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CASE STUDY

Central airway stenosis in a patient with autoimmune pancreatitis

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ABSTRACT: **Autoimmune pancreatitis is a unique form of chronic pancreatitis characterised by a high-serum immunoglobulin (Ig)G4 concentration involving various extra pancreatic lesions.**

A 63-yr-old female with autoimmune pancreatitis complained of cough. Chest computed tomography revealed an irregular stenosis of the central airway, lung hilar and mediastinal lymph node swelling, and a marked thickness of the bronchovascular bundle. Bronchoscopic examination revealed an irregular tracheobronchial stenosis accompanied with an oedematous mucosa and engorged vessels. Lung hilar and mediastinal lymph node swelling, central airway stenosis and bronchoscopic findings remarkably resembled those of sarcoidosis.

Bronchial biopsy specimens demonstrated diffuse infiltrations of plasma cells, lymphocytes and eosinophils with fibrosis. Immunostaining showed infiltration of several IgG4-positive plasma cells. The patient was treated with oral prednisolone at 1 mg·kg^{-1}·day^{-1} for pancreatic lesions. A month later, the lung lesions, including central airway stenosis, lung hilar and mediastinal lymph node swelling, and bronchovascular bundle thickness, had dramatically improved along with improvement of pancreatitis, thus indicating a close association between the two conditions.

This is the first report of a patient with autoimmune pancreatitis showing central airway stenosis similar to that of sarcoidosis.

KEYWORDS: **Airway, autoimmune disease, bronchoscopy, immunopathology**

Autoimmune pancreatitis is a unique form of chronic pancreatitis characterised by a high-serum immunoglobulin (Ig)G4 concentration and various extra pancreatic complications, including sclerosing sialoadenitis, sclerosing cholangitis, retroperitoneal fibrosis, tubulointerstital nephritis [1] and lung lesions, such as hilar lymphadenopathy, lung nodule [2] and interstitial pneumonia [3]; thus suggesting that this disease consists of a spectrum of systematic diseases. These extra pancreatic lesions exhibit similar histopathological findings to those in the pancreas, including dense infiltration of IgG4-positive plasma cells and T lymphocytes with fibrosis [1], and have sometimes been misdiagnosed as the specific diseases of corresponding organs, such as Sjoegren's syndrome for lachrymal and salivary gland lesions and primary sclerosing cholangitis for bile duct lesion, leading to incorrect therapy [4]. Herein, the present authors report a patient with autoimmune pancreatitis showing central airway stenosis in which special care was needed to differentiate it from sarcoidosis.

CASE REPORT

A 63-yr-old female was admitted to the present authors' hospital (Shinshu University School of Medicine, Matsumoto, Japan) complaining of cough, a loss of appetite and pale stool. She had undergone resection of a right submandibular gland neoplasm at age 58 yrs. The peripheral blood cell count was normal and a biochemistry examination revealed the following: total bilirubin 0.49 mg·dL^{-1}; alanine aminotransferase 77 IU·L^{-1}; aspartate aminotranferase 28 IU·L^{-1}; alkaline phosphatase 425 U·L^{-1} (normal range: 124–367 U·L^{-1}); γ-glutamyltransferase 127 IU·L^{-1} (normal range: 6–30 IU·L^{-1}); and amylase 48 IU·L^{-1} (normal range: 44–127 IU·L^{-1}). The IgG level was elevated to 2,889 mg·dL^{-1}. At the same time, the patient was diagnosed with diabetes. Other serological tests were within the normal range. Radiological studies showed diffuse enlargement of the pancreas, a circumference of lymphadenopathy and an irregular narrowing of the main pancreatic duct; these findings corresponded to those of autoimmune pancreatitis. Serum IgG4 was measured and

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revealed an elevated value of 1,660 $mg \cdot dL^{-1}$. An immunohistochemical examination of biopsy specimens from the bile duct showed infiltration of IgG4-positive plasma cells. Therefore, the patient was diagnosed to have autoimmune pancreatitis.

The left submandibular gland swelling and lung hilar and mediastinal lymph node swelling were detected by chest computed tomography (CT) and thought to be the extra pancreatic lesions of autoimmune pancreatitis. In addition, the patient had suffered from cough and chest CT revealed an irregular stenosis of the central airway and a marked thickness of the bronchovascular bundle (fig. 1a). A lung function test revealed a vital capacity of 3.11 L (129% predicted), forced expiratory volume in one second (FEV1) of 2.17 L (106.4% pred), FEV1/forced vital capacity (FVC) of 67.8% and a diffusing capacity of the lung for carbon monoxide of 17.7 $mL \cdot min^{-1} \cdot mmHg^{-1}$ (84.3% pred).

Bronchoscopic examination unveiled an irregular tracheobronchial stenosis accompanied with mucosal oedema and engorged vessels (fig. 2), which closely mimicked the findings observed in sarcoidosis. Analysis of the bronchoalveolar lavage fluid revealed a cell count of $4.0 \times 10^4 \cdot mL^{-1}$, with 60.6% macrophages, 39% lymphocytes (CD4/8 ratio 4.48), 0% eosinophils and 0.4% neutrophils, which were also similar to the findings of sarcoidosis. Bronchial biopsy specimens demonstrated diffuse inflammatory infiltrates consisting mainly of plasma cells, lymphocytes and scattered eosinophils with fibrosis.

An immunohistochemical examination showed infiltration of several IgG4-positive plasma cells (fig. 3). The number of IgG4-positive cells was superior to that of IgG1-positive cells, and the number identified per high power field (HPF) was scored as severe (>30 per HPF) according to KAMISAWA *et al.* [5], which has been described elsewhere [6]. The present authors did not identify any specific finding in the biopsy specimens from right S2 and S3. At this time, the serum angiotensin-converting enzyme (ACE) level was 5.3 $U \cdot I^{-1}$ (7.0~25.0 $U \cdot I^{-1}$). These findings were similar to the findings of the bile duct and were considered to be an IgG4-related change. The patient was treated with oral prednisolone at 1 $mg \cdot kg^{-1} \cdot day^{-1}$. One month later, the enlargement of the pancreas, the circumference of lymphadenopathy, the irregular narrowing of the main pancreatic duct and left submandibular gland swelling had all dramatically improved. A chest CT revealed a significant improvement of the central airway stenosis (fig. 1b), lung hilar and mediastinal lymph node swelling, and the thickness of the bronchovascular bundles. The IgG4 level decreased to 515 $mg \cdot dL^{-1}$. The results of the lung function tests were also improved. The vital capacity was 3.23 L (134% pred), FEV1 was 2.86 L (140.2% pred), FEV1/FVC was 89.9% and diffusing capacity of the lung for carbon monoxide was 25.3 $mL \cdot min^{-1} \cdot mmHg^{-1}$ (120.2% pred).

DISCUSSION

Mediastinal lymphadenopathy [1, 2], interstitial pneumonia [3] and inflammatory pseudotumour of the lung [3, 7] have been previously reported as pulmonary manifestations of autoimmune pancreatitis; however, no pulmonary lesion of central airway stenosis has been reported. In previous studies, histological examinations of the lung lesions showed a dense infiltration of lymphocytes and plasma cells in the thickened interstitium of the alveoli, around the bronchioles and the peribronchovascular region. In addition, immunostaining showed an infiltration of IgG4-positive plasma cells in these areas [2, 3, 7]. ZEN *et al.* [7] reported an inflammatory pseudotumour with IgG4-positive plasma cells. They also observed an irregular narrowing of bronchioles entrapped in nodules and an interstitial pneumonia pattern at the boundaries of nodules. The present case showed the central airway stenosis and thickness of the bronchovascular bundles. In addition, the immunohistochemical findings of bronchial mucosal biopsy specimens were similar to those observed in previous studies, but with no special findings in bronchioles.

