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CD24 gene exon 2 dimorphism does not affect disease susceptibility in Japanese sarcoidosis patients

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Abstract. *Background:* CD24 proteins are expressed on several inflammatory cells, and play an important role for the T-cell activation. *Objectives:* The aim of this study is to investigate the relationship of a CD24 gene polymorphism to disease susceptibility or clinical findings including bronchoalveolar lavage (BAL) cell profiles in Japanese sarcoidosis patients. *Methods:* A previously reported functional single nucleotide polymorphism (SNP) of CD24 gene exon 2 was examined in 186 Japanese sarcoidosis patients and 146 sex and age-matched healthy controls using restriction fragment length polymorphism method. The distribution of genotypes was compared between the two groups. The association between genotypes or alleles and clinical features or BAL cell profiles was also examined. *Results:* There were no significant differences in the distribution of genotypes or allele frequencies between sarcoidosis and controls. There were also no significant differences in clinical features or BAL cell profiles among patients with different genotypes of CD24. *Conclusions:* There was no relationship between a CD24 exon 2 SNP and disease susceptibility or clinical findings in Japanese sarcoidosis patients. *(Sarcoidosis Vasc Diffuse Lung Dis 2010; 27: 64-69)*

Key words: CD24, single nucleotide polymorphisms, sarcoidosis

Introduction

Sarcoidosis is a systemic granulomatous disease of unknown cause. A combination of genetic and environmental factors is thought to cause the disease. Two robust lines of evidence support the hypothesis of a genetic component in the pathogenesis of sarcoidosis, namely, ethnic variation in its epidemiology, and familial clustering of cases (1, 2). The pathogenesis of sarcoidosis consists of CD4+ T cell activation by antigen and subsequent granuloma formation at the site of involvement. Genes involved in the processes of T cell regulation are candidates for the susceptibility genes of sarcoidosis.

Activation of naive T-cells is critically dependent on two signals: one mediated by interaction of the T-cell antigen receptor (TCR) with specific antigen in association with MHC molecules, and the second an antigen-independent, costimulatory signal provided by interaction between CD28 on the T-cell surface and its ligands B7-1 (CD80), and B7-2 (CD86) on the antigen-presenting cell (APC). CD28 homologue CTLA-4, like CD28, binds members of the B7 family, plays an essential role in the down regulation of T-cell responses (3). We have reported that B7 and CTLA-4 gene polymorphisms

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do not affect disease susceptibility in Japanese sarcoidosis patients (4, 5). Following these reports, genome-wide screening and linkage analysis revealed that butyrophilin-like 2 (BTNL2), a homologue of B7 molecule, was a disease susceptibility gene of sarcoidosis (6). CD24 is a glycosylphophatidylinositol (GPI)- anchored cell surface protein with expression in several inflammatory cells, including activated T cells, B cells and macrophages (7-9). It is well known that CD24 mediates a CD28-independent costimulatory pathway that promotes activation of CD4+ T cells (10). Recently, a CD24 gene exon 2 C/T dimorphism was reported to be a genetic risk factor for susceptibility to and progression of multiple sclerosis (MS), a chronic inflammatory neurodegenerative disease (11). Following that report, an association between this polymorphism and disease susceptibility has also been identified in other inflammatory diseases such as systemic lupus erythematosis (SLE) and giant cell arteritis (12, 13). In the present study, we hypothesized that a CD24 gene exon 2 dimorphism might affect the activation of alveolar lymphocytes with subsequent development of the disease in sarcoidosis patients. In the present study, we investigated the influence of CD24 gene exon 2 dimorphism on disease susceptibility or clinical findings including bronchoalveolar lavage (BAL) cell profiles in Japanese sarcoidosis patients.

Materials and methods

Study design and populations

The study population comprised 186 Japanese sarcoidosis patients, who were followed in Kyoto University hospital and Kyoto Central Clinic/Clinical Research Center. Diagnosis was based on histological findings consistent with sarcoidosis (non-caseating epithelioid cell granulomas) in the lung, lymph 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. Subjects with concurrent autoimmune diseases were excluded from the study. CD24 genotypes were determined in these patients. At their first presentation to the hospital, SACE measurement and classification of chest radiographs was performed as follows: (Stage 0, Normal; Stage I, Bilateral hilar lymphadenopathy (BHL); Stage II, BHL with pulmonary infiltrations; Stage III, Pulmonary infiltrates without BHL). Extrapulmonary lesions were also investigated (14). The control group for genotype analysis consisted of 146 unrelated healthy Japanese subjects without clinical evidence or family history of sarcoidosis. All participants (n=332) were Japanese and from the central part of Japan.

