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ORIGINAL ARTICLE—LIVER, PANCREAS, AND BILIARY TRACT

K-ras mutation in the major duodenal papilla and gastric and colonic mucosa in patients with autoimmune pancreatitis

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Abstract
Background Pancreatic cancer occurs in some patients with autoimmune pancreatitis (AIP). Significant K-ras mutations are frequently detected in the pancreas of AIP patients. AIP may be a pancreatic lesion of IgG4-related systemic disease. Gastric and colonic cancer can occur during the follow up of AIP patients. We examined K-ras mutations in the major duodenal papilla and gastric and colonic mucosa of AIP patients.
Methods K-ras analysis and/or immunohistochemical study was performed on the tissues of the major duodenal papilla ($n = 8$), gastric mucosa ($n = 5$), colonic mucosa ($n = 3$), pancreas ($n = 5$), common bile duct ($n = 5$), and gallbladder ($n = 4$) of 12 AIP patients.
Results Significant K-ras mutations were detected in the major duodenal papilla of 4 of 8 cases [GAT ($n = 4$)], in the gastric mucosa of 2 of 4 cases [AGT ($n = 2$)], and in the colonic mucosa of 2 of 3 cases [GAT ($n = 2$)]. Significant K-ras mutations were detected in the pancreas of all 5 cases [GAT ($n = 5$), in the common bile duct of 4 cases (GAT ($n = 2$), TGT ($n = 1$), and GCT/TGT ($n = 1$)], and in the gallbladder epithelium of 3 cases [GAT ($n = 1$), GCT ($n = 1$), and GTT ($n = 1$)]. K-ras mutations were detected in the organs associated with IgG4-related fibroinflammation with abundant infiltration of T lymphocytes and forkhead box P3-positive cells.
Conclusions Significant K-ras mutations were frequently detected in the major duodenal papilla and gastric and colonic mucosa of AIP patients. AIP patients may have risk factors for gastric and colonic cancer, but the mechanisms of K-ras mutation and its clinical implications are not clear.

Keywords Autoimmune pancreatitis · K-ras · IgG4 · Foxp3

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Introduction

Autoimmune pancreatitis (AIP) is a type of pancreatitis with presumed autoimmune etiology. AIP is characterized radiologically by enlargement of the pancreas and irregular narrowing of the main pancreatic duct, serologically by the elevation of serum IgG4 levels, histopathologically by fibrosis with dense infiltration of T lymphocytes and IgG4-positive plasma cells in the peripancreatic and interlobular areas of the pancreas, and clinically by a preponderance of elderly males and good responsiveness to steroid therapy. However, AIP cases may be difficult to differentiate from pancreatic cancer, and some AIP patients in whom pancreatic cancer is suspected undergo unnecessary laparotomy or pancreatic resection [1, 2].

AIP responds well to steroid therapy and appears to be reversible, unlike ordinary chronic pancreatitis [3]. However,

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several reports of AIP associated with pancreatic cancer occurring simultaneously or during follow up have recently been described [2, 4–8]. To assess the relationship between AIP and pancreatic cancer, we previously demonstrated high K-ras mutation levels in the pancreas of 8 patients, in the common bile duct of 5 AIP patients, and in the gallbladder epithelium of 4 AIP patients [9]. We also reported that K-ras mutations were detected in the fibroinflammatory pancreas, bile duct, and gallbladder with abundant infiltrating IgG4-positive plasma cells and forkhead box P3 (Foxp3)-positive cells of AIP patients with elevated serum IgG4 levels [9].

AIP is frequently associated with various extrapancreatic lesions. We have found dense infiltrations of IgG4-positive plasma cells and CD4- or CD8-positive T lymphocytes, as well as fibrosis, in extrapancreatic lesions, such as sclerosing cholangitis, sclerosing sialadenitis, and retroperitoneal fibrosis. Furthermore, abundant infiltration of IgG4-positive plasma cells was detected in various organs, such as the liver, stomach, major duodenal papilla, colon, dermis, lymph node, and bone marrow of AIP patients. Both the pancreatic and extrapancreatic lesions of AIP respond well to steroid therapy. Therefore, we have proposed the existence of a novel clinicopathological entity, an "IgG4-related sclerosing disease", and suggested that AIP is a pancreatic lesion of this systemic disease [10–12]. From the standpoint of the systemic disease, we investigated K-ras mutations in the major duodenal papilla and gastric and colonic mucosa of AIP patients.

Materials and methods

Study patients and study materials

Between 1989 and 2009, 63 patients were diagnosed as having AIP, based on the Asian diagnostic criteria for AIP [13]. They were followed up for 3.8 ± 2.3 years (mean ± SD). With respect to associated malignancies, pulmonary cancer occurred in 2 patients (9 years after onset of AIP and simultaneously with AIP, respectively); esophageal cancer occurred 1 year after onset of AIP, and prostatic cancer occurred 1 year after AIP.

K-ras analysis and/or immunohistochemical study was performed on the tissues of the major duodenal papilla ($n = 8$), gastric mucosa ($n = 5$), and colonic mucosa ($n = 3$) of 12 AIP patients (9 males and 3 females; age range, 25–79 years; average age, 59.1 ± 18.5 years). One patient had a history of heavy drinking and smoking and 2 had a history of heavy smoking. Nine patients showed diffuse enlargement of the pancreas, and 3 patients showed segmental enlargement of the pancreatic head. Five patients underwent pancreatoduodenectomy on suspicion of pancreatic cancer, and the histopathological findings of the resected pancreas were compatible with lymphoplasmacytic sclerosing pancreatitis. The other 7 patients received steroid therapy and responded well. As to the long-term outcome, 9 patients have been followed up until now, 1 patient was lost to follow up 3 years after the operation, and 2 patients died, of pulmonary cancer and pneumonia, respectively (Table 1).