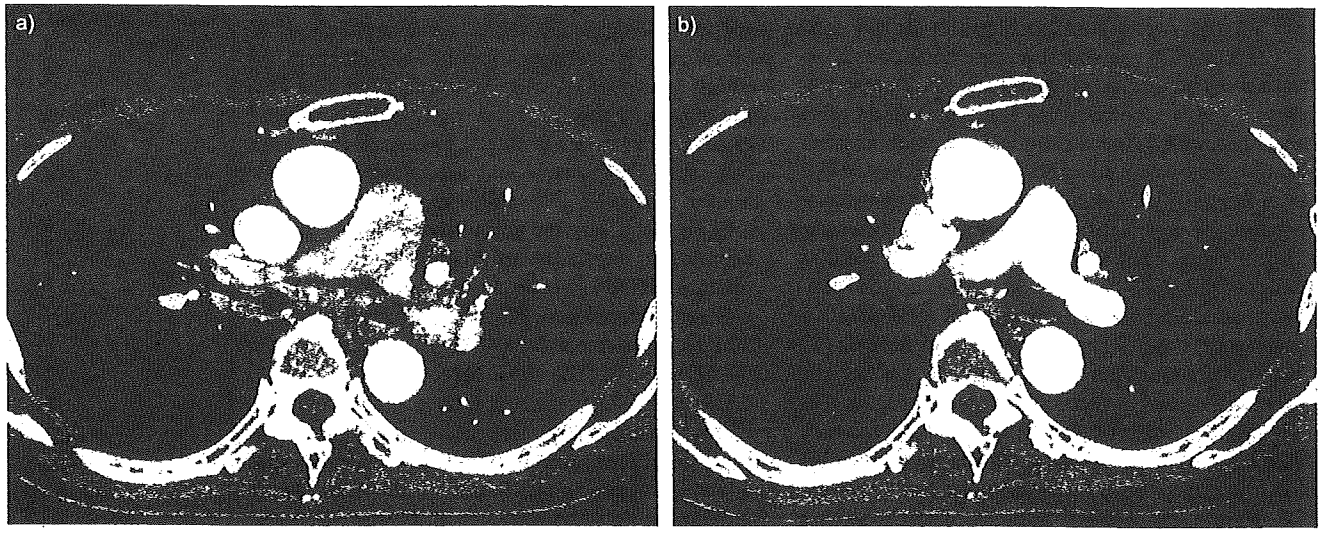

FIGURE 1. a) An irregular stenosis of bilateral central airway with marked thickness of peribronchial area and mediastinal lymph node swelling were seen in pre-treatment of chest computed tomography. b) An improvement of central airway stenosis and mediastinal lymph node swelling was seen after prednisolone treatment.

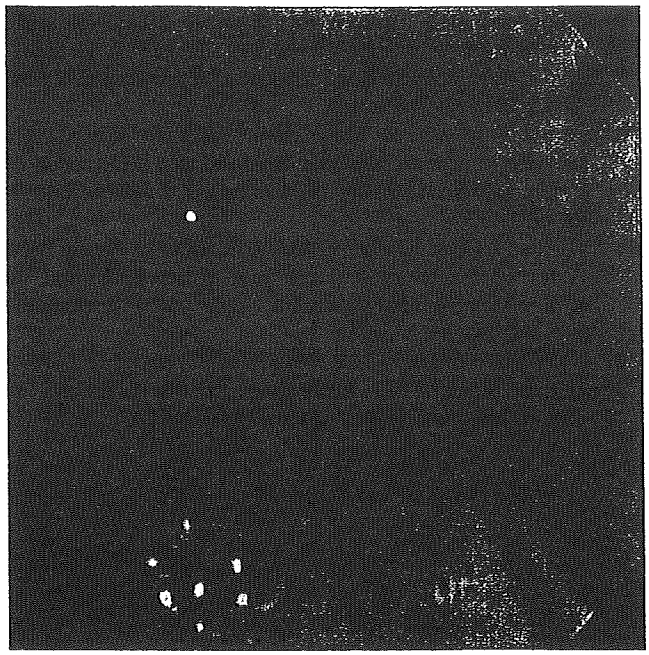

FIGURE 2. An irregular tracheobronchial stenosis accompanied with mucosal oedema and engorged vessels were seen by bronchoscopy.

Steroid therapy dramatically improved the central airway stenosis, thus indicating a close association between central airway stenosis and autoimmune pancreatitis. Accordingly, the pulmonary manifestations of central airway stenosis found in the present case are considered to be IgG4-related lesions. Lung lesions of autoimmune pancreatitis, including the interstitium of the alveoli, around the bronchioles, the peribronchovascular region and central airway, were all in the broad category of the interstitium.

Lung hilar and mediastinal lymph node swelling are characteristics of sarcoidosis and central airway stenosis has been reported to be one of the clinical phenotypes [8]. In sarcoidosis, granulomas and serum ACE elevation were often observed [9]; however, in the present case, granulomatous change was not detected in the central airway specimens and a normal ACE value was shown. In addition, the present authors did not observe any IgG4-positive plasma cells in the bronchial biopsy specimens of four sarcoidosis patients with a thickened bronchial wall who were examined at Shinshu University School of Medicine. Therefore, these clinical findings were able to help differentiate between IgG4-related pulmonary lesion and sarcoidosis. However, IgG4-related lesions have sometimes been found without any pancreatic manifestations. The solitary manifestation of an IgG4 related pulmonary lesion may show findings similar to those of sarcoidosis. Therefore, special care is required to accurately differentiate between these two conditions.

Some studies found high serum concentrations of IgG4 in patients with atopic dermatitis, asthma and some parasitic diseases, which are a direct response to an exogenous antigen [10]. Patients with severe asthma sometimes showed irregular tracheobronchial stenosis accompanied with an oedematous

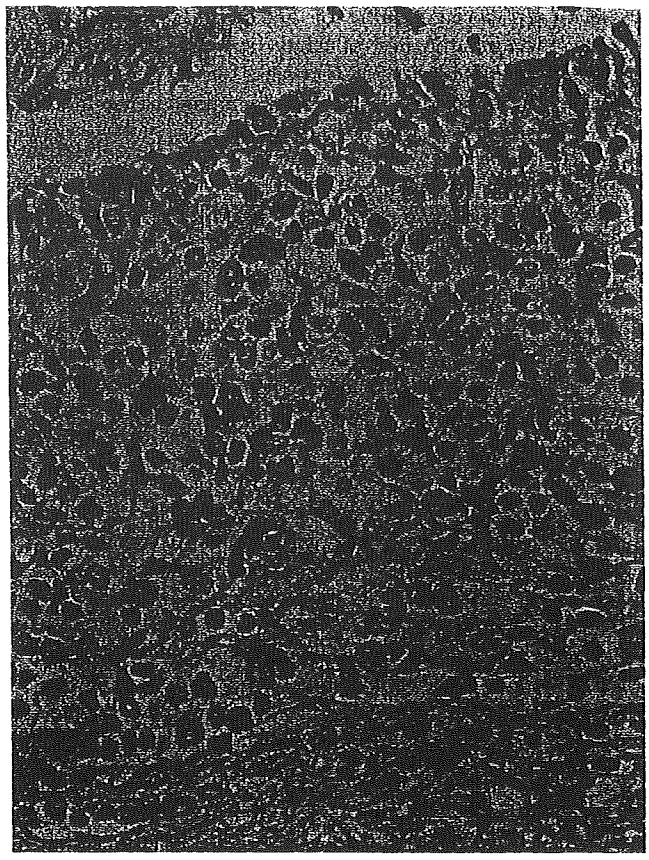

FIGURE 3. An immunohistochemical examination of bronchial biopsy specimen shows dense infiltration of immunoglobulin G4-positive plasma cells in submucosal area. Scale bar=50 μm.

change of the mucosa; however, the present patient never had any episodes of bronchial asthma. In addition, IgG4-positive cell infiltration has been found in pancreatic cancer and chronic pancreatitis [11, 12]. However, IgG4-positive cells in these diseases are fewer than in autoimmune pancreatitis patients and the tissue specimens of these cases were not associated with lymphoplasmacytic sclerosing pancreatitis, which was the characteristic attribute of autoimmune pancreatitis.

In conclusion, the present authors experienced an impressive case of central airway stenosis with autoimmune pancreatitis. The characteristic findings of this case were central airway stenosis, submucosal immunoglobulin G4 positive plasma cell involvement and an obvious relief of the stenosis by corticosteroids. As a result, such cases need to be carefully differentiated from sarcoidosis.

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ORIGINAL ARTICLE

Pulmonary involvement of autoimmune pancreatitis

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ABSTRACT

Background A wide variety of systemic lesions have been seen in patients with autoimmune pancreatitis. The pulmonary involvement of autoimmune pancreatitis was analysed to clarify the clinicopathological features of pulmonary lesions in comparison with pulmonary sarcoidosis.

Materials and methods Nineteen patients had autoimmune pancreatitis and eight had pulmonary sarcoidosis. The symptoms, laboratory data, chest computed tomography, Gallium-67 scintigraphy, pulmonary function testing and bronchoscopy findings, including the histological IgG4-immunostaining and IgG subclasses in the bronchoalveolar lavage in autoimmune pancreatitis, were collected to compare them with pulmonary sarcoidosis.

