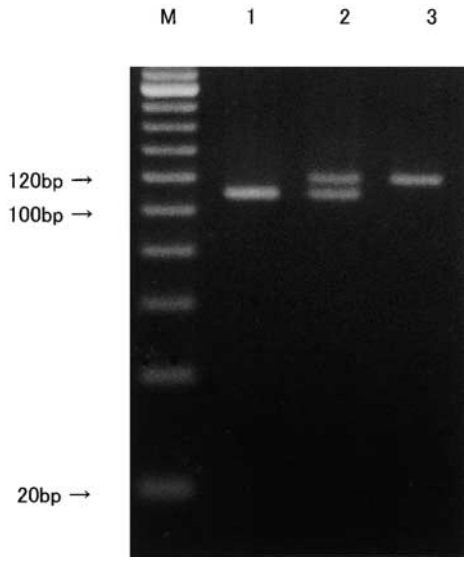

Figure 1. Detection of guanine-adenine substitution at position 38 (G38A) downstream from the transcription initiation site by allele-specific polymerase chain reaction. G/G (*lane 1*) and A/A (*lane 3*) genotypes showed a single band of 120 and 110 base pairs (bp), respectively, and the G/A genotype (*lane 2*) showed two bands of 110 and 120 bp.

TABLE 3. PATIENTS CHARACTERISTICS ACCORDING TO THE CLARA CELL 10-kD PROTEIN G38A GENOTYPES

	CC10 G38A Genotype			
	G/G	G/A	A/A	p Value*
Number of subjects	80	131	54	
Sex, male†	25 (31.3)	40 (30.5)	20 (37.0)	0.76
Nonsmoker†	45 (56.3)	80 (61.1)	30 (55.6)	0.74
Age at onset, yr‡	47.4 (18.4)	44.5 (16.2)	45.5 (17.7)	0.81
Stage, 0, 1, 2, 3, +4†	7/50/16/7 (9/63/20/9)	8/84/30/9 (8/62/23/7)	3/33/15/3 (6/61/28/6)	0.99
Number of affected organs‡	2.1 (0.9)	2.2 (0.9)	2.3 (0.9)	0.86
Serum ACE, IU/L, 37°C‡	22.5 (9.5), n = 68	26.4 (12.3), n = 102	28.4 (14.6), n = 41	0.13

Definition of abbreviations: ACE = angiotensin-converting enzyme; CC10 = Clara cell 10-kD protein.

* Chi-square contingency table analysis or analysis of variance was used where appropriate.

† Values are number (%).

‡ Mean (SD).

Clinical parameters at the time of diagnosis were evaluated according to the genotypes.

TABLE 4. BRONCHOALVEOLAR LAVAGE CHARACTERISTICS ACCORDING TO THE CLARA CELL 10-kD PROTEIN G38A GENOTYPES

	CC10 G38A Genotype			
	G/G	G/A	A/A	p Value*
Nonsmoker				
Number of subjects†	28	42	21	
Total cells, 10^5/ml‡	2.0 (1.5)	2.2 (2.1)	2.4 (1.5)	0.70
Macrophages, %‡	60.4 (14.4)	58.5 (17.8)	65.8 (14.4)	0.25
Lymphocytes, %‡	37.7 (16.9)	39.2 (18.5)	32.8 (14.4)	0.37
Neutrophils, %‡	1.2 (2.2)	1.2 (2.1)	1.1 (1.3)	0.93
Eosinophils, %‡	0.4 (1.4)	0.4 (0.6)	0.3 (0.5)	0.88
CD4, %‡	72.4 (12.1)	74.4 (11.8)	71.2 (15.2)	0.64
CD8, %‡	16.0 (8.1)	15.8 (10.2)	18.6 (13.4)	0.58
CD4/CD8‡	6.6 (4.9)	7.5 (5.4)	6.2 (4.5)	0.62
Smoker				
No of subjects†	20	37	14	
Total cells, 10^5/ml‡	2.5 (1.6)	2.2 (1.4)	1.7 (0.9)	0.17
Macrophages, %‡	75.3 (16.6)	74.6 (16.6)	73.6 (16.1)	0.96
Lymphocytes, %‡	22.8 (16.6)	24.2 (15.9)	24.4 (16.1)	0.94
Neutrophils, %‡	1.0 (1.3)	0.8 (1.1)	1.4 (1.7)	0.37
Eosinophils, %‡	0.8 (1.3)	0.3 (0.7)	0.5 (0.9)	0.20
CD4, %‡	69.7 (11.4)	67.7 (16.5)	68.4 (10.6)	0.89
CD8, %‡	21.8 (9.2)	22.0 (15.8)	22.3 (12.0)	0.99
CD4/CD8‡	4.2 (3.1)	5.2 (5.3)	4.4 (3.0)	0.61