The study materials consisted of 5 stomachs, and 4 major duodenal papillae obtained from pancreatoduodenectomies, 4 endoscopically biopsied major duodenal papillae, and 3 endoscopically biopsied colonic mucosa [rectum ($n = 2$) and sigmoid colon ($n = 1$)] specimens. Endoscopically, the major duodenal papilla was swollen in 1 case (Case 6), and a biopsy was performed prospectively for examining the infiltration of IgG4-positive plasma cells. Biopsy specimens were taken from an area of colitis in the sigmoid colon of Case 11, from a reddish area in the rectum of Case 10, and from a tiny polypoid lesion in the rectum of Case 12. The duration between the clinical onset

Table 1 Clinical and radiological findings in 12 patients with autoimmune pancreatitis

Case	Age (years)	Sex	Type	Therapy	Long-term outcome
1	65	m	Segmental (H)	Pancreatoduodenectomy	Currently alive 9 years after the operation
2	71	m	Segmental (H)	Pancreatoduodenectomy	Died of pneumonia 1 year after the operation
3	65	f	Diffuse	Pancreatoduodenectomy	Currently alive 13 years after the operation
4	69	m	Diffuse	Pancreatoduodenectomy	Died of pulmonary cancer 10 years after the operation
5	79	m	Diffuse	Pancreatoduodenectomy	Lost to follow up 3 years after the operation
6	66	f	Diffuse	Steroid therapy	Currently alive 2 years after the diagnosis
7	76	m	Diffuse	Steroid therapy	Currently alive 4 years after the diagnosis
8	66	m	Diffuse	Steroid therapy	Currently alive 2 years after the diagnosis
9	64	m	Segmental (H)	Steroid therapy	Currently alive 3 years after the diagnosis
10	26	m	Diffuse	Steroid therapy	Currently alive 2 years after the diagnosis
11	25	m	Diffuse	Steroid therapy	Currently alive 1 year after the diagnosis
12	37	f	Diffuse	Steroid therapy	Currently alive 10 years after the diagnosis

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of AIP and the tissue sampling was within 1.5 months in all cases. The 5 pancreases, common bile ducts, and gallbladders obtained from pancreatoduodenectomies were also examined; all of these specimens were the subject of a previous report [9].

The surgical, biopsied, and autopsied tissues were fixed in 10% neutral buffered formalin. Each 1-cm-wide tissue section was examined. Tissue blocks were routinely processed and embedded in paraffin. Serial sections were cut at a thickness of 3 μm. All sections were stained with hematoxylin and eosin (H&E) and examined immunohistochemically.

All subjects provided their written informed consent. This study was approved by the relevant institutional review boards.

Immunohistochemical study

Immunohistochemistry was performed on an average of 2 representative sections from each case, using antibodies against IgG4 (The Binding Site, Birmingham, UK), CD4 T-cell subset (Novocastra, Newcastle, UK), CD8 T-cell subset (Nichirei Bio Science, Tokyo, Japan), and Foxp3 (clone 22509; Abcam, Oxford, UK). All sections were stained by the avidin–biotin horseradish peroxidase method (Vectastain Elite ABC kit; Vector, Burlingame, CA, USA). The additional staining procedures used have all been previously reported [10, 11, 14]. The degrees of infiltrating IgG4-positive plasma cells and Foxp3 were classified as (3+) [more than 30/high power field (HPF)], (2+) (10–30/HPF), (1+) (5–10/HPF), (+/−), (1–4/HPF), and (−) (0/HPF) [11].

K-ras mutation analysis

Paraffin blocks were prepared for DNA extraction. Two lesions from the gastric mucosa, major duodenal papilla, pancreatic duct epithelium, common bile duct epithelium, and gallbladder mucosal epithelium of pancreatoduodenectomy specimens, and biopsy specimens taken endoscopically from the major duodenal papilla and colonic mucosa were used for DNA extraction. The target lesions were microdissected, using a 20-G needle, by comparing an H&E-stained section in the same position. DNA was extracted from paraffin-embedded tissue using a QIAamp DNA FFPE Tissue Kit (QIAGEN, Hilden, Germany). DNA was extracted from the pancreatic juice using a QIAamp DNA Mini Kit (QIAGEN). Mutation of K-ras codon 12 was analyzed and results were compared by enriched polymerase chain reaction-enzyme linked mini-sequence assay (PCR-ELMA) [9, 15].

K-ras was amplified by PCR and then detected and quantitated using a microtiter plate reader (MULTISKAN JX; ThermoFisher Scientific, Yokohama, Japan). The results of the semiquantitative analysis were scored as (3+), (2+), (1+), (+/−), and (−) according to the percentage of mutant ras gene. In general, (3+), (2+), (1+), (+/−), and (−) represented approximately more than 20%, 2%–20%, 0.2%–2%, less than 0.2%, and none (not detected) of the mutant, respectively, according to the manufacturer [9, 15].

Results

Histopathological and immunohistochemical findings

Histopathologically, marked lymphoplasmacytic infiltration with mild to moderate fibrosis was detected in the major duodenal papilla, but there were no atypical changes in the epithelium of the major duodenal papilla (Fig. 1a). Most infiltrated lymphocytes were CD4- or CD8-positive T lymphocytes. Infiltration of IgG4-positive plasma cells in the major duodenal papilla was abundant (3+ or 2+) in 4 cases (Fig. 1b) and mild (1+) in 4 cases. Infiltration of Foxp3-positive cells in the major duodenal papilla was abundant (2+) in 2 cases, mild (1+) in 3 cases, and few (+/−) in 3 cases.

Histologically, marked lymphoplasmacytic infiltration was detected in the gastric mucosa in 2 cases (Cases 1 and 3), but there were no atypical changes in the epithelium of the gastric mucosa. Infiltration of IgG4-positive plasma cells in the gastric mucosa of the antrum was abundant (2+) in 2 cases (Fig. 1c), and few (+/−) in 2 cases. Infiltration of Foxp3-positive cells in the gastric mucosa was mild (1+) in 1 case, and few (+/−) in 3 cases.

Histologically, marked lymphoplasmacytic and neutrophilic infiltration was detected in the colonic mucosa in 1 case (Case 11), but there were no atypical changes in the epithelium of the colonic mucosa. Infiltration of IgG4-positive plasma cells in the colonic mucosa was abundant (2+) in 2 cases (Fig. 1d) and none (−) in 1 case. Infiltration of Foxp3-positive cells in the colonic mucosa was abundant (2+) in 1 case (Fig. 1e), mild (1+) in 1 case, and none (−) in 1 case.