Results The serum total protein, IgG and IgG4 levels were found to be significantly elevated in comparison with pulmonary sarcoidosis. In autoimmune pancreatitis, 17 patients showed bilateral hilar lymphadenopathy, while eight showed pulmonary nodules on chest computed tomography. Eighteen of 19 patients on Gallium-67 scintigraphy showed accumulation spots in either the hilar or mediastinal lymph nodes. Six patients with pulmonary nodules demonstrated accumulation spots in the corresponding lesions on chest computed tomography. All eight patients with pulmonary sarcoidosis showed accumulation spots in either the hilar or mediastinal lymph nodes. Bronchoalveolar lavage IgG4 in autoimmune pancreatitis showed a significant increase in comparison with pulmonary sarcoidosis. The histological findings obtained by a transbronchial lung biopsy showed the infiltration of lymphocytes and plasma cells in the thickened interstitum and alveoli with IgG4-positive plasma cell infiltration in patients with autoimmune pancreatitis.

Conclusion IgG4 in the bronchoalveolar lavage was seen at remarkably increased levels and IgG4-positive plasma cells were identified in the pulmonary lesions of patients with autoimmune pancreatitis.

Keywords Autoimmune pancreatitis, bronchoalveolar lavage fluid, chest computed tomography, IgG4, pulmonary involvement.

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Introduction

Autoimmune pancreatitis is a unique form of chronic pancreatitis that presents in association with clinically obstructive jaundice, an irregular narrowing of the main pancreatic duct and swelling of the pancreas. In addition, it responds favourably to corticosteroid therapy [1,2]. The other characteristic features include a high serum IgG4 concentration and an abundant IgG4-bearing plasma cell infiltration in the pancreatic tissue [3,4]. This condition also causes a variety of extra-pancreatic complications [5,6] including sclerosing cholangitis, retroperitoneal fibrosis, hilar lymphadenopathy, sialoadenitis and thyroiditis [7,8]. As a result, autoimmune pancreatitis has been regarded as a systemic disease. Abundant IgG4-positive plasma cells were identified in extra-pancreatic lesions, thus suggesting that similar underlying mechanisms are also involved in pancreatic lesions [3,5,6].

Patients with this disease have been monitored for novel clinical conditions. Taniguchi and Nieminen reported a patient with interstitial changes of the bilateral lower lung fields [9,10]. A transbronchial lung biopsy showed marked thickening of the alveolar septum with infiltration of the IgG4-positive plasma cells. Zen reported inflammatory pseudo-tumours of the lung with IgG4-positive plasma cell infiltration and thus suggested that clinicopathological similarities exist between inflammatory pseudo-tumours and autoimmune pancreatitis [11]. Hirano reported that four of 30 patients with autoimmune pancreatitis developed pulmonary involvement and two of these patients

demonstrated severe hypoxia [12]. As Gallium (Ga)-67 scintigraphy can also generally identify the progression of this disease, Saegusa therefore routinely performed Ga-67 scintigraphy in patients with autoimmune pancreatitis. Some patients with autoimmune pancreatitis have demonstrated Ga-67 accumulation in both their hilar lymph nodes and salivary glands [13]. This uptake pattern is usually seen in pulmonary sarcoidosis.

The clinical diagnosis of autoimmune pancreatitis and pulmonary sarcoidosis is clearly based on the diagnostic criteria. In this study, we selected patients with hilar, mediastinum lymphadenopathy or lung field accumulation and we evaluated them to clarify the clinical and pathological features of pulmonary involvement which is associated with autoimmune pancreatitis in comparison with those features in pulmonary sarcoidosis. The analyses included examining clinical symptoms, laboratory data, chest computed tomography (CT) scans, pulmonary function testing, bronchoalveolar lavage (BAL) and transbronchial lung biopsies (TBLB).

Patients and methods

Our research protocol included the use of invasive procedures approved by the human ethics committee of Shinshu University School of Medicine. All patients gave their written informed consent for the drawing of blood samples and the performance of bronchoscopy.

Patients

All enrolled patients with autoimmune pancreatitis and pulmonary lesions were admitted to Shinshu University School of Medicine from 2005 to 2007. The diagnosis of autoimmune pancreatitis was based on the revised version of the diagnostic criteria proposed by the Japan Pancreas Society in 2002 [14]. All enrolled patients were confirmed to have either bilateral or unilateral mediastinal lymphadenopathy and/or small nodules in the lung fields on chest CT scans.

The enrolled sarcoidosis patients with bilateral hilar lymphadenopathy and/or parenchymal infiltrate (small nodules) were admitted to our hospital from 2005 to 2007 to receive an accurate diagnosis. The diagnosis of pulmonary sarcoidosis was based on consistent clinical features and a BAL fluid analysis, according to the American Thoracic Society/European Respiratory Society/World Association of Sarcoidosis and other Granulomatous Disorders guidelines [15,16]. This diagnosis was confirmed by the histological evidence of noncaseating epithelioid cell granulomas and the exclusion of other diseases capable of producing a similar histological or clinical picture.

The patients filled out a questionnaire that addressed the respiratory symptoms, smoking history, past and present histories and the demographic data. The questions about respiratory symptoms were related to cough, sputum and shortness of breath. The questions and the ratings of the responses related to the above symptoms included: (i) the frequency of these symptoms (none, intermittently and almost every day); (ii) the duration (within 1 week, within 1 month and over 3 months) and the onset of these symptoms; and (iii) whether the symptoms were progressive or persistent. Blood samples were obtained from these patients and chest X-rays, chest CT scans, Ga-67 scintigraphy, pulmonary function tests and bronchoscopy were all performed. In addition, none of the enrolled patients had been receiving any concurrent treatments for pulmonary diseases.

Laboratory data

The following blood tests were performed after the presence of pulmonary lesions was observed: total protein (normal range, 6·8–8·3 g dL^{-1}), albumin (normal range, 4·2–5·1 g dL^{-1}), lactate dehydrogenase (LDH) (normal range, 114–220 IU L^{-1}), C-reactive protein (CRP) (normal range, < 0·1 mg dL^{-1}), IgG (normal range, 870–1700 mg dL^{-1}) and soluble interleukin-2 receptor (sIL2r) (normal range, 198–493 U mL^{-1}) at Shinshu University Hospital and IgG4 (normal range, < 108 U mL^{-1}) at the Special Reference Lab. Inc (Hachioji, Tokyo, Japan).

Chest X-ray, Chest CT scan and Ga-67 scintigraphy

Chest X-rays and CT scans were obtained from all patients. We used a helical CT scanner (Hi Speed advantages; GE Medical Systems; Milwaukee, WI, USA) fluoroscopy (150 mA, 120 kV, 1-mm section thickness). All CT images were viewed in cine-mode formats on a computer workstation by one radiologist (S.K.) and were displayed under three display conditions to adequately examine both the lungs (width 1000 Hounsfield units (HU), level – 700 HU) and the lymph nodes in the hilar and mediastinum regions (width 300 HU, level 20 HU). Significant lymphadenopathy showed lymph nodes with an enlargement of more than 1 cm in the hilar and mediastinal images of HRCT scans. Ga-67 scintigraphy was performed before bronchoscopy. A whole-body scan with a single-head rectangular gamma camera (SNC-510R; Shimazu, Japan) was obtained at 48 h after the intravenous injection of 111MBq of Ga-67 citrate. A positive image was defined as the finding of a normal or greater than normal hepatic uptake.

Pulmonary function testing

Pulmonary function testing was performed within 15–45 min after inhaling a β2 stimulant using our routine method [17]. The vital capacity (VC), forced vital capacity (FVC), maximal mid-expiratory flow (MMF), and forced expiratory volume in one second (FEV_1) were measured using a Chestac-65V (Chest Co. Ltd., Tokyo, Japan). The MMF, residual volume (RV) and total lung capacity (TLC) was calculated and the peak expiratory flow rate (PEFR) and flow rate at 25% of the forced vital

capacity (V_{25}) and V_{25}/height (V_{25}/HT) were calculated from the maximum expiratory flow-volume curve. The thoracic gas volume (TGV) was measured using a body plethysmograph. The functional residual capacity (FRC) with the helium dilution method and the diffusing capacity for carbon monoxide (DLco) were measured using the single-breath method (Pulmocorder model R1551S; Anima Co., Tokyo, Japan).