Definition of abbreviation: CC10 = Clara cell 10-kD protein.
* Analysis of variance was used.
† Value are number.
‡ Mean (SD).

insignificant, the A/A genotypes tended to have decreased levels of serum CC10 compared with the G/A genotypes.

Modulation of Transcription of the CC10 Gene by G38A SNP

To examine whether G38A SNP influences promoter activity of the CC10 gene, the promoter sequence, connected to a luciferase reporter gene, was transfected into human lung adenocarcinoma NCI-H441 cells in RMPI-1640 containing 2–10% fetal calf serum (Figure 3). The reporter activities were compared between two constructs containing either G or A at position 38 downstream from the transcription initiation site within the noncoding region of exon 1 in the presence of IFN-γ (1 to 1,000 U/ml). Higher concentrations of fetal calf serum showed low luciferase activities. The result was in accordance with the prior observation (28–30). When examined in RPMI-1640 containing 2% fetal calf serum, 100 and 1,000 U/ml IFN-γ influenced the viability of transfected cells. In the absence of IFN-γ or in the presence of 1 U/ml IFN-γ, there were no significant differences in luciferase reporter activities detected for the 38G and 38A constructs. Significantly lower luciferase reporter activities were detected for the 38A construct in the presence of 10-U/ml IFN-γ than for the 38G construct ($p = 0.0177$). These results suggest that the A allele of G38A may be associated with the decreased transcriptional activity of the CC10 gene when enhanced IFN-γ release occurs in the lung.

DISCUSSION

This study demonstrates a significant increase in the A allele frequency of CC10 G38A SNP in patients with sarcoidosis compared with healthy control subjects. Although there were no significant differences of clinical parameters at the time of diagnosis among the three genotypes, the A/A genotype patients with sarcoidosis had significantly lower serum and BAL fluid CC10 levels than the G/G and G/A genotypes. More importantly, the A allele frequency in patients with progressive disease, but not in patients with regressive disease, was significantly higher than that in control subjects, when analyzed in the 223 patients with sarcoidosis who had follow-up periods of 3 years or more. The evidence suggests that CC10 G38A polymorphism may influence CC10 protein expression and be associated with the development of sarcoidosis and risk of its progression.

CC10-deficient mice are powerful models to investigate the functional roles of CC10 in the development of lung inflammation in asthma (31, 32). As compared with wild-type mice, intensive eosinophilic inflammatory response was provoked by sensitization and challenge with ovablumin in association with elevated levels of Th2 cytokines and eotaxin (31). In another asthma model, airway reactivity and lung inflammation were enhanced in the lung (32). Infiltration of neutrophils was enhanced in an early stage and alucian blue-periodic acid-Schiff–positive mucosubstances were also increased in the airways after ovalbumin exposure (32). The evidence suggests that CC10 modulates lung inflammation and airway responsiveness to inhaled allergens *in vivo*. Clinical investigations demonstrated decreased levels of CC10 in the serum (9, 20) and BAL fluid of patients with asthma (33). Immunohistochemical analysis demonstrated that CC10-positive epithelial cells were decreased in the small airways in patients with asthma (34). It is of note that the accumulation of T cells and mast cells negatively correlated with the ratios of CC10-positive epithelial cells to total epithelial cells in the small airways. In addition, transgenic mice overexpressing interleukin-4 in the airways showed decreased CC10 expression (35). Thus, decreased CC10 may have important implications in the development of chronic lung inflammation in asthma. In contrast, increased levels of serum CC10 were observed in patients with sarcoidosis, and there were no significant elevations of CC10 in the BAL fluid of patients with sarcoidosis and healthy subjects (8, 19). The elevations of CC10 in the serum of patients with sarcoidosis may result from an increased intravascular leakage