Marked lymphoplasmacytic infiltration, periductal and interlobular fibrosis, and obliterative phlebitis in the pancreas, and transmural fibrosis with marked lymphoplasmacytic infiltration in the common bile duct were detected in the tissues from all AIP cases. Transmural fibrosis with lymphoplasmacytic infiltration was detected in the gallbladder wall of 4 cases (Cases 1–4). There were no atypical changes in the epithelium of the pancreatic and biliary ducts and gallbladder. Abundant infiltration of IgG4-positive plasma cells (2+ or 3+) in the pancreas, bile duct, and gallbladder was detected in all cases. Abundant (2+) and mild (1+) infiltration of Foxp3-positive cells in the

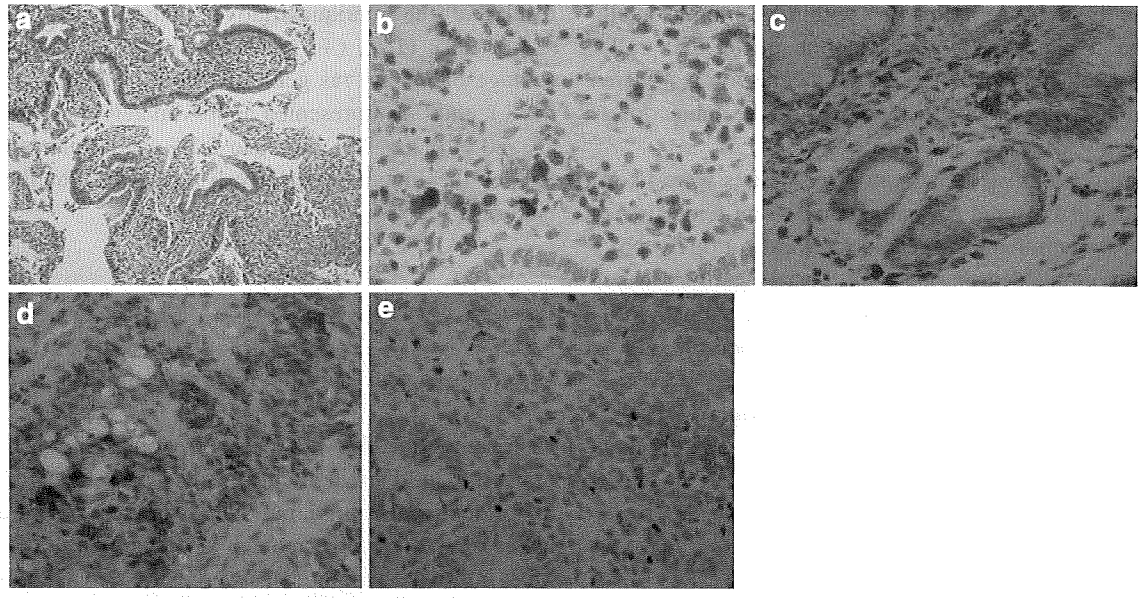

Fig. 1 Histopathological and immunohistochemical findings in the major duodenal papilla and gastric and colonic mucosa of patients with autoimmune pancreatitis. **a** Marked lymphoplasmacytic infiltration with mild fibrosis was detected in the major duodenal papilla (Case 1). **b** Abundant infiltration of IgG4-positive plasma cells detected in the major duodenal papilla (Case 1). **c** Abundant infiltration of IgG4-positive plasma cells detected in the gastric mucosa (Case 3). Abundant infiltration of **d** IgG4-positive plasma cells and **e** forkhead box P3 (Foxp3)-positive cells detected in the colonic mucosa (Case 11)

pancreas, bile duct, and gallbladder was detected in 60 and 40%, 40 and 60%, and 0 and 40%, respectively (Table 2).

K-ras mutation

Significant K-ras mutations (3+) were detected in the major duodenal papilla of 4 of 8 cases [GAT ($n = 4$)] (Fig. 2a). Significant K-ras mutations (3+) were detected in the gastric mucosa of 2 of 4 cases [AGT ($n = 2$)] (Fig. 2b). Significant K-ras mutations (2+) were detected in the colonic mucosa of 2 of 3 cases [GAT ($n = 2$)] (Fig. 2c).

Significant K-ras mutations (3+) were detected in the pancreas of all 5 cases [GAT ($n = 5$)]. Significant K-ras mutations (3+) were detected in the common bile duct of 4 cases [GAT ($n = 2$), TGT ($n = 1$), and GCT/TGT ($n = 1$)]. Significant K-ras mutations (3 +or 2+) were detected in the gallbladder epithelium of 3 cases [GAT ($n = 1$), GCT ($n = 1$), and GTT ($n = 1$)].

Abundant or mild infiltration of IgG4-positive plasma cells and Foxp3-positive cells was also detected in the major duodenal papilla and gastric and colonic mucosa of all cases with significant K-ras mutations (Table 2).

In comparisons of the distribution of K-ras mutation and the density of IgG4-positive or Foxp3-positive cells in the major duodenal papilla with those in the stomach of the same patient (Cases 1, 3, and 4), significant K-ras mutation of different mutant types with abundant infiltration of IgG4-positive plasma cells and mild or few Foxp3-positive cells was detected in both the major duodenal papilla and stomach in Case 3. However, in Cases 1 and 4, the distribution of K-ras mutation and the densities of IgG4-positive plasma cells and Foxp3-positive cells were different in the major duodenal papilla and stomach.

Discussion

K-ras mutation is believed to occur at a relatively early stage during the multistep carcinogenesis process. K-ras mutations were detected in more than 95% of pancreatic cancers [16] and in 27% of hyperplastic pancreatic duct epithelium in chronic pancreatitis [17]. Furthermore, as the cumulative risk of pancreatic cancer in subjects with chronic pancreatitis was reported to be 1.8% after 10 years and 4.0% after 20 years, chronic pancreatitis is considered to be a risk factor for the development of pancreatic cancer [18].