In addition, we retrospectively analyzed BAL results in the subgroup of 84 patients who fulfilled the following criteria: no history of systemic corticosteroid treatment before BAL, no history of smoking in the preceding six months, no history of acute respiratory illness in the preceding four weeks. Characteristics of patients and controls are shown in Table 1.

The study was approved by Ethics Committee of Kyoto University and Kyoto Central Clinic/Clinical Research Center, and all subjects gave written informed consent.

Genotyping of CD24 gene exon 2 C/T dimorphism

The target SNP for CD24 is a replacement of T at nucleotide 226 by C (T/C) in the coding region of exon 2 (GEnbank accession no. NM_013230), which results in a substitution of Ala at amino acid 57 by Val near the GPI anchorage site of the mature protein. DNA was prepared from 10 mL whole blood, using the QIAamp DNA blood mini kit (Qiagen, Hilden, Germany). Amplification of target DNA in exon 2 of the CD24 gene was carried out using the PCR with primers of sequence 5'-TTG TTG CCA CTT GGC ATT TTT GAG GC-3' and 5'-GGATTGGGTTTAGAAGATGGGGA AA-3'. The reaction was performed in a final volume of 50 μl containing 30 ng genomic DNA, 0.5 μmol/L of each primer, 1.25 U Taq polymerase, and 200 μmol/L dNTPs in a buffer of 10 mM Tris-HCl pH 9, with 1.5 mM $MgCl_2$, and 50 mM KCl. Amplification was performed for 30 cycles of 94°C for 60s, 55°C for 60s, and 72°C for 60s. The amplicon was 685bp in length and was visualised on a 3% agarose gel stained with ethidium bromide. The G allele in the dimorphic position contained a Bbr restriction site. Restriction fragment length polymorphism analysis was performed on 5 μL of PCR product, digested in a final volume of 10 μL with 1U

Table 1. Profiles and characteristics of study subjects

	Whole sarcoidosis patients (1)	Patients who underwent BAL (2)	Controls (3)	p value		
				1vs.2	1vs.3	2vs.3
Number of patients	186	84	146			
Sampling age, years	52.4±14	53.4±13	48.9±19	NS	NS	NS
Onset age, years	42.6±16	46.6±13	–	NS	–	–
Male/Female	78/108	28/56	65/81	NS	NS	NS
Chest radiography stage, 0/I/II/III	7/94/71/14	2/45/31/6	–	NS	–	–
Extrathoracic lesions, +/–	150/36	66/18	–	NS	–	–
SACE, IU/L/37°C	25.3±11.3	27.5±12.3	–	NS	–	–

Data are expressed as mean ± SD. SACE = serum angiotensin-converting enzyme (Normal range, 8.3-21.4 IU/l); NS = not significant

Bbv◦ enzyme using the manufacturer's recommended buffer (New England Biolabs, Hertford, UK) at 37°C for 16 hrs. The resulting digestion products were visualized on a 3% agarose gel stained with ethidium bromide.

Bronchoscopy and processing of BAL

Bronchoscopy was performed using a flexible fiberoptic bronchoscope (Olympus IT 200; Olympus Optical Co., Ltd., Tokyo, Japan). Subjects received atropine (0.5 mg i.m.) and hydroxyzine (25 mg i.m.). Fifteen minutes prior to bronchoscopy, local anesthesia was achieved by inhalation of 10 ml aerosol solution of 2% lidocaine. Prewarmed saline (150 ml) was instilled in 3 50 ml aliquots into the right middle lobe or the left lingual and recovered by gentle suction into siliconized containers. Cells were washed in MEM (Nissui, Tokyo, Japan) into the final cell concentration of 2 x 10^6/mm^3, and cytospin preparations were stained with Giemsa for differential counting. All procedures were performed on ice (cell wash and preparation of cytospin specimens)

Evaluation of lymphocyte subpopulations

Flow cytometry was performed with a dual FACScan equipped with an Ar+laser (Becton Dickinson, Mountain View, Calif., USA). Data were collected and analyzed using the CELLQuest programs (Becton Dickinson). The information was collected on a logarithmic scale. The lymphocyte population evaluated was based on detection of CD45 expression by side scatter.