Bronchoscopy

BAL and TBLB were performed according to our laboratory methods [18]. The total cell counts of unfractionated BAL fluid were evaluated using a haemocytometer counting chamber. The number of BAL cells counted for each differential was counted 300 cells/sample. Differential counts were obtained using a smear of May–Giemsa stain. The pellets in the BAL fluid were analysed for lymphocyte subsets by flow cytometry using CD4-fluorescein isothiocyanate (FITC) and CD8-phycoerythrin (PE) monoclonal antibodies (eBioscience Inc., San Diego, CA, USA), while amylase and IgG were analysed using an enzyme-linked immunosorbent assay (ELISA). The specimens obtained by TBLB were fixed with 10% formalin and embedded in paraffin. Four micrometre-thick sections were cut from each paraffin block and some were stained with haematoxylin–eosin while the remaining sections were used for immunostaining of IgG4 (The Binding Site Limited, Birmingham, UK). IgG4-positive plasma cells were counted in 10 different high-power fields in the more prominently inflamed areas in the biopsied cases.

ELISA methods

Micro ELISA plates (Nunc immunoplate 446612, Cosmo Bio Co., LTD, Tokyo, Japan) were coated overnight with 100 μL of goat polyclonal antibodies for anti-IgG1, IgG2, IgG3 and IgG4 (The Binding Site Limited) (1 μg per well) in 0·14 mol of sodium bicarbonate per litre (pH 7·6) at 4 °C. After discarding the immunoglobulin solution, the plate was blocked with 1% bovine serum albumin in phosphate-buffered saline containing 10 mM of ethylene diaminetetraacetic acid. After discarding the blocking solution, 100 μL of the serum samples were diluted with a solution containing phosphate-buffered saline containing 0·5% bovine serum albumin and 0·1% sodium azide to 1 : 5000. After mixing and incubation for 1 h at room temperature, the wells were rinsed five times with phosphate-buffered saline containing 0·01% Tween 20, and immobilized complex was then incubated with 100 μL of peroxidase-conjugated anti-goat monoclonal secondary antibody for anti-IgG1, anti-IgG2, anti-IgG3 and anti IgG4 antibodies in enzyme conjugate stabilizer solution (Stab-ELISA- horseradish peroxidase diluent/stabilizer; Cygnus Technologies I-035, Southport, NC, USA) (1 : 2000 dilution) for 1 h to allow the complex to bind to the peroxidase-conjugated antibody. After rinsing, the enzyme which bound to the wells was incubated in the dark with 100 μL of tetramethylbenzidine substrate solution (TMB One-component Microwell Peroxidase Substrate; Kirkegaard & Perry Laboratories 53-00-01, Gaithersburg, ML, USA) for 20 min. The reaction was then stopped by the addition of 100 μL of stop solution (TMB One-Component Stop Solution Kirkegaard & Perry Laboratories 50-85-05). After brief mixing, the plate was read at 450 nm. To construct the standard curve, serial dilution of pooled BAL of patients with autoimmune pancreatitis were measured and crossover points for the optical density and each IgG subclass value were plotted. The optical density of the bound IgG1-4 concentration in each BAL sample was converted to an absolute IgG1-4 value using this standard curve.

Treatment

All patients with autoimmune pancreatitis were orally treated with prednisolone (initial dose; 0·5 mg kg^{-1} day^{-1}) and then underwent follow-up blood tests, chest X-rays and chest CT scans after 3 months of treatment.

Statistics

The values are given as the mean ± standard deviation (SD). The values of the two groups were compared using the unpaired Student's *t*-test and Fisher's exact test. We considered *P*-values of less than 0·05 to be significant.

Results

Clinical findings

Nineteen patients with autoimmune pancreatitis and eight with pulmonary sarcoidosis were enrolled in our study, three patients with autoimmune pancreatitis showed obstructive jaundice, two showed liver dysfunction, 17 showed swelling of the pancreas and two showed swelling of the liver on abdomen CT scans. As shown in Tables 1, 4 of these patients complained of a productive cough. One patient complained of productive sputum, and one patient complained of dyspnoea with Fletcher-Hugh-Jones II. One patient with pulmonary sarcoidosis complained of a productive cough. Patients with autoimmune pancreatitis showed significantly higher levels of the serum total protein, IgG and IgG4 compared with pulmonary sarcoidosis. In addition, the serum albumin level was significantly lower in patients with autoimmune pancreatitis than in those with pulmonary sarcoidosis and six patients were positive for ANA. After the administration of prednisolone, no patients with autoimmune pancreatitis had any respiratory symptoms and the serum levels of IgG (1594 ± 742 mg dL^{-1}) (P = 0·042) and IgG4 (273 ± 89 mg dL^{-1}) (P = 0·049) significantly decreased.

As shown in Table 2, all patients with autoimmune pancreatitis had lymphadenopathy, eight had pulmonary nodules and four demonstrated reticular shadows on chest CT scans (Fig. 1).

Table 1 Characteristics and data

	(A) Autoimmune pancreatitis (n = 19)	(B) Sarcoidosis (n = 8)	P-value [(A) vs. (B)]
Age (years)	65·6 ± 9·9	53·6 ± 15·7	0·11
Sex, M/F	15/4	7/1	0·38
Symptoms – no. of subjects			
Cough (n)	4	1	0·52
Sputum (n)	1	0	0·70
Shortness of breath	1	0	0·70
Laboratory data			
Total protein (g dL^{-1})	8·7 ± 1·1	7·4 ± 0·6	0·046
Albumin (g dL^{-1})	3·4 ± 0·3	4·3 ± 0·5	0·003
LDH (IU L^{-1})	221 ± 82	193 ± 49	0·61
CRP (mg dL^{-1})	0·64 ± 0·4	0·25 ± 0·4	0·32
Soluble IL-2r (U mL^{-1})	1370 ± 1039	1190 ± 452	0·86
Positive numbers of ANA	6	0	0·092
IgG (mg dL^{-1})	2747 ± 1121	1498 ± 427	0·012
IgG4 (mg dL^{-1})	1185 ± 991	31·9 ± 18	0·008

LDH, lactate dehydrogenase; CRP, C-reactive protein; ACE, angiotensin-converting enzyme; IL2r, interleukin 2 receptor; RF, rheumatoid factor; ANA, antinuclear antibody; M, male; F, female.

Table 2 Image findings

	(A) Autoimmune pancreatitis (n = 19)	(B) Sarcoidosis (n = 8)	P-value [(A) vs. (B)]
Chest CT findings (n)			
BHL/unilateral	17/2	8/0	> 0·99
Pulmonary nodules	8	4	0·30
Reticular shadow	4	0	0·22
Ground glass opacity	0	0	> 0·99
Ga-67 scintigraphy accumulation			
Pancreas	17	0	< 0·001
Hilar and mediastinal LN	18	8	0·70
Lung nodules	6	4	0·23
Lung fields	1	0	0·70

BHL, bilateral hilar lymphadenopathy; LN, lymph node; Ga, gallium; n, number.

Eighteen patients on Ga-67 scintigraphy showed accumulation spots in either the hilar or mediastinal lymph nodes and 17 showed them in the pancreatic body. Six patients with pulmonary nodules demonstrated accumulation spots in the corresponding lesions on chest CT scans, while two patients had a salivary gland uptake in the Ga-67 scintigraphy findings. The uptake of the lymph nodes in the hilar and mediastinum regions and the nodules in the lung field in Ga-67 scintigraphy tended to demonstrate higher serum IgG4 levels than that of the lymph nodes in the hilar and mediastinum in Ga-67. All patients with sarcoidosis showed bilateral hilar lymphadenopathy (BHL), and four patients showed pulmonary nodules on chest CT scans. The patterns of the chest CT findings and the uptake of Ga-67 scintigraphy of patients with autoimmune