TABLE 5. ASSOCIATION OF CLARA CELL 10-kD PROTEIN G38A POLYMORPHISM WITH RISK OF DISEASE PROGRESSION IN SARCOIDOSIS*

	G38A Genotype					
	G/G	G/A	A/A	OR	95% CI	p Value
Healthy subject, n = 258	110 (42.6)	116 (45.0)	32 (12.4)			
Progressive group, n = 55	4 (7.3)	24 (43.6)	27 (49.1)	4.55	2.97–6.97	< 0.0001
Regressive group, n = 168	66 (39.2)	87 (51.8)	15 (8.9)	1.00	0.73–1.37	0.985

Definition of abbreviations: CI = confidence interval; OR = odds ratio.
* Association of Clara cell 10-kD protein G38A polymorphism with risk of disease progression in sarcoidosis was evaluated in 223 patients with sarcoidosis who had follow-up periods of 3 years or more.
The odds ratio (95% confidence interval) was calculated for the presence of 38A allele, using chi-square contingency table analysis.

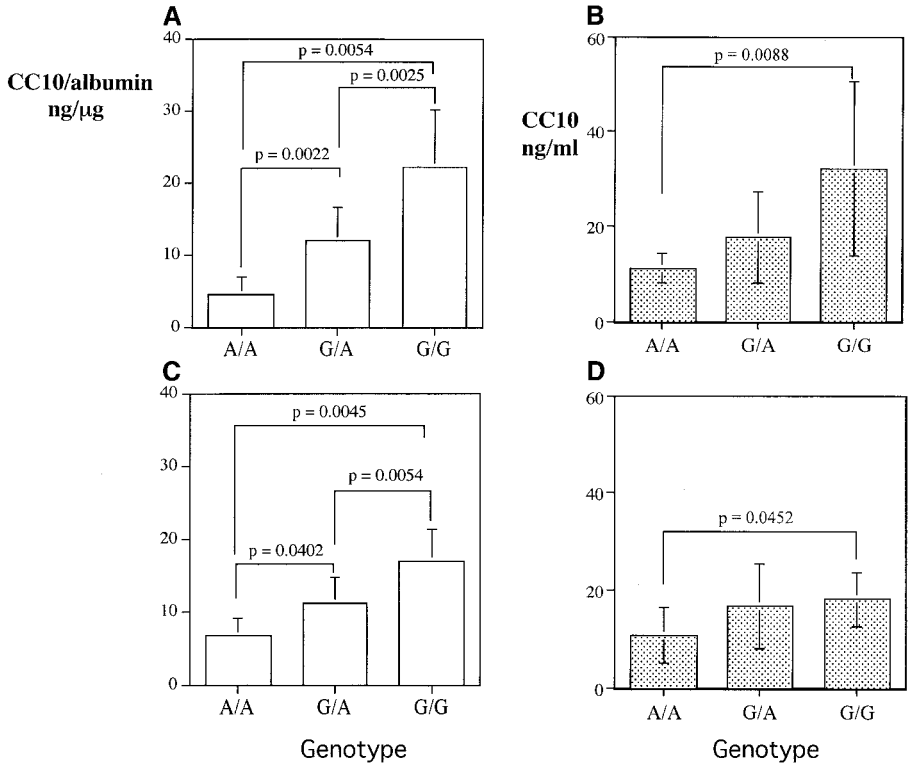

Figure 2. Clara cell 10-kD protein (CC10) levels in the BAL fluid (*A* and *C*) and serum (*B* and *D*) among the three G38A genotypes. *A* and *B* show sarcoid nonsmokers (G/G, n = 12; G/A, n = 19; A/A, n = 8), and *C* and *D* show sarcoid smokers (G/G, n = 9; G/A, n = 16; A/A, n = 6). BAL fluid CC10 levels are normalized for albumin. The Mann-Whitney *U* test was used. *Bars* indicate SD.