Lymphocyte subsets were identified by staining with monoclonal antibodies as follows: CD3, total T cells; CD4, T helper cells, and CD8, T suppressor/cytotoxic cells. Monoclonal antibodies were directly conjugated to either phycoerythrin (RD1) or fluorescein isothiocyanate. Cells were incubated for 10 min at 4 with monoclonal antibodies, washed with 1% FCS/PBS and analyzed within 24 hr.

Measurement of SACE

SACE activity was measured by Kasahara's method using optical density measurements at 505 nm and 800 nm with a spectrophotometer. Serum samples were considered to be positive if they contained more than 21.4 IU/L (15).

Statistical analysis

Statistical analyses were performed using Statview® software (SAS Institute, Inc., Cary, NC, USA). χ^2 test or Fisher exact probability test were used for comparisons of categorical data among different groups. The Man-Whitney-U test was used to compare age of different groups. A p value of less than 0.05 was considered to be statistically significant. Statistical power calculations were carried out using the PS Program (16).

Results

Distribution of CD24 gene exon 2 dimorphism in sarcoidosis patients and healthy controls

There were no significant differences in genotype and allele frequencies between sarcoidosis patients and control subjects (Table 2). The distribution of these polymorphisms fulfilled Hardy-Weinberg equilibrium expectations in both the sarcoidosis

Table 2. CD24 polymorphism in Japanese subjects with sarcoidosis and controls.

	Sarcoidosis n = 186	Controls n = 146
Genotype frequencies		
CC	71 (38%)	52 (36%)
CT	79 (42%)	69 (47%)
TT	36 (19%)	25 (17%)
P value	NS	
Allele frequencies		
C	221 (59%)	173 (59%)
T	151 (41%)	119 (41%)
P value	NS	

Comparison between the two groups was made using the χ^2 test for genotype frequencies, and the Fisher exact probability test for allele frequencies. No significant differences were found in the distribution of genotype and allele frequencies between the two groups

patients and control subjects. According to power calculations based on our sample size and frequencies of polymorphisms, statistical significance would be reached when the odds ratios attributable to rarer alleles equaled 1.74 for the investigated CD24 SNP.

CD24 gene exon 2 dimorphism and Clinical features

There were no significant differences in onset age, chest radiographic stage, sACE and frequencies of extrathoracic lesions among patients with different genotypes of CD24 exon 2 dimorphism. There were also no significant differences in frequencies of ocular, skin, heart or nervous system lesions among them. Neither the C nor the T allele was associated with any of the clinical features analyzed (data not shown).

CD24 gene exon 2 dimorphism and BAL fluid cell profile

Of 125 patients who had undergone BAL since disease onset, 84 met the inclusion criteria. Of the 41patients excluded, 27 had smoked in the preceding six months, and 14 had received systemic corticosteroid treatment before BAL, including two current smokers. One had acute respiratory infection at BAL, and in the other, BAL recovery was insufficient for analysis. Lymphocyte, T cell and CD4+ cell counts of BAL fluid were not different between patients with different genotypes of CD24 exon 2 dimorphism (Table 3), while a slight distortion of Hardy-Weinberg equilibrium was observed (p=0.041).

Discussion

In this study, we showed that CD24 gene exon 2 dimorphism, a previously reported disease susceptibility gene of MS, was not associated with disease susceptibility or clinical profiles including BAL cell profiles of sarcoidosis.