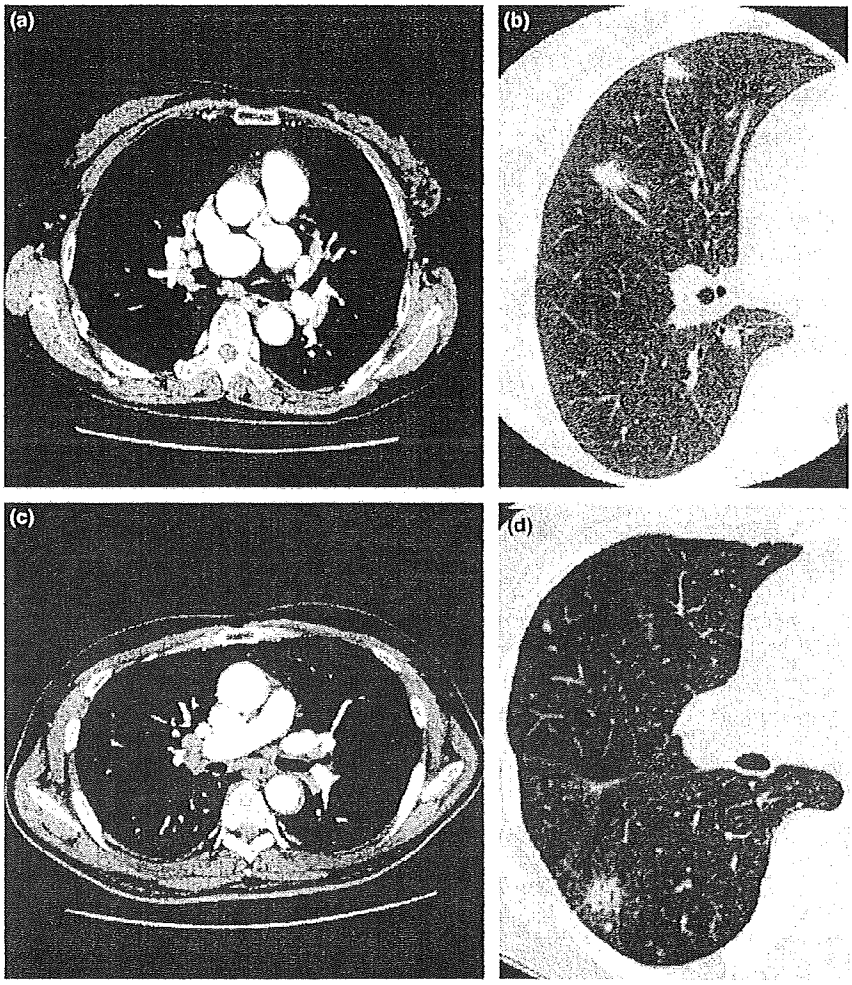

Figure 1 (a) A 78-year-old female with autoimmune pancreatitis showed bilateral hilar lymphadenopathy and an enlargement of the mediastinal lymph nodes on chest CT scans. (b) The same patient showed consolidated nodules with speculation in the right lower lobe and the inguinal lobe. (c) A 65-year-old male with autoimmune pancreatitis showed unilateral lymphadenopathy and enlargement of the mediastinal lymph nodes on chest CT scans. (d) A 73-year-old female with autoimmune pancreatitis showed nodule with spiculation in the right lower lobe and fine nodule in the right upper lobe.

pancreatitis were radiographically very similar to patients with pulmonary sarcoidosis. However, the morphology of pulmonary nodules and the size of the lymphadenopathy in autoimmune pancreatitis were similar to that of lung adenocarcinoma (Fig. 1a,d). After treatment, all peripheral nodules and lymphadenopathy, either decreased in size or were completely absent on the follow-up chest CT scans.

In patients with autoimmune pancraetitis,pulmonary function testing showed the %VC, %FEV_1, FEV_1% and %DLco to be within the normal range. Six patients showed increased levels of TGV-FRC in comparison with the normal levels in our laboratory (normal range; < 0·35L) (Table 3). We therefore suspected that these patients may thus have had a small-airway flow limitation. All six patients with increased levels of TGV-FRC showed a positive histology for IgG4.

As shown in Table 4, in patients with autoimmune pancreatitis, the BAL showed increased total cell counts with a lymphocyte predominance. One patient showed a predominance of eosinophils in the BAL, however, this patient was also associated with bronchial asthma. The CD4/CD8 lymphocyte ratio in the BAL was significantly higher than that of the normal patients (data not shown), but it was lower than that of pulmonary sarcoidosis (range; 5·22 ± 1·6) in our laboratory. Eight patients with autoimmune pancreatitis had a CD4/CD8 ratio of more than 3·5 (the normal upper limit at our laboratory). The percentages of BAL CD4-positive T lymphocytes ranged from 60% to 88%. The BAL showed an especially notable increase IgG, especially the IgG4 levels, a characteristic finding in autoimmune pancreatitis, not pulmonary sarcoidosis. The BAL IgG4 level was observed to correlate with serum IgG4 level (r = 0·71, P = 0·049). The histological findings obtained by TBLB showed the infiltration of abundant lymphocytes and mild-to-moderate infiltration of plasma cells in the thickened interstitium and alveoli. As shown in Fig. 2a, the histological findings

Table 3 Pulmonary function data

	(A) Autoimmune pancreatitis (n = 19)	(B) Sarcoidosis (n = 8)	P-value [(A) vs. (B)]
VC (L)	3·63 ± 0·7	2·58 ± 0·40	0·002
VC% predicted (%)	118 ± 15	89·7 ± 38	0·046
FEV1 (L)	2·69 ± 0·47	2·19 ± 0·33	0·02
FVC (L)	3·60 ± 1·1	2·64 ± 0·44	0·032
FEV1/FVC (%)	76·2 ± 8·8	83·3 ± 4·9	0·057
FEV1% predicted (%)	115 ± 21	112 ± 16	0·72
V25/height (%)	3·72 ± 1·02	3·24 ± 1·1	0·36
%MMF	72·8 ± 34	98·3 ± 31	0·12
%PEFR	101 ± 23	105 ± 17	0·67
%DLco	87·0 ± 13	76·3 ± 6·1	0·049
RV/TLC (%)	99·4 ± 36	130 ± 24	0·056
TGV-FRC (L)	0·32 ± 0·14	0·30 ± 0·31	0·84

VC, vital capacity; FVC, forced vital capacity; MMF, maximal mid-expiratory flow; FEV_1, forced expiratory volume in one second; PEFR, peak expiratory flow rate; V_{25}, flow rate at 25% of the forced vital capacity; FRC, functional residual capacity; DLco, diffusing capacity for carbon monoxide; RV, residual volume; TGV, thoracic gas volume; TLC, total lung capacity.

Table 4 Bronchoscopy

	(A) Autoimmune pancreatitis (n = 19)	(B) Sarcoidosis (n = 8)	P-value [(A) vs. (B)]
Bronchoalveolar lavage			
Recovery ratio (%)	57·6 ± 7·6	54·1 ± 16	0·54
Total cell counts ($\times 10^4$ per mL^{-1})	134 ± 57	69·6 ± 42	0·17
Macrophages (%)	56·1 ± 18	64·4 ± 20	0·15
Lymphocytes (%)	35 ± 17	33·5 ± 19	0·44
Neutrophils (%)	2·8 ± 3·2	1·8 ± 1·5	0·85
Eosinophils (%)	6 ± 15	0·56 ± 0·5	0·28
CD4/CD8 ratio	3·2 ± 2·3	5·22 ± 1·6	0·24
Amylase ($U\ mL^{-1}$)	342 ± 988	21·1 ± 20	0·37
IgG ($mg\ dL^{-1}$)	6·9 ± 6·2	2·44 ± 1·4	0·05
IgG4 ($\mu g\ dL^{-1}$) (n = 8)	2132 ± 1932	9·0 ± 0·5	< 0·001
Lung biopsy			
Numbers of IgG4 positive	9	0	0·02

CD, clusters of differentiation; n, number.

in autoimmune pancreatitis showed mononuclear cell aggregation, not typical granuloma, as is normally seen in sarcoidosis. In nine of 19 patients, the plasma cells stained positive for IgG4 (P = 0·02). Three of eight patients with an increased CD4/CD8 ratio showed positive staining of IgG4; however, all patients showed increased levels of IgG4 in the BAL. Five of six patients with a positive accumulation in pulmonary nodules via Ga-67 scintigraphy also showed positive immunostaining for IgG4. Four of 12 patients with only lymphadenopathy showed positive immunostaining for IgG4 in the specimens obtained from

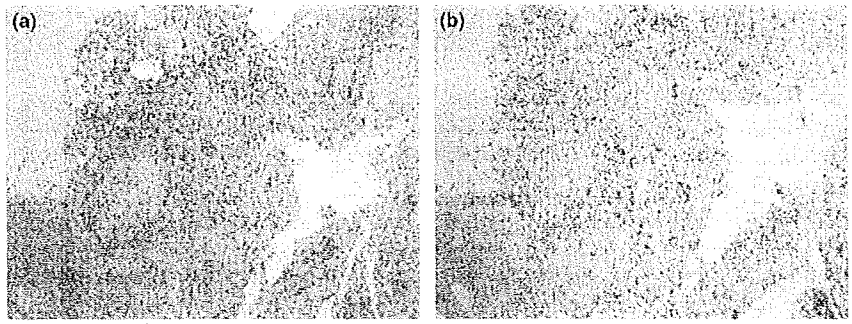

Figure 2 (a) A 71-year-old male with autoimmune pancreatitis showed granulation tissue and severe infiltration of lymphocytes and plasma cells (H–E stain ×10). (b) The same patient showed IgG4-positive plasma cells (IgG4 antibody immunostaining ×10)

routine lobes (right inguinal lobe). The histological findings of these four patients revealed IgG4-positive plasma cells in the bronchiolus and in the interstitum, not in the alveoli. One patient with Ga-67 positive nodules showed negative IgG4 plasma cell infiltration in the biopsied lung fields, but unfortunately sufficient lung specimens could not be obtained by TBLB to stain IgG4. This patient, however, had notably increased levels of IgG4 in the BAL and in serum. None of the patients with sarcoidosis showed positive immunostaining for IgG4.