of the protein across the air–blood barrier (19). It is noteworthy that CC10 levels in sarcoidosis at the time of diagnosis were significantly increased in the serum and BAL fluid of patients with regressive disease compared with healthy control subjects, and they were significantly decreased in the serum and BAL fluid of patients with progressive disease compared with those with regressive disease (8). Several investigations have demonstrated that administration of IFN-γ enhances CC10 expression in airway epithelial cells (13–15). This evidence may help clarify the mechanism of elevations of CC10 in Th1 cytokine-mediated inflammatory lung diseases such as sarcoidosis.

The structure of the human CC/UG gene is very similar to those of other mammalian species; the two proteins show 53%, 55%, and 61% sequence homology in monkey, rat, and human, respectively, as well as similarities in their tertiary structure (9, 16). The exon–intron boundaries of the human CC10/UG gene are very similar to those of rabbit and mouse. The TATA box and two Octamer-like regions are located in the 5′- flanking region in human, rabbit, and mouse. Several groups have reported transcription factors of the CC10 gene; Forkhead box A (also called hepatocyte nuclear factor-3), activation protein-1, Octamer, and thyroid transcription factor-1 (or NKX 2.1) are involved in the positive regulation of the CC10 gene (9, 36). IFN-γ has been reported to increase CC10 expression in airway epithelial cells (13–15). Ramsay and colleagues (15) demonstrated the molecular mechanism by which IFN-γ stimulates the expression of the CC10 gene in mouse transformed Clara cells and transgenic mice. Deletion mapping and linker-scanning mutations demonstrated that IFN-γ–induced expression of CC10 is regulated, in part, at the level of transcription. *In vitro* and *in vivo* studies verified that the minimal IFN-γ–responsive segment was localized in the proximal 166 bp of the 5′-flanking region. Additionally, the induced expression of CC10 was mediated indirectly through an interferon regulatory factor-1–mediated increase in hepatocyte nuclear factor-3β.

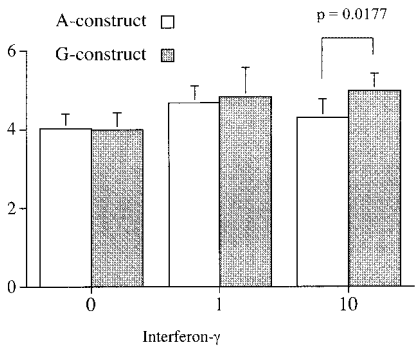

Figure 3. Human CC10 gene reporter assay and G38A polymorphism. Relative luciferase activities of constructs harboring the human CC10 gene sequence from −960 to +55 bp, with either G (38G) or A (38A) at 38 bp, were compared in transient transfection studies using NCI-H441 cells.Luciferase activities are shown; values are based on the activity obtained with the pGL3-Basic vector as 1. Data are means of five experiments. *Bars* indicate SD. Paired Student's *t* test was used.

Human CC10/UG is encoded by a 3-kilobase single-copy gene, which contains three exons and two introns and is localized in the long arm of chromosome 11 (11q12.3-q13.1) (9). There have been several reports associating IgA nephropathy and bronchial asthma with CC10/UG G38A SNP (21–25). Associations of G38A SNP with an increased risk of asthma have been debated. Laing and colleagues (21) studied the CC10 G38A SNP in a matched case control cohort of 67 children with asthma and 46 unaffected children. They reported a 6.9-fold increased risk of developing asthma in the A/A genotypes and a 4.2-fold increased risk in the G/A genotypes. Similar studies of British and Japanese subjects have failed to replicate the G38A genotype association with development of asthma (24). It is of note that serum CC10 levels in the A/A genotype patients with asthma were significantly decreased compared with the G/G and /GA genotypes (21). Very recently, Sengler and coworkers (25) re-