CD24 or its polymorphism plays a critical role in the pathogenesis of some autoimmune diseases. We targeted a C/T dimorphism at position 170 from the CD24 translation start site (P170) in the CD24 putative cleavage site for the GPI anchor. The $P170^{TT}$ genotype expresses higher cell-surface CD24 than the $P170^{CT}$ or $P170^{CC}$ genotypes, and has an increased risk and more rapid progression of multiple sclerosis (MS), an autoimmune neurodegenerative disease of the central nervous system (11). While

Table 3. Results for the subgroup of patients undergoing BAL and who met the following criteria

	Whole	CC	CT	TT	p-value
Number of patients	84	36	31	17	-
BALF cell recovery %	66.4±34.3	64.0±33.7	67.3±35.1	69.4±35.8	NS
Recovered cells, _105/ml	1.89±1.14	1.92±1.15	2.03±1.21	1.55±0.87	NS
Macrophages, %	66.0±24.9	68.9±18.9	60.9±29.1	69.4±27.1	NS
Lymphocytes, %	32.0±22.0	30.6±18.9	34.8±25.6	29.8±22.0	NS
Neutrophils, %	1.7±9.1	0.3±0.9	4.0±14.8	0.4±0.8	NS
Eosinophils, %	0.2±0.4	0.2±0.3	0.3±0.5	0.3±0.4	NS
Lymphocyte count, $_10^5$/ml	0.71±0.71	0.66±0.59	0.84±0.87	0.56±0.57	NS
T-cell count, $_10^5$/ml	0.61±0.61	0.59±0.53	0.69±0.74	0.47±0.48	NS
CD4+ cell count, $_10^5$/ml	0.54±0.55	0.52±0.50	0.63±0.64	0.39±0.42	NS
CD4+/CD8+ ratio	8.0±8.8	8.0±5.8	9.2±12.5	5.6±4.3	NS

Inclusion criteria: no history of systemic corticosteroid treatment before BAL, no history of smoking in the preceding six months, no history of acute respiratory illness in the preceding four weeks. BALF=bronchoalveolar lavage fluid; NS=not significant. P-values are for comparisons among patients with CC, CT and TT genotypes

sarcoidosis is no longer regarded as an autoimmune disease, it has certain clinical features in common with autoimmune diseases. In addition, sarcoidosis and autoimmune diseases sometimes co-exist (17). Furthermore, the activation of type 1 helper T cells (Th1) is essential for the development of sarcoidosis as well as MS or SLE (18, 19). Sarcoidosis and MS also have a common genetic risk factor. For example, the major histocompatibility human leukocyte antigen (HLA)-DRB1*15 (DR2) haplotype is strongly associated with risk of both diseases (20). In addition, the association of a BTNL2 polymorphism with disease susceptibility of sarcoidosis and MS was reported, although it is thought to be secondary to HLA-DRB1*15, at least in MS (21).

According to the results of the present study, CD24 may not be involved in the mechanism of lymphocyte activation in sarcoidosis, consistent with a pathogenesis of sarcoidosis different from autoimmune diseases such as SLE, MS, and giant cell arteritis. We believe that comparison of disease susceptibility genes between sarcoidosis and autoimmune diseases may provide clues to elucidate similarities and differences in the pathogenesis of these disease entities.

A limitation of this study is that we investigated only one CD24 polymorphism. It has also been demonstrated that another CD24 polymorphism, a dinucleotide deletion in the 3'UTR, conferred a significant protection against the risk of progression of MS and SLE (22). However, a more recent report showed a high degree of linkage disequilibrium between this deletion polymorphism and the exon 2 dimorphism that we studied here (13, 22). A slight distortion of Hardy-Weinberg equilibrium was observed in the patient population undergoing BAL ($p=0.041$). Is is most likely that this distortion was due to the small number of subjects (n=84), although there may be some selection bias in the population. Furthermore, the present study was limited to a Japanese population. Genetic susceptibility to sarcoidosis is different among different ethnicities (23). Thus, further study is required in other populations with different ethnic and ancestral backgounds.

Despite the limitation, we first showed that CD24 exon 2 dimorphism does not affect disease susceptibility or clinical features including BAL cell profiles of sarcoidosis using adequate numbers of Japanese patients.

In conclusion, we demonstrated that C/T dimorphism in exon 2 of CD24 gene had no significant association with disease susceptibility or clinical features including BAL cell profiles in Japanese sarcoidosis patients.