Discussion

We focused on the pulmonary involvement that can occur with autoimmune pancreatitis in comparison with pulmonary sarcoidosis. Those clinical findings were generally similar to those of pulmonary sarcoidosis. However, the patients presenting with autoimmune pancreatitis had elevated serum levels of total protein, IgG and IgG4. Hamano *et al.* reported the serum IgG4 concentration to be significantly and specifically elevated in patients with autoimmune pancreatitis [4]. In the absence of a diagnostic serological marker for AIP, AIP should be diagnosed based on the combination of characteristic findings [19]. In our study, IgG4 in the BAL was also detected at extremely high levels in the autoimmune pancreatitis patients.

The chest CT scan findings in autoimmune pancreatitis were apparently different from those of Hirano's report [12]. In our study, 17 patients with lymphadenopathy resembled those for pulmonary sarcoidosis and small pulmonary nodules on the chest CT scan. However, the findings for autoimmune pancreatitis differed from those for pulmonary sarcoidosis with regard to the fact that the lymphadenopathy was smaller in size. In addition, the morphology of pulmonary nodules and unilateral lymphadenopathy resembled that of lung cancer with speculation as shown in Fig. 1c. One patient with unilateral large lymphadenopathy reached diagnosis of autoimmune pancreatitis due to video-assisted thoracoscopic surgery and due to a suspicion of lung cancer.

Patients with Ga-67 accumulation in the hilar lymph nodes also showed a similar pattern of Ga-67 accumulation to that of pulmonary sarcoidosis. Patients who showed an accumulation in the lung nodules on Ga-67 scintigraphy, already exhibited the infiltration of positive IgG4-plasma cells in the lung, but the patients who had only accumulation in the hilar or mediastinal lymph nodes did not have any infiltration of plasma cells in the lung fields. However, IgG4-positive plasma cells could mainly be seen in the bronchiolus, but not in the alveoli. We can therefore surmise that patients with autoimmune pancreatitis might develop hilar and mediastinal lymphadenopathy at an earlier phase with lung involvement as the disease progresses through lymph apparatus in the bronchus. As shown in the correlation of the distribution of the uptake in Ga-67 scintigraphy, it was found to be a useful tool for detecting the progression of this disease.

The BAL findings showed increased total cell counts with a predominance of lymphocytes. However, no significant differences were observed in BAL differentiation and the CD4-to-CD8 lymphocyte ratio in comparison with the patients with pulmonary sarcoidosis, as seen in our laboratory data. The IgG level in the BAL was also significantly higher than that of pulmonary sarcoidosis. As some active pulmonary sarcoidosis patients showed elevated levels of IgG in the BAL, we thought that IgG in the BAL was therefore not a point of discrimination between the two diseases. As a characteristic finding, BAL IgG4 in autoimmune pancreatitis showed extremely higher levels than that of pulmonary sarcoidosis. Accordingly, it was possible to distinguish between autoimmune pancreatitis and sarcoidosis based on the data obtained from BAL, although BAL IgG4 could not be measured using a commercialized kit.

Generally, autoimmune pancreatitis is histologically characterized by a diffuse lymphoplasmacytic infiltration, irregular fibrosis, obliterative phlebitis and the severe infiltration of IgG4-positive plasma cells [3,20]. The pathohistological findings obtained by TBLB showed the infiltration of lymphocytes, foamy macrophages and plasma cells in the bronchiolus and in the interstitium. Moreover, the histological findings of pulmonary involvement provided no evidence of non-caseating epithelioid cell granulomas. IgG4-positive plasma cells were histologically dominant in the pulmonary involvement of

autoimmune pancreatitis in comparison to pulmonary sarcoidosis. The inflammatory infiltrate consisted of lymphocytes, macrophages, plasma cells and eosinophils, but not neutrophils. Moderate plasma cell infiltration was seen in the bronchioles, and in the interstitium of patients with autoimmune pancreatitis, but plasma cell infiltration was absent in patients with pulmonary sarcoidosis. As shown in Table 4, these pathological findings including IgG4-positive plasma cell infiltration could therefore differentiate between autoimmune pancreatitis and pulmonary sarcoidosis.

All of the patients had a remission of symptoms and a resolution of abnormalities on imaging studies after 3 months of steroid therapy. After treatment, the serum IgG4 level was significantly lower than the initial line, although we did not re-measure IgG4 in the BAL after treatment for ethical reasons. We believe that the serum IgG4 level therefore correlates with the IgG4 levels in the BAL as well as in the decrease in the size of lymph nodes and nodules observed on the chest CT findings. Therefore, for autoimmune pancreatitis complicated with pulmonary involvement, the serum IgG4 is also considered to be a predictive marker of therapeutic effectiveness.

In conclusion, we herein showed that the pulmonary involvement of patients with autoimmune pancreatitis resembled lymphadenopathy of typical pulmonary sarcoidosis and the pulmonary nodules of lung cancer observed on chest CT findings. Similar to the pancreas, IgG4-positive plasma cells were histologically observed to infiltrate the lung and also increases in the IgG4 levels in the serum and BAL were identified. Similar mechanisms may therefore be involved in both pancreatic and extra-pancreatic lesions in such complicated cases.

Conflict of interests

None.

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Association analysis of Toll-like receptor 4 polymorphisms with autoimmune pancreatitis

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ABSTRACT

Autoimmune pancreatitis (AIP) is characterized by lymphoplasmocytic inflammation, high serum IgG4 concentrations, and a favorable response to corticosteroid treatment. Although long-term follow-up studies have shown that a relapse rate of 30–40% can occur in AIP after remission with corticosteroids, there are few genetic characteristic predictors of relapse in AIP patients. Toll-like receptor (TLR) is an important mediator in both innate and adaptive immunity. Polymorphisms in *TLR4* gene have been linked with several autoimmune and allergic diseases. We therefore investigated the genetic association between *TLR4* polymorphisms and AIP susceptibility and relapse in a Japanese population. Eight SNPs in *TLR4* (rs10759930, rs1927914, rs1927911, rs12377632, rs2149356, rs11536889, rs7037117, and rs7045953) were genotyped in 59 patients with AIP and 126 healthy controls using a TaqMan assay. Analysis of allelic frequencies revealed no statistical association with either susceptibility or relapse of AIP. These data indicate that *TLR4* polymorphisms do not play an important role in the development of AIP.

1. Introduction

Autoimmune pancreatitis (AIP) is characterized by irregular narrowing of the main pancreatic duct, swelling of the pancreas, histologic evidence of lymphoplasmocytic inflammation, and a favorable response to corticosteroid treatment [1–4]. We and others have previously reported that IgG4 concentrations are significantly and specifically higher in patients with AIP, suggesting that IgG4 plays a major role in AIP pathogenesis [5,6]. In addition, abundant IgG4-bearing plasma cells have been found infiltrating the pancreas in AIP [7,8]. This disease is also characterized by systemic complications involving various extra-pancreatic lymphoplasmocytic inflammation and IgG4-bearing plasma cell infiltration; thus AIP has been recognized as a systemic inflammatory condition [7–10]. Furthermore, we previously reported three susceptibility genetic markers [11–14]. However, because none of the genetic markers currently identified can sufficiently explain disease etiology, additional genes that influence immune tolerance are likely to be involved. Zen *et al.* recently reported that in patients with AIP, the Th2 and regulatory immune reactions were upregulated in the affected tissues [15]. These investigators indicated that the predominance of Th2 and regulatory immune reactions in AIP might reflect an allergic nature in the pathogenesis. According to recent studies on AIP, susceptibility and relapse of AIP are influenced by genetic factors, specific HLA alleles, amino acid sequences at the presentation site of the HLA molecule, and cytotoxic T-lymphocyte antigen 4 *(CTLA4)* SNPs [13,16].