ported that the A allele frequency of G38A was not associated with the development of asthma in a population of Germany children. However, in the children with asthma, $PC_{20}FEV_1$ values were significantly lower in the A/A and G/A genotypes than in the G/G genotypes. A significantly greater decrease was observed in FEV_1 of the A/A genotypes after exercise than was seen in the G/G and G/A genotypes. Several lines of evidence suggest that G38A SNP may influence CC10 protein levels and bronchial hyperreactivity and may be a genetic determinant of disease severity of asthma. Th2 cytokines, including interleukin-4 and interleukin-13, may affect CC10 expression (35, 37). The associations among G38A polymorphism, transcriptional activity of CC10 gene, and Th2 cytokines need to be elucidated.

In this study, the reporter gene assay demonstrated that the point mutation of G to A at 38 bp in the human CC10 gene decreased the reporter luciferase activities in the presence of 10-U/ml IFN-γ, but the point mutation did not influence the reporter luciferase activities in the absence of IFN-γ. In the microenvironment of enhanced IFN-γ release in the lung (Th1 cytokine-mediated inflammatory lung diseases such as sarcoidosis), the mutation of G38A polymorphism may decrease the affinity of a particular nuclear protein to the CC10 gene promoter, resulting in reduced transcriptional activity and ultimately leading to a lower expression of CC10 protein. If patients have no mutation in the CC10 gene, CC10 enhanced by IFN-γ release from inflamed lesions could exert negatively feedback on IFN-γ production leading to immunoregulatory action. In this context, CC10 G38A SNP may influence CC10 protein expression and may be a good marker to predict the risk of disease progression in sarcoidosis.

Conflict of Interest Statement: T.O. has no declared conflict of interest; N.S. has no declared conflict of interest; I.K. has no declared conflict of interest; S.I. has no declared conflict of interest; S-i.I. has no declared conflict of interest; A.Y. has no declared conflict of interest; Y.U. has no declared conflict of interest; Y.I. has no declared conflict of interest; S.A. has no declared conflict of interest; Y.H. has no declared conflict of interest; N.S. has no declared conflict of interest.