Several reports of AIP associated with pancreatic cancer occurring simultaneously or during follow up have recently been described [2, 4–8]. We have demonstrated that significant K-ras mutations occurred frequently in the pancreas, common bile duct, and gallbladder with fibroinflammation with abundant infiltrating IgG4-positive plasma cells and Foxp3-positive cells in AIP patients, although the mechanism of the K-ras mutation was unclear [9].

Table 2 Serum IgG4 levels, distribution of IgG4- and Foxp3-positive cells, and K-ras mutations in the pancreas, common bile duct, gallbladder, major duodenal papilla, stomach, and colon of 12 patients with autoimmune pancreatitis

	Serum IgG4 (mg/dl)	Pancreas	Common bile duct	Gallbladder	Duodenal papilla	Stomach	Colon
1	505	K-ras: 3+ (GAT) IgG4+: 3+ Foxp3+: 2+	K-ras: 3+ (TGT) IgG4+: 3+ Foxp3+: 1+	K-ras: 2+ (GAT) IgG4+: 3+ Foxp3+: +/−	K-ras: 3+ (GAT) IgG4: 2+ Foxp3: 2+	K-ras: +/− (CGT) IgG4+: 2+ Foxp3+: +/−	NE
2	550	K-ras: 3+ (GAT) IgG4+: 3+ Foxp3+: 2+	K-ras: 3+ (GAT) IgG4+: 3+ Foxp3+: 1+	K-ras: 3+ (GTT) IgG4+: 3+ Foxp3+: 1+	NE	K-ras: +/− (GTT) IgG4+: +/− Foxp3+: +/−	NE
3	1240	K-ras: 3+ (GAT) IgG4+ : 3+ Foxp3+: 2+	K-ras: 3+ (GCT/TGT) IgG4+: 3+ Foxp3+: 2+	K-ras: 3+ (GCT) IgG4+: 3+ Foxp3+: 1+	K-ras: 3+ (GAT) IgG4+: 3+ Foxp3+: 1+	K-ras: 3+ (AGT) IgG4+: 2+ Foxp3+: 1+	NE
4	150	K-ras: 3+ (GAT) IgG4+: 3+ Foxp3+: 1+	K-ras: 3+ (GAT) IgG4+: 3+ Foxp3+: 2+	NA IgG4+: 2+ Foxp3+: −	K-ras: +/− (AGT) IgG4+: 2+ Foxp3+: 1+	K-ras: 3+ (AGT) IgG4+: +/− Foxp3+: +/−	NE
5	43	K-ras: 3+ (GAT) IgG4+ : 3+ Foxp3+: 1+	NA IgG4+: 2+ Foxp3+: 1+	+/− (GAT) IgG4+: 2+ Foxp3+: −	K-ras: 3+ (GAT) IgG4+: 1+ Foxp3+: 1+	NA IgG4+: − Foxp3+: −	NE
6	1230	NE	NE	NE	K-ras: 3+ (GAT) IgG4+: 2+ Foxp3+: 2+	NE	NE
7	1160	NE	NE	NE	K-ras: − IgG4+: 1+ Foxp3+: +/−	NE	NE
8	395	NE	NE	NE	K-ras: − IgG4+: 1+ Foxp3+: +/−	NE	NE
9	867	NE	NE	NE	K-ras: − IgG4+: 1+ Foxp3+: +/−	NE	NE
10	647	NE	NE	NE	NE	NE	K-ras: 2+ (GAT) IgG4+: 2+ Foxp3+: 1+
11	45	NE	NE	NE	NE	NE	K-ras: 2+ (GAT) IgG4+: 2+ Foxp3+: 2+
12	11	NE	NE	NE	NE	NE	K-ras: +/− (AGT) IgG4+: − Foxp3+: −

NE Not examined, *NA* not amplified, *Foxp3* forkhead box P3

Because dense infiltration of IgG4-positive plasma cells and CD4- or CD8-positive T lymphocytes, as well as fibrosis, was detected in extrapancreatic lesions, and abundant infiltration of IgG4-positive plasma cells was also detected in various other organs in AIP patients, we have proposed a new clinicopathological entity, an "IgG4-related sclerosing disease", and suggested that AIP is a pancreatic lesion of this systemic disease [10–12]. This concept led us to investigate K-ras mutation in the major duodenal papilla, and in the gastric and colonic mucosa of AIP patients.

In the present study, significant K-ras mutations were detected in the major duodenal papilla of 4 (3 resected and 1 biopsied cases) of 8 AIP cases. All major duodenal papillae with significant K-ras mutations were associated with abundant or mild infiltration of IgG4-positive plasma cells and Foxp3-positive cells. The mutant type of ras gene was GAT in all 4 cases, which was quite similar to the

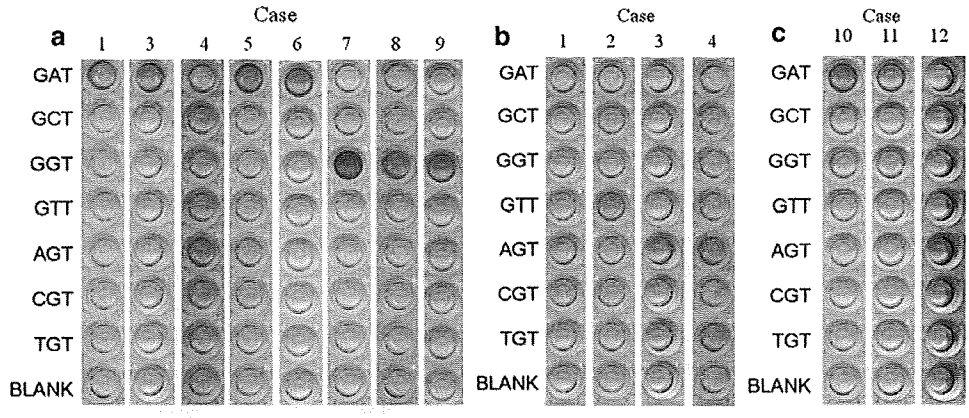

Fig. 2 Actual images of microwells in semiquantitative analysis of mutant K-ras gene by polymerase chain reaction-enzyme linked minisequence assay (PCR-ELMA) in **a** the major duodenal papilla (Cases 1, 3–9), **b** the gastric mucosa (Cases 1–4), and **c** the colonic mucosa (Cases 10–12). Hybridization was performed using both wild-type (GGT) and 6 kinds of mutant specific probes (GAT, GCT, GTT, AGT, CGT, and TGT) that were immobilized to the microtiter plate well. The type and semiquantitation of the K-ras gene were identified as mutant type (3+) or (2+), when the signal was observed exclusively in the mutant specific probe well

K-ras mutation type in the pancreas. It was reported that K-ras mutation was detected in about 40% of cancers of the major duodenal papilla, and the mutations were mainly GAT or GTT [19, 20]. However, because histological examination of the resected pancreas showed inflammation of the major duodenal papilla followed by inflammation of the pancreatic head in AIP cases, it seems that the K-ras mutation in the major duodenal papilla might be the consequence of K-ras mutation in the pancreas.