Toll-like receptors (TLRs) are transmembrane proteins expressed by cells of the innate immune system, which recognize pathogen-associated molecular patterns and play important roles in immune and inflammatory responses to destroy the invaders. Among TLR family members, TLR4 (Toll-like receptor-4) has been the most thoroughly investigated. Apart from its involvement in the recognition of lipopolysaccharide, TLR4 also interacts with endogenous ligands, including heat-shock proteins. Some studies have reported that allergic diseases, including bronchial asthma and atopic dermatitis, are associated with single-nucleotide polymorphisms (SNPs) in the *TLR4* gene [17–19]. However, no study has comprehensively evaluated risk factors for AIP relapse and investigated the association between *TLR4* SNPs and AIP. Therefore, we examined the potential involvement of *TLR4* SNPs in the susceptibility and relapse of AIP.

2. Subjects and methods

2.1. Subjects

Between September 1994 and September 2007, we recruited 59 patients with AIP (49 men and 10 women), 38–76 years old (median, 63 years old), and 126 healthy control subjects. The diagnosis

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of AIP was based on criteria released by the Japan Pancreas Society using clinical data, imaging tests, and/or histopathologic findings, as reported previously [20]. Of the 59 patients with AIP, 37 (63%) had concurrent autoimmune diseases, including hypothyroidism (11 patients) and sclerosing cholangitis (34 patients); these diagnoses were described in prior studies [9,21]. All control subjects had indicated the absence of major illnesses on a standard questionnaire. This group was formed by enrolling volunteers from hospital staff. All racial/ethnic backgrounds were Japanese.

Serum levels of IgG4 were determined by single radial immunodiffusion kits (normal, <135 mg/dl) as reported previously [5]. High serum IgG4 concentrations (median, 730.0 mg/dl; interquartile range, 265.0–1037.5 mg/dl) were found in 55 of the 59 patients with AIP. Of the patients, 52 were treated with 40 mg prednisolone daily for 4 weeks; the dose was then reduced by 5 mg per week over a period of several weeks. All 52 patients responded favorably to corticosteroid therapy, resulting in improvements in clinical, laboratory, and imaging findings. We found no high concentrations of serum IgG4 in healthy subjects. All patients and controls were negative for the hepatitis B surface antigen and antibodies to hepatitis C in the serum [22].

In total, we followed the 55 patients with high IgG4 levels, including 52 patients who were treated with corticosteroids every month for a period of at least 12 months (median, 72 months; range, 12–178 months). Patients underwent regular follow-up visits with an interview every month; laboratory tests every 2–3 months, and imaging tests, including computed tomography or magnetic resonance imaging, every 6 months or, in the event of relapse, until September 2007. Of the 55 patients, 16 (29%) experienced relapse during follow-up. A relapse was defined as a recurrent attack of pancreatic swelling that resulted in irregular narrowing of the pancreatic duct or stenosis of the common bile duct, as reported previously [23].

All participants provided written informed consent for tests with DNA samples. After receiving permission, serum samples were obtained from patients and normal subjects. This study was approved by the institutional ethics committee.

2.2. TLR4 genotyping

Genomic DNA was isolated from whole blood of patients and healthy individuals using QuickGene-800 (Fujifilm, Tokyo, Japan). The concentration of genomic DNA was adjusted to 10–15 ng/μl for the TaqMan SNP genotyping assay. TLR4 is composed of four exons and has four transcript isoforms. We evaluated eight SNPs (rs10759930, rs1927914, rs1927911, rs12377632, rs2149356, rs11536889, rs7037117, and rs7045953) that were localized within the exons and introns of the *TLR4* gene. These SNPs were selected from among previous reports [24–26] and public information sources, such as the NCBI dbSNP (http://www.ncbi.nlm.nih.bov/SNP/), Applied Biosystems (http://www.appliedbiosystems.com), and HapMap (http://www.hapmap.org/) databses, and had minor allele frequencies >5%. The SNP spans approximately 1–5 kb, and includes 5 kb of the predicted 5′-untranslated region (UTR) and 6 kb of the predicted 3′ UTR in the *TLR4* gene. Genotyping of all SNPs was performed by a TaqMan 5′ exonuclease assay using primers supplied by (Applied Biosystems, Foster City, CA). The probe fluorescence signals were detected with a TaqMan Assay for Real-Time PCR (7500 Real Time PCR System, Applied Biosystems), according to the manufacturer's instructions.

2.3. HLA typing

HLA class I and II alleles, and *DRB1* and *DQB1* alleles were identified, as reported previously [27,28]. These HLA typings had been done before, not for the purpose of this manuscript.

2.4. Statistical analysis

The Hardy-Weinberg equilibrium (HWE) test was done for each SNP among controls and patient groups. The pairwise linkage disequilibrium (LD) patterns, haplotype block structure, and haplotype frequency analysis for all SNPs were assessed by the block definition of Gabriel *et al.*, and was based on 95% CI of D′ with implementation of Haploview version 3.32 software [29,30] (http://www.broad.mit.edu/mpg/haploview/index.php). The significance of allele distribution between patients with AIP and healthy subjects was tested using the χ^2 test for 2 × 2 or 2 × 3 comparisons. When the number of subjects was less than 5, Fisher's exact test was used. A value of $p < 0.05$ was considered statistically significant. The corrected p value (p_c) was calculated by the Bonferroni's correction where the coefficient was the total number of the contingency tables tested.

3. Results

3.1. TLR4 genotyping in patients with AIP and healthy subjects

Eight SNPs in TLR4 were genotyped in 59 patients with AIP and in 126 healthy subjects (Table 1). In controls, the genotype distributions of all SNPs exhibited Hardy-Weinberg equilibrium, and the minor allele frequencies of all SNPs were more than 5%. However, in patients, the genotype distribution of one SNP (rs2149356) differed significantly from the expected Hardy-Weinberg values ($p < 0.05$) (Table 1). All eight SNPs were located in 1 haplotype block, and the magnitude of LD between each SNP was high (Fig. 1). Analysis of allelic frequencies (Table 2) revealed a significant difference between patients with AIP and healthy subjects for SNP rs2149356: Positivity for G was significantly higher in patients with AIP than in healthy subjects ($\chi^2 = 8.58$, $p = 0.014$). The G/T genotype was significantly increased in patients with AIP compared with healthy subjects. This SNP preliminary showing statistical significance was later confirmed as not significant after correction for multiple testing. No other SNPs were significantly associated with AIP. The statistical power of this study was 0.9349 and enough for analysis.

Table 1
Allele frequencies of SNPs of the TLR4 gene in AIP patients and controls

dbSNP	Alleles (1/2)	Position (bp)	Gene location	Patients ($n = 59$)		Controls ($n = 126$)	
				MAF (%)	HWE	MAF (%)	HWE
rs10759930	T/C	119,501,442	5′-UTR	38.1	0.276	33.3	0.801
rs1927914	A/G	119,504,546	5′-UTR	38.1	0.276	32.9	0.698
rs1927911	G/A	119,509,875	Intron	35.6	0.629	32.9	0.235
rs12377632	C/T	119,512,551	Intron	35.6	0.629	32.5	0.602
rs2149356	G/T	119,514,020	Intron	31.4	0.007	32.1	0.511
rs11536889	C/C	119,517,952	3′-UTR	26.3	1.000	26.2	1.000
rs7037117	A/G	119,523,484	3′-UTR	20.3	0.505	17.5	0.282
rs7045953	A/G	119,525,616	3′-UTR	10.2	1.000	7.5	0.961

1, major allele; 2, minor allele; bp, base pair.
Position is distance from short arm telomere.

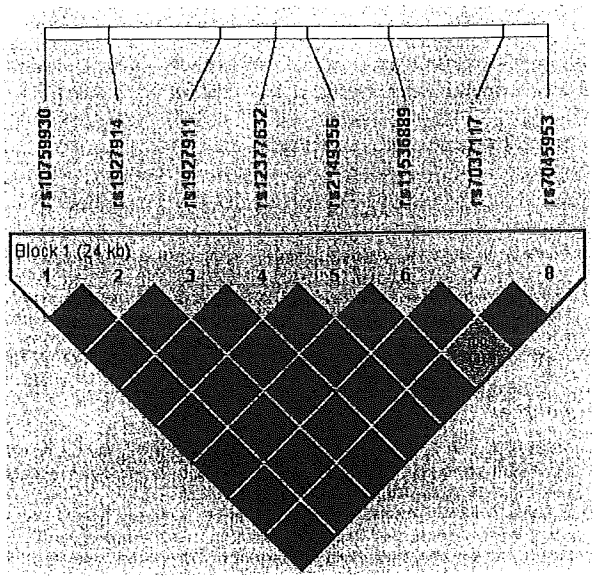

Fig. 1. Structure of linkage disequilibrium (LD) plot of 8 SNPs of the *TLR4* gene in the controls. The D′ value and r^2 value (in parentheses) corresponding to each SNP pair are express as a percentage and shown within the respective square. Higher D′ is indicated by a brighter red. The 8 SNPs constitute a haplotype block spanning 24 kb of the *TLR4* gene.