References

1. Baughman RP, Lower EE, du Bois RM. Sarcoidosis. *Lancet* 2003;361: 1111–1118.
2. du Bois RM. The genetic predisposition to interstitial lung disease: functional relevance. *Chest* 2002;121:14S–20S.
3. Sato H, Grutters JC, Pantelidis P, Mizzon AN, Ahmad T, Van Houte AJ, Lammers JW, Van Den Bosch JM, Welsh KI, du Bois RM. HLA-DQB1*0201: a marker for good prognosis in British and Dutch patients with sarcoidosis. *Am J Respir Cell Mol Biol* 2002;27:406–412.
4. Grutters JC, Sato H, Pantelidis P, Lagan AL, McGrath DS, Lammers JW, van den Bosch JM, Wells AU, du Bois RM, Welsh KI. Increased frequency of the uncommon tumor necrosis factor −857T allele in British and Dutch patients with sarcoidosis. *Am J Respir Crit Care Med* 2002;165:1119–1124.
5. Hutyrova B, Pantelidis P, Drabek J, Zurkova M, Kolek V, Lenhart K, Welsh KI, du Bois RM, Petrek M. Interleukin-1 gene cluster polymorphisms in sarcoidosis and idiopathic pulmonary fibrosis. *Am J Respir Crit Care Med* 2002;165:148–151.
6. Yamaguchi E, Itoh A, Hizawa N, Kawakami Y. The gene polymorphism of tumor necrosis factor-β, but not that of tumor necrosis factor-α, is associated with the prognosis of sarcoidosis. *Chest* 2001;119:753–761.
7. Zissel G, Homolka J, Schlaak J, Schlaak M, Muller-Querrheim J. Anti-inflammatory cytokine release by alveolar macrophages in pulmonary sarcoidosis. *Am J Respir Crit Care Med* 1996;154:713–719.
8. Shijubo N, Itoh Y, Shigehara K, Yamaguchi T, Itoh K, Shibuya Y, Takahashi R, Ohchi T, Ohmichi M, Hiraga Y, *et al.* Association of Clara cell 10-kDa protein, spontaneous regression and sarcoidosis. *Eur Respir J* 2000;16:414–419.
9. Mukherjee AB, Chilton BS. The uteroglobin/Clara cell protein family. *Ann N Y Acad Sci* 2000;923:1–356.
10. Reynolds SD, Reynolds PR, Pryhuber GS, Finder JD, Stripp BR. Secretoglobins SCGB3A1 and SCGB3A2 define secretory cell subsets in mouse and human airways. *Am J Respir Crit Care Med* 2002;166: 1498–1509.
11. Okutani R, Itoh Y, Yamaguchi T, Kawai K, Singh G. Preparation and characterization of human recombinant protein 1/Clara cell 10 kDa protein. *Eur J Clin Chem Clin Biochem* 1996;34:691–696.
12. Dierynck I, Bernard A, Roels H, Ley MD. Potent inhibition of both human interferon-γ production and biological activity by the Clara cell protein CC16. *Am J Respir Cell Mol Biol* 1995;12:205–210.
13. Yao XL, Ikezono T, Cowan M, Logun C, Angus CW, Shelhamer JH. Interferon-γ stimulates human Clara cell secretory protein production by human airway epithelial cells. *Am J Physiol* 1998;274:L864–L869.
14. Magdaleno SM, Wang G, Jackson KJ, Ray MK, Welty S, Costa RH, DeMayo FJ. Interferon-γ regulation of Clara cell gene expression: in vivo and in vitro. *Am J Physiol* 1997;272:L1142–L1151.
15. Ramsay PL, Luo Z, Magdaleno SM, Whitbourne SK, Cao X, Park MS, Welty SE, Yu-Lee LY, DeMayo FJ. Transcriptional regulation of CCSP by interferon-γ *in vitro* and *in vivo*. *Am J Physiol Lung Cell Mol Physiol* 2003;284:L108–L118.
16. Hermans C, Bernard A. Lung epithelium-specific proteins: characteristics and potential applications as markers. *Am J Respir Crit Care Med* 1999;159:646–678.
17. Lesur O, Bernard A, Arsalane K, Lauwerys R, Bégin R, Cantin A, Lane D. Clara cell protein (CC16) induces a phospholipase A2-mediated inhibition of fibroblast migration in vitro. *Am J Respir Crit Care Med* 1995;152:290–297.
18. Shijubo N, Itoh Y, Yamaguchi T, Sugaya F, Hirasawa M, Yamada T, Kawai T, Abe S. Serum levels of Clara cell 10-kDa protein are decreased in patients with asthma. *Lung* 1999;177:45–52.
19. Hermans C, Petrek M, Kolek V, Weynand B, Pieters T, Lambert M, Bernard A. Serum Clara cell protein (CC16), a marker of the integrity of the air-blood barrier in sarcoidosis. *Eur Respir J* 2001;18:507–514.