Significant K-ras mutations were detected in the gastric mucosa of 2 of 4 resected cases in the present study, and the mutation type was AGT in the 2 cases. Lee et al. [21] reported that K-ras mutations were detected in 7.9% of gastric cancers, and the mutation type was AGT in 78% of them. Gong et al. [22] reported that the AGT transition was important for the progression of gastric mucosal cells to a more advanced premalignant stage. Watari et al. [23] reported that K-ras mutations in chronic gastritis with GAT and TGT types disappeared after *Helicobacter pylori* eradication, but AGT remained in most cases after treatment, and mutations with AGT were considered more likely to be advantageous in K-ras gene alterations.

Significant K-ras mutations were detected in the colonic mucosa of 2 of 3 biopsied cases in the present study, and the mutation type was GAT in the 2 cases. One specimen was taken from an area of colitis in the sigmoid colon, and the other was taken from a reddish area in the rectum. It has been reported that K-ras mutations were detected in 32%–44% of colonic cancers, and the mutant types were mainly GAT and GTT [24, 25].

In the present AIP patients, high levels of significant K-ras mutation were frequently detected in the major duodenal papilla, gastric mucosa, and colonic mucosa, associated with abundant infiltration of IgG4-positive plasma cells, T lymphocytes, and Foxp3-positive cells, similar to the findings in the pancreas, bile duct, and gallbladder. K-ras mutation types in the major duodenal papilla, gastric mucosa, and colonic mucosa were similar to those in cancers of each organ. It has been reported that gastric cancer and colonic cancer developed simultaneously or during the follow up of AIP [26, 27]. In AIP patients, the major duodenal papilla, gastric mucosa, and colonic mucosa, as well as the pancreas, bile duct, and gallbladder, may be the sites of a premalignant lesion. However, the mechanism of K-ras mutation in the various organs of AIP patients is still unclear. The epithelia of these organs did not show any atypical changes, although there was a report of a stepwise increase in K-ras mutation that correlated with the grade of dysplasia in pancreatic intraepithelial neoplasia (PanIN) [28]. Foxp3-positive regulatory T cells, producing interleukin 10 and transforming growth factor β, which was followed by IgG4 class switching and fibroplasias, were increasingly detected in the pancreas and the biliary tract of AIP patients [1, 29]. It has been reported that Foxp3-positive regulatory T cells were increased locally in pancreatic cancer [30], and Foxp3-positive regulatory T cells and inflammation played an essential and important role in K-ras-mediated lung tumorigenesis in mice [31]. Brembeck et al. [32] showed dramatic lymphocytic infiltration around the periductal area in transgenic mice fused to mutant K-ras and suggested that these lymphocyte infiltrations might act as an adaptive immune response to activated ras-mediated signaling. Watari et al. [23] reported that, because K-ras mutations sometimes occurred in gastric intestinal metaplasia with *H. pylori* infection, but some mutations disappeared after *H. pylori* eradication, early events in K-ras mutations in gastric intestinal metaplasia were unstable in some cases and may have been related to the lymphocyte infiltration caused by *H. pylori* infection.

AIP appears to be a pancreatic lesion of an IgG4-related systemic disease. AIP is usually diagnosed in the active stage, but IgG4-related fibroinflammation may have persisted subclinically in various organs for a long time. In AIP patients, K-ras mutation may occur in the organs showing persistent IgG4-related fibroinflammation with abundant infiltration of T lymphocytes and Foxp3-positive cells.

The present study is the first to have demonstrated frequent and significant K-ras mutations in the major duodenal papilla and gastric and colonic mucosa of AIP patients, but the number of cases and the number of samples in which K-ras mutation was investigated were small. However, we believe that the data presented here, as a pilot study, are very interesting and will generate interest to extend the studies. Further investigations are thus required using a larger series of samples with a longer-term follow up to determine the possible role of K-ras mutation as a precancerous lesion in AIP patients. Furthermore, investigations of K-ras mutation in other organs in AIP patients and changes in K-ras mutation after steroid therapy should also be informative.

In conclusion, in AIP patients, significant K-ras mutations were frequently detected in the major duodenal papilla, and in the gastric and colonic mucosa, as well as in pancreatobiliary regions associated with IgG4-related fibroinflammation with abundant infiltration of T lymphocytes and Foxp3-positive cells. AIP patients may have risk factors for gastric and colonic cancer, but the mechanism of K-ras mutation and its clinical implications are not clear.

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Standard steroid treatment for autoimmune pancreatitis

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ABSTRACT

Objective: To establish an appropriate steroid treatment regimen for autoimmune pancreatitis (AIP).

Methods: A retrospective survey of AIP treatment was conducted in 17 centres in Japan. The main outcome measures were rate of remission and relapse.

Results: Of 563 patients with AIP, 459 (82%) received steroid treatment. The remission rate of steroid-treated AIP was 98%, which was significantly higher than that of patients without steroid treatment (74%, 77/104; $p<0.001$). Steroid treatment was given for obstructive jaundice (60%), abdominal pain (11%), associated extrapancreatic lesions except the biliary duct (11%), and diffuse enlargement of the pancreas (10%). There was no relationship between the period necessary to achieve remission and the initial dose (30 mg/day vs 40 mg/day) of prednisolone. Maintenance steroid treatment was given in 377 (82%) of 459 steroid-treated patients, and steroid treatment was stopped in 104 patients. The relapse rate of patients with AIP on maintenance treatment was 23% (63/273), which was significantly lower than that of patients who stopped maintenance treatment (34%, 35/104; $p = 0.048$). From the start of steroid treatment, 56% (55/99) relapsed within 1 year and 92% (91/99) relapsed within 3 years. Of the 89 relapsed patients, 83 (93%) received steroid re-treatment, and steroid re-treatment was effective in 97% of them.