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Reference

1. Grunewald J. Genetics of sarcoidosis. Curr Opin Pulm Med 2008; 14: 434-9.
2. Rossman MD, Kreider ME, State of the Art. Lesson learned from ACCESS (A case controlled etiologic study of sarcoidosis). Proc Am Thorac Soc 2007; 4: 453-6.
3. Chambers CA, Krummel MF, Boitel B Hurwits A, Sullivan TJ et al. The role of CTLA-4 in the regulation and initiation of T-cell responses. Immunol Rev 1996; 153: 27-46.
4. Handa T, Nagai S, Ito I, et al. Cytotoxic T-lymphocyte antigen-4 (CTLA-4) exon 1 polymorphism affects lymphocyte profiles in bronchoalveolar lavage of patients with sarcoidosis. Sarcoidosis Vasc Diffuse Lung Dis 2003; 20: 190-6.
5. Handa T, Nagai S, Ito I, et al. Polymorphisms of B7 (CD80 and CD86) genes do not affect disease susceptibility to sarcoidosis. Respiration 2005; 72: 243-8.
6. Valentonyte R, Hampe J, Huse K, et al. Sarcoidosis is associated with a truncating splice site mutation in BTNL2. Nat Genet 2005; 37: 357-64.
7. Hubbe M, Altevogt P. Heat-stable antigen/CD24 on mouse T lymphocytes: evidence for a costimulatory function. Eur J Immunol 1994; 24: 731-7.
8. Liu Y, Jones B, Aruffo A, et al. Heat-stable antigen is a costimulatory molecule for CD4 T cell growth. J Exp Med 1995; 175: 437-45
9. De Bruijn ML, Peterson PA, Jackson MR. Induction of heat-stable antigen expression by phagocytosis is involved in in vitro activation of unprimed CTL by macrophages. J Immunol 1996; 156: 2686-92.
10. Wu Y, Zhou Q, Zheng P, Liu Y. CD28-independent induction of T helper cells and immunoglobulin class switches requires costimulation by the heat-stable antigen. J Exp Med 1998; 187: 1151-6.
11. Zhou Q, Rammohan K, Lin S, et al. CD24 is a genetic modifier for risk and progression of multiple sclerosis. Proc Natl Acad Sci USA 2003; 100: 15041.

12. Sanchez E, Abelson AK, Sabio JM, et al. Association of a CD24 gene polymorphism with susceptibility to systemic lupus erythematosus. Arthritis Rheum 2007; 56: 3080-6.
13. Rueda B, Miranda-Filloy JA, Martin J, Gonzalez-Gay MA. Association of CD24 gene polymorphisms with susceptibility to biopsy-proven giant cell arteritis. J Rheumatol 2008; 35: 850-4.
14. Statement on sarcoidosis. Joint Statement of the American Thoracic Society (ATS), the European Respiratory Society (ERS) and the World Association of Sarcoidosis and Other Granulomatous Disorders (WASOG) adopted by the ATS Board of Directors and by the ERS Executive Committee, February 1999. Am J Respir Crit Care Med 1999; 160: 736-55.
15. Kasahara Y, Ashihara Y. Colorimetry of angiotensin-I converting enzyme activity in serum. Clin Chem 1981; 27: 1922-5.
16. Dupont WD, Plummer WD. Power and Sample Size Calculations for Studies Involving Linear Regression, Controlled Clinical Trials 1998; 19: 589-601.
17. Sharma OP. Sarcoidosis and other autoimmune disorders. Curr Opin pilm Med 2002; 8: 452-6.
18. Ando DG, Clayton J, Kono D, Urban JL, Sercarz EE. Encephalitogenic T cells in the B10. PL model of experimental allergic encephalomyelitis (EAE) are of the Th-1 lymphokine subtype. Cell Immunol 1989; 124: 132-43.
19. Zissel G, Prasse A, Müller-Quernheim J. Sarcoidosis - immunopathogenetic concepts. Semin Respir Crit Care Med 2007; 28: 3-14.
20. Larsen CE, Alper CA. The genetics of HLA-associated disease. Curr Opin Immunol 2004; 16: 660-7.
21. Traherne JA, BArcellos LF, Sawcer SJ, et al. Association of the truncationg splice site mutation in *BTNL2* with multiple sclerosis is secondary to *HLA-DRB1*15*. Hum Mol Genet 2006; 15: 155-61.
22. Wang L, Lin S, Rammohan KW, et al. A dinucleotide deletion in CD24 confers protection against autoimmune diseases. PLoS Genet 2007; 3: e49.
23. Westney GE, Judson MA. Racial and ethnic disparities in sarcoidosis: from genetics to socioeconomics. Clin Chest Med 2006; 27: 453-62.