20. Johnston CJ, Mango GW, Finkelstein JN, Stripp BR. Altered pulmonary response to hyperoxia in Clara cell secretory protein deficient mice. *Am J Respir Cell Mol Biol* 1997;17:147–155.
21. Laing IA, Hermans C, Bernard A, Burton PR, Goldblatt J, Le Souef PN. Association between plasma CC16 levels, the A38G polymorphism, and asthma. *Am J Respir Crit Care Med* 2000;161:124–127.
22. Matsunaga A, Numakura C, Kawakami T, Itoh Y, Kawabata I, Masakane I, Suzuki T, Suzuki M, Goto T, Itoh K, *et al.* Association of the uteroglobin gene polymorphism with IgA nephropathy. *Am J Kidney Dis* 2002;39:36–41.
23. Laing IA, Goldblatt J, Eber E, Hayden CM, Rye PJ, Gibson NA, Palmer LJ, Burton PR, Le Souef PN. A polymorphism of the CC16 gene is associated with an increased risk of asthma. *J Med Genet* 1998;35: 463–467.
24. Mansur AH, Fryer AA, Hepple M, Strange RC, Spiteri MA. An association study between the Clara cell secretory protein CC16 A38G polymorphism and asthma phenotypes. *Clin Exp Allergy* 2002;32:994–999.
25. Sengler C, Heinzmann A, Jerkic SP, Haider A, Sommerfeld C, Niggemann B, Lau S, Forster J, Schuster A, Kamin W, *et al.* Clara cell protein 16 (CC16) gene polymorphism influences the degree of airway responsiveness in asthmatic children. *J Allergy Clin Immunol* 2003;111: 515–519.
26. Yamaguchi T, Yamada T, Okutani R, Shijubo N, Singh G, Itoh Y. Characterization of monoclonal antibodies to human protein 1/Clara cell 10 kilodalton protein. *Clin Chem Lab Med* 1999;37:631–637.
27. Robin M, Dong P, Hermans C, Bernard A, Bersten AD, Doyle IR. Serum levels of CC16, SP-A and SP-B reflect tobacco-smoke exposure in asymptomatic subjects. *Eur Respir J* 2002;20:1152–1161.
28. Braun H, Suske G. Combinatorial action of HNF3 and Sp family transcription factors in the activation of the rabbit uteroglobin/CC10 promoter. *J Biol Chem* 1998;273:9821–9828.
29. Ray MK, Magdaleno SW, Finegold MJ, DeMayo FJ. Cis-acting elements involved in the regulation of mouse Clara cell-specific 10-kDa protein gene: *in vitro* and *in vivo* analysis. *J Biol Chem* 1995;270:2689–2694.
30. Stripp BR, Huffman JA, Bohinski RJ. Structure and regulation of the murine Clara cell secretory protein gene. *Genomics* 1994;20:27–35.
31. Chen LC, Zhang Z, Myers AC, Huang SK. Cutting edge: altered pulmonary eosinophilic inflammation in mice deficient for Clara cell secretory 10-kDa protein. *J Immunol* 2001;167:3025–3028.
32. Wang SZ, Rosenberger CL, Espindola TM, Barrett EG, Tesfaigzi Y, Bice DE, Harrod KS. CCSP modulates airway dysfunction and host responses in an OVA-challenged mouse model. *Am J Physiol Lung Cell Mol Physiol* 2001;281:L1303–L1311.
33. Van Vyve T, Chanez P, Bernard A, Bousquet J, Godard P, Lauwerijs R, Sibille Y. Protein content in bronchoalveolar lavage fluid of patients with asthma and control subjects. *J Allergy Clin Immunol* 1995;95: 60–68.
34. Shijubo N, Itoh Y, Yamaguchi T, Imada A, Hirasawa M, Yamada T,

Kawai T, Abe S. Clara cell protein-positive epithelial cells are reduced in small airways of asthmatics. *Am J Respir Crit Care Med* 1999;160: 930–933.
35. Jain-Vora S, Wert SE, Temann UA, Rankin JA, Whitsett JA. Interleukin-4 alters epithelial cell differentiation and surfactant homeostasis in the postnatal mouse lung. *Am J Respir Cell Mol Biol* 1997;17: 541–551.
36. Cassel TN, Berg T, Suske G, Nord M. Synergistic transactivation of the differentiation-dependent lung gene Clara cell secretory protein (secretoglobin 1a1) by the basic region leucine zipper factor CCAAT/enhancer-binding protein α and the homeodomain factor Nkx2.1/thyroid transcription factor-1. *J Biol Chem* 2002;277:36970–36977.
37. Kim S, Shim JJ, Burgel PR, Ueki IF, Dao-Pick T, Tam DC, Nadel JA. IL-13-induced Clara cell secretory protein expression in airway epithelium: role of EGFR signaling pathway. *Am J Physiol Lung Cell Mol Physiol* 2002;283:L67–L75.