Conclusions: The major indication for steroid treatment in AIP is the presence of symptoms. An initial prednisolone dose of 0.6 mg/kg/day, is recommend, which is then reduced to a maintenance dose over a period of 3–6 months. Maintenance treatment with low-dose steroid reduces but dose not eliminate relapses.

Autoimmune pancreatitis (AIP) is a newly described entity in which autoimmune mechanisms seem to be involved. It is characterised clinically by obstructive jaundice as a frequent initial symptom and an association with diabetes mellitus (DM) and various extrapancreatic lesions. Radiologically, AIP is characterised by enlargement of the pancreas and irregular narrowing of the main pancreatic duct. Serologically, it is characterised by serum immunoglobulin G4 (IgG4) elevation and the presence of autoantibodies. Histopathologically, dense infiltration of lymphocytes and IgG4-positive plasma cells with fibrosis are seen in the pancreas.[1–4] Since the fibroinflammatory process of AIP responds well to steroids, many AIP patients receive steroid treatment.[5–10] Although there are some published reports dealing with steroid treatment for AIP, these previous studies involved only small numbers of patients, and there is little consensus on a steroid treatment regimen. To establish the appropriate steroid treatment regimen, a survey of AIP treatment was conducted in 17 centres in Japan.

METHODS

A retrospective survey of AIP treatment, focusing on steroid treatment, was conducted in the 17 centres that participated in this study. The majority of these centres are major referral centres across Japan with established expertise in the diagnosis and management of AIP. Tokyo Metropolitan Komagome Hospital and Tohoku University served as the coordinating centres.

The diagnosis of AIP was made according to the Asian diagnostic criteria for AIP.[11] To make the diagnosis of AIP, the imaging criterion, consisting of enlargement of the pancreas and irregular narrowing of the main pancreatic duct, must be present, together with the serological criterion (elevated serum IgG or IgG4 levels, or detection of autoantibodies) and/or the histopathological criterion (lymphoplasmacytic sclerosing pancreatitis). AIP can be also diagnosed with fulfilment of both the imaging criterion and a good response to steroid treatment.

Data regarding induction of remission by steroid treatment, maintenance steroid treatment and relapse of AIP were analysed. Remission was defined as the disappearance of clinical symptoms and resolution of the pancreatic and/or extrapancreatic manifestations on imaging studies.[8 12–14] For follow-up after remission, laboratory tests and imaging studies were performed periodically, usually every 3–6 months in the first year. Relapse of AIP was defined as reappearance of symptoms with the development or reappearance of pancreatic and/or extrapancreatic (including bile duct, salivary gland and retroperitoneum) abnormalities on imaging studies and/or marked elevation of serum IgG or IgG4 levels.[8 12–14] Re-elevation of serological levels alone without clinical symptoms or abnormal imaging was not considered to be relapse.

Statistical analysis was performed using Fisher's exact test and Mann–Whitney's U test. Differences with p values of <0.05 were considered significant. The period from the start of steroid treatment to relapse was evaluated using the Kaplan–Meier curve.

After analysis of the data, a consensus meeting was held involving members of the 17 centres to propose a consensus regarding steroid treatment for AIP.

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RESULTS

Study subjects

A total of 563 cases of AIP (439 men and 124 women, average age 63.0 years) were confirmed to fulfil the Asian diagnostic criteria for AIP, and they were enrolled in the analyses for steroid treatment of AIP. Of these, 459 (82%) patients with AIP (374 men and 85 women, average age 62.3 years) received steroid treatment. Of the others, 56 patients underwent surgical procedures, and 48 patients were followed-up conservatively.

No patients received any other immunosuppressive treatments such as azathioprine or ulsodeoxycholic acid.

Induction of remission by steroid treatment

The remission rate of patients with AIP was significantly higher in patients who received steroid treatment (98%, 451/459) than in those not given steroid treatment (74%, 77/104; $p<0.001$) (table 1).

Steroid treatment was administered mainly for obstructive jaundice (247/459 patients, 60%), abdominal pain (51 patients, 11%), associated extrapancreatic lesions such as retroperitoneal fibrosis (50 patients, 11%), diffuse enlargement of the pancreas (45 patients, 10%) and confirmation or differentiation of the diagnosis after a negative investigation of pancreatic cancer (24 patients, 5%).

In patients with DM, before steroid administration, blood glucose levels were controlled using insulin in 104 patients and using oral antidiabetic medicines in 39 patients. Endoscopic or transhepatic biliary drainage was performed in 242 (77%) of 314 patients with obstructive jaundice due to associated sclerosing cholangitis. Endoscopic or transhepatic biliary drainage was performed for patients showing hyperbilirubinaemia of >3 mg/dl in two-thirds of centres.

The initial oral prednisolone dose was 20 mg/day (n = 8, 2%), 30 mg/day (n = 283, 62%), 40 mg/day (n = 160, 35%), 60 mg/day (n = 4, 1%) and others (n = 4, 1%). The initial dose was administered for 2 weeks in three-quarters of cases, and for 3–4 weeks in the remainder. The initial dose was gradually tapered by 5 mg every 1–2 weeks to the maintenance dosage, based on changes in the clinical manifestations, biochemical blood tests (such as serum liver enzymes and IgG or IgG4 levels) and repeated imaging findings. The dose was tapered more gradually, such as 2.5 or 5 mg every 2–8 weeks, after the dose reached 15 mg/day.

The period necessary to achieve remission from the start of initial administration was 6.82 (6.11) months (mean (SD)) in the patients treated with an initial prednisolone dose of 30 mg/day, which was not significantly different from the period (6.34 (8.13) months) in those treated with an initial prednisolone dose of 40 mg/day ($p = 0.401$) (table 2).