The haplotype frequency of the eight SNPs was estimated with the expectation-maximization algorithm. Nine unique SNP haplotypes were found altogether, and five had frequencies greater than 5% (Table 3). Association analysis using haplotypes calculated by expectation-maximization algorithms showed none of haplotypes were associated with either susceptibility or resistance to AIP.

We previously reported that the *HLA DRB1**0405-*DQB1**0401 haplotype was associated with AIP [11,12]; thus, we further investigated the genetic association between HLA haplotype and *TLR4* SNPs in patients with AIP. Analysis of allelic frequencies revealed no significant difference in SNP5 ($\chi^2 = 0.52$, $p = 0.77$) between patients with and without the *HLA DRB1**0405-*DQB1**0401 haplotype.

3.2. Associations among TLR4 SNPs, patient characteristics, and AIP relapse

Next, we examined associations between the *TLR4* SNPs and clinical parameters. We found no associations between any of the eight *TLR4* SNPs and age, gender, or serum IgG4 concentrations (data not shown). In particular, the median serum IgG4 concentration was not significantly different between patients with and without the SNP5G allele (730 vs 728 mg/dl; $p = 0.94$).

Previous studies found that AIP was associated with the autoimmune diseases, sclerosing cholangitis (34/44; 77%) and hypothyroidism (11/50; 22%) [13]. Thus, we evaluated whether sclerosing cholangitis or hypothyroidism were associated with any of the eight *TLR4* SNPs. We found no significant association between the *TLR4* SNPs and the two diseases (data not shown). Other studies found an association between relapse of AIP and genetics [13,19]; thus, we further analyzed the relationship between *TLR4* SNPs and risk of AIP relapse. In our cohort, 16 of 55 patients (29%) experienced relapse during the follow-up period. However, we found no significant associations between the SNPs or the haplotypes and the relapse of AIP.

4. Discussion

In previous studies, we determined that the *HLA DRB1**0405-*DQB1**0401 haplotype correlated with an increased prevalence of AIP in the Japanese population [11,12]. However, none of the previously identified genetic markers were sufficient to fully explain the disease etiology. Therefore, we suspected that a number of genes outside the major histocompatibility complex region might play a role in AIP susceptibility. For instance, we previously identified polymorphisms of the *FCRL3* and *CTLA4* genes that correlated with an increased prevalence of AIP in the Japanese population [13,14]. However, these findings have not been examined and confirmed in other ethnicities. *TLR4* is an interesting candidate for a gene related to AIP susceptibility because it has previously been implicated in other autoimmune diseases and allergic diseases, including rheumatoid arthritis, Behçet's disease, bronchical asthma, and atopic dermatitis [16–18,31,32].

Of the two co-segregating missense mutations in the gene encoding *TLR4*, A896G and C1196T (which result in Asp299Gly and Thr399Ile amino acid changes, respectively), only A896G interrupted TLR-4 signaling [10]. Most studies that reported disease associations with *TLR4* SNPs have shown significantly higher frequencies of SNPs related to the A896G and C1196T mutations. However, we did not detect polymorphisms on these two SNPs in 100 Japanese healthy controls, consistent with other reports including HapMap data. In the present study, we examined eight SNPs. Only rs2149356 SNP was statistically associated with AIP before correction of the *p* value. However, this SNP, among all SNPs

Table 2
TLR4 polymorphisms in 59 patients with AIP and 126 healthy subjects

dbSNP	Frequency (%)		p	OR	95% CI
	AIP ($n = 59$)	Controls ($n = 126$)			
rs10759930					
Allele frequency					
C allele	38.1	33.3	0.37	1.24	0.78–1.94
T allele	61.9	66.7			
rs1927914					
Allele frequency					
G allele	38.1	32.9	0.33	1.26	0.80–1.98
A allele	61.9	67.1			
rs1927911					
Allele frequency					
A allele	35.6	32.9	0.61	1.13	0.71–1.78
G allele	64.4	67.1			
rs12377632					
Allele frequency					
C allele	64.4	67.5	0.57	1.15	0.72–1.81
T allele	35.6	32.5			
rs2149356					
Genotype frequency					
G/G	39.0	47.6	0.014*		
G/T	59.3	40.5			
T/T	1.7	11.9			
Allele carrier frequency					
G (G/G+G/T)	98.3	88.1	0.021**	7.84	1.01–60.82
T (G/T+T/T)	61.0	52.4	0.27	1.43	0.76–2.67
Allele frequency					
G allele	68.6	67.9	0.98	1.04	0.65–1.66
T allele	31.4	32.1			
rs11536889					
Allele frequency					
A allele	26.3	26.2	0.91	1.00	0.61–1.65
G allele	73.7	73.8			
rs7037117					
Allele frequency					
A allele	79.7	82.5	0.60	1.21	0.69–2.10
G allele	20.3	17.5			
rs7045953					
Allele frequency					
G allele	89.8	92.5	0.52	1.39	0.65–2.96
T allele	10.2	7.5			

AIP, autoimmune pancreatitis; OR, odds ratio; 95% CI, 95% confidence interval.
p Value was calculated by χ^2 test 2 × 2 contingency table (df = 1), or test 3 × 2 contingency table (df = 2).
*p_c (corrected *p* value) = 0.042, **p_c = 0.17.

Table 3
TLR4 haplotypes in patients with AIP and healthy subjects

Haplotype	8 SNPs within a haplotype block spanning 24 Kb								Proportion of indicated haplotype (%)		p value
	1 rs10759930	2 rs1927914	3 rs1927911	4 rs12377632	5 rs2149356	6 rs11536889	7 rs7037117	8 rs7045953	AIP (n = 118)	Controls (N = 252)	
HP1	T	A	G	C	G	G	A	A	33.9	41.4	0.21
HP2	T	A	G	C	G	C	A	A	28.0	24.0	0.43
HP3	C	G	A	T	T	G	A	A	11.0	16.1	0.32
HP4	C	G	A	T	T	G	G	A	10.2	9.5	0.85
HP5	C	G	A	T	T	G	G	G	10.2	6.9	0.26

AIP, autoimmune pancreatitis.
Values for n indicate two times the number of individuals since each person carries two haplotypes.

tested in the patients group, significantly deviated from HWE in the patient group. The deviation might be explained by collecting samples of affected individuals selectively, because HWE failure is generally caused by migration, mutation, gene flow, genetic drift, nonrandom mating, and natural selection. This phenomenon is thought to be the cause of decreasing homozygous TT and GG carriers in contrast to increasing heterozygous TG carriers in the patients. The functional effects of increased TG heterozygotes in patients are still uncertain. However, as there were only a small number of patients in our cohort, it will be necessary to confirm this association in future studies with larger cohorts. Although the statistical power was enough as 0.9349 in this study, type II error was also a possible explanation for the lack of association between TLR4 SNPs and AIP.

Although previous work has shown that serum IgG4 concentrations were closely associated with AIP, we found no significant correlations between serum IgG4 concentrations and *TLR4* SNPs or haplotypes in this study. In addition, our analysis of patients with AIP that also had sclerosing cholangitis and hypothyroidism indicated that there were no associations between extrapancreatic complications and *TLR4* SNPs or haplotypes.

We checked whether the *HLA DRB1**0405-*DQB1**0401 haplotype and rs2149356 SNP was independently associated with AIP. However, we found no confounding association between the *HLA DRB1**0405-*DQB1**0401 haplotype and rs2149356 SNP. This result was similar to that shown in a previous study that found no association between the *HLA DRB1**0405-*DQB1*0401* haplotype and *FCRL3*-110 alleles, although each was independently associated with AIP [14].

Long-term follow-up studies have shown that a 30-40% rate of AIP relapse can occur after remission with corticosteroids [13,19,23,33]. However, no characteristic risk factors or predictive markers have been identified that might be associated with a relapse of AIP. We recently found that patients that experienced relapse had high associations with *CTLA4* +49A/A or +6230A/A genotypes [13]. Furthermore, substitution of aspartate to a non-aspartate residue at HLA DQβ1 57 was associated with a relapse of AIP in a Korean cohort [19]. In the present study, we examined whether any of the *TLR4* SNPs were associated with an AIP relapse but found no associations. Further studies are required to identify predictive markers of AIP relapse.

In conclusion, we found that *TLR4* gene polymorphisms were not significantly associated with susceptibility to AIP in Japan. However, the connection between genetic variations and susceptibility to AIP and AIP relapse remains to be addressed in future investigations.

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