At remission, the enlarged pancreas returned to near-normal size in 239 (80%) of 300 patients. It became atrophic in 58 patients (20%), and showed persistent focal enlargement in 3 patients. Elevated serum IgG4 levels decreased in all patients after the start of steroid treatment, but they failed to normalise (<135 mg/dl) in 115 (63%) of 182 patients. At remission, irregularity of the pancreatic ducts and/or some degree of bile duct stenosis remained in 67 (58%) of 115 patients with persistent elevation of serum IgG4 levels, while it remained in only 18 (27%) of 67 patients with normalised serum IgG4 levels ($p<0.001$).

Table 1 Remission and relapse rate in patients with autoimmune pancreatitis treated with and without steroid

	With steroid	Without steroid	p Value
Remission rate	451/459 (98%)	77/104 (74%)	<0.0001
Relapse rate	110/451 (24%)	32/77 (42%)	0.003

Maintenance steroid treatment

Maintenance steroid treatment was performed after remission in 377 (82%) of 459 patients treated with steroid. The maintenance oral prednisolone dose was 10 mg/day (n = 27, 7%), 7.5 mg/day (n = 13, 3%), 5 mg/day (n = 238, 63%), 2.5 mg/day (n = 78, 21%) and others. Of the 377 patients who underwent maintenance treatment, the maintenance treatment was stopped in 104 patients (28%) in whom complete radiological and serological improvement was obtained.

Relapse of AIP

The relapse rate of patients with AIP was significantly lower in those who received steroid treatment (24%, 110/451) than in those not given steroid treatment (42%, 32/77; $p = 0.003$) (table 1). In the patients who received steroid treatment, relapse occurred in the pancreas (n = 57, 52%), bile duct (n = 37, 34%) and extrapancreatic lesions (n = 19; salivary gland swelling (n = 10), interstitial pneumonia (n = 4), interstitial nephritis (n = 2) and others).

There was no correlation between the relapse rate and the initial prednisolone dose (40 mg/day: 19% (31/160) vs 30 mg/day: 23% (65/283), $p = 0.402$) (table 2). As regards the period from the start of steroid treatment to relapse, 32% (32/99) relapsed within 6 months, 56% (55/99) relapsed within 1 year, 76% (75/99) relapsed within 2 years and 92% (91/99) relapsed within 3 years after starting medication (fig 1). The relapse rate of patients with AIP on maintenance treatment was 23% (63/273), which was significantly lower than that of patients who stopped maintenance treatment (34%, 35/104; $p = 0.048$). The doses of prednisolone at the time of relapse were 10 mg/day (n = 10, 16%), 7.5 mg/day (n = 7, 11%), 5 mg/day (n = 29, 46%), 2.5 mg/day (n = 8, 13%) and others.

The relapse rate of AIP was significantly higher in patients with persistent elevation of serum IgG4 levels (30%, 34/115) than in those with normalised serum IgG4 levels (10%, 7/69; $p = 0.003$). Although serum IgG4 levels fluctuated by >30 mg/dl in 94 (55%) of 172 patients during maintenance treatment, re-elevation of serum IgG4 levels was detected in 37 (69%) of 54 patients who relapsed during maintenance treatment.

Of the 89 relapsed patients, 83 (93%) of 89 received steroid re-treatment (prednisolone: 60 mg/day (n = 4, 5%), 40 mg/day (n = 19, 23%), 30 mg/day (n = 39, 47%), 20 mg/day (n = 9, 11%) and others). Steroid re-treatment was effective in 91 (97%) of 94 relapsed patients. Of the 77 patients initially managed without steroid treatment relapses occurred in 32 (42%), and the relapses were treated with steroid with a 100% response rate.

Steroid-related complications

After steroid treatment, mildly or moderately worse glucose tolerance occurred in several patients, but they could be controlled by oral antidiabetic medication or insulin injection. Osteoporosis developed in 10 patients, in whom compression fractures of lumbar vertebrae (n = 5) and avascular necrosis of the femoral head (n = 3) occurred. They were treated with reduction of dosage or cessation of medication. Pneumonia occurred in 3 patients, and they were treated with antibiotics.

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Table 2 Period to yield a remission and relapse rate in patients with autoimmune pancreatitis treated with initial prednisolone of 40 and 30 mg/day

	40 mg/day	30 mg/day	p Value
Period to remission (mean (SD), months)	6.34 (8.13)	6.82 (6.11)	0.401
Relapse rate	31/160 (19%)	65/283 (23%)	0.402

There were no deaths attributable to complications of steroid treatment.

DISCUSSION

In the analysis of 563 patients with AIP, 82% received oral steroid treatment. The remission rate of patients with AIP was significantly higher in those who received steroid treatment (98%) than in those who did not receive steroid treatment (74%). The relapse rate was significantly lower in patients who received steroid treatment than in those not given steroid treatment. Therefore, the administration of oral steroid appears to be standard treatment for inducing remission in AIP. However, it is most important to distinguish AIP from pancreatic cancer before starting steroid treatment. Facile use of steroids for cases in which the diagnosis of AIP is questionable should be prohibited.[15 16]

The indications for steroid treatment in patients with AIP are thought to be symptoms such as obstructive jaundice due to sclerosing cholangitis, abdominal pain and hydronephrosis due to associated retroperitoneal fibrosis. DM is often (67%[17] to 76%[18] of cases) observed in patients with AIP. Pancreatic exocrine function is also impaired in 88%[19] to 91%[20] of AIP patients. However, as pancreatic exocrine or endocrine dysfunction improves in some patients with AIP after steroid treatment,[17 19 20] steroid treatment may be indicated in patients showing diffuse enlargement of the pancreas, even if they are asymptomatic.

In patients with DM, blood glucose levels should be controlled using insulin before starting steroid treatment. The major presenting complaint of patients with AIP is obstructive jaundice due to associated sclerosing cholangitis (65%[18] to 86%[21] of cases). As steroid treatment may trigger or worsen cholangitis, jaundice is usually managed by endoscopic or transhepatic biliary drainage in patients with obstructive jaundice (usually total bilirubin ≥3 mg/dl) due to associated sclerosing cholangitis before steroid administration. In the literature, most patients with AIP were treated with 30 or 40 mg/day as the initial prednisolone dose.[5–10] There was no relationship between the period necessary to achieve remission and the initial prednisolone dose. In Japan, 30 mg/day is usually used as an initial dose in patients with standard body weight (~50 kg; 0.6 mg/kg) and 40 mg/day is used in larger patients (body weight >60 kg; 0.67 mg/kg). Therefore, we would recommend that a general initial dose of prednisolone should be 0.6 mg/kg/day.

Since pancreatic enlargement begins to improve from 1 to 2 weeks after medication,[10 12] morphological and serological evaluation for effectiveness of steroid treatment should be performed 2 weeks after starting steroid treatment. A poor response to steroid treatment should raise the possibility of pancreatic cancer and the need for re-evaluation of the diagnosis. When steroid treatment is effective, the dose is tapered by 5 mg every 1–2 weeks until the dose reaches 15 mg/day, while carefully monitoring the patient's symptoms, as well as the biochemical, serological and imaging findings. After that, the dose is tapered more gradually, and the amount of steroid is reduced to a maintenance dose over a period of 3–6 months.

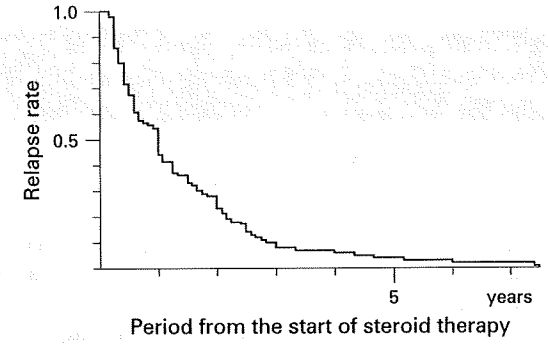

Figure 1 Relapse rate of autoimmune pancreatitis and the period from the start of steroid treatment to relapse.

Relapse occurred in 24% of patients with AIP treated with steroid, and it occurred within 6 months after medication in 32%, within 1 year in 56% and within 3 years in 92%. Although it still remains unclear what are useful predictive findings for relapse, it has been reported that markedly elevated serum IgG4 levels and the presence of bile duct stenosis were predictive factors for relapse of AIP.[8] To our knowledge, patients complicated with extrapancreatic lesions such as stenosis of the proximal extrahepatic or intrahepatic bile duct or retroperitoneal fibrosis seem to take longer to achieve remission. In the present study, the patients with elevation of serum IgG4 levels during remission showed persistent abnormalities of the pancreatic and/or bile ducts and more frequent relapses than in those with normalised serum IgG4 levels. Furthermore, in 69% of relapsed patients during maintenance treatment, re-elevation of serum IgG4 levels was detected. Serum IgG4 levels at remission and during follow-up may accordingly be useful to predict or detect relapse earlier.

To prevent relapse, maintenance treatment (5 mg/day) is recommended in almost all patients treated with steroid for at least about 6 months. However, as patients with AIP are typically elderly and are at high risk of developing steroid-related complications, such as osteoporosis, DM and pneumonia, cessation of the medication should be tried. In patients showing complete improvement of cholangiopancreatogram, 1 year after initial administration of steroid, maintenance treatment can be withdrawn. Stopping maintenance treatment should be planned within at least 3 years, because of a lower relapse rate after 3 years. After stopping medication, patients should be followed-up for relapse of AIP. In most recurrent cases, re-administration or dose-up of steroid is effective. In these cases, longer maintenance treatment is necessary to prevent repeated relapse. Therefore, we think that it is now necessary to reduce the relapse rate. Careful, long-term follow-up is also necessary, since pancreatic cancer developed in three patients 3, 3.5 and 5 years after onset of AIP, respectively. On the other hand, 23% of patients with AIP relapsed despite maintenance treatment. We need to identify more useful alternative approaches to maintain remission.

In the Japanese diagnostic criteria for AIP,[15] seronegative AIP cases without histological examination cannot be diagnosed as AIP even if they fulfil the imaging criterion. Kim *et al*[22] reported that a steroid trial was useful for differentiating seronegative AIP from pancreatic cancer, based on marked improvement of pancreatic ductal narrowing, which is evident as early as 2 weeks after the beginning of steroid treatment. When

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Box 1 Standard steroid treatment for autoimmune pancreatitis

- Oral steroids is the standard treatment for AIP.
- It is most important to distinguish AIP from pancreatic cancer before starting steroid treatment.
- The indications for steroid treatment in patients with AIP are symptoms such as obstructive jaundice, abdominal pain and hydronephrosis.
- Before steroid treatment, blood glucose level should be controlled using insulin in patients with diabetes mellitus.
- Before steroid treatment, jaundice is usually managed by endoscopic or transhepatic biliary drainage in patients with obstructive jaundice.
- As initial dose of oral prednisolone of 0.6 mg/kg/day is recommended.
- Morphological and serological evaluation for effectiveness of steroid treatment is performed 2 weeks after starting steroid treatment. A poor response to steroid treatment should raise the possibility of pancreatic cancer and the need for re-evaluation of the diagnosis. When steroid treatment is effective, the dose is tapered by 5 mg every 1–2 weeks until the dose reaches 15 mg/day, while carefully monitoring the patient's symptoms, as well as the biochemical, serological and imaging findings. After that, the dose is tapered more gradually to a maintenance dose over a period of 3–6 months.
- To prevent relapse, maintenance treatment (5 mg/day) is recommended in almost all patients treated with steroid for at least about 6 months. In patients showing complete remission 1 year after initial administration of steroid, maintenance treatment can be withdrawn. Stopping maintenance treatment should be planned within at least 3 years.
- In relapsed cases, re-administration or dose-up of steroid is effective.
- A steroid diagnostic trial is not generally recommended. It should only be performed by pancreatologists with extreme caution in limited cases after a negative investigation for pancreatic cancer, including endoscopic ultrasound-guided fine needle aspiration.

response to steroid treatment is added to the diagnostic criteria, the diagnostic sensitivity is increased. However, we are concerned that the facile use of steroid trials will result in delaying pancreatic cancer surgery, which could lead to cancer progression in some cases. Therefore, as described in the Asian diagnostic criteria,[11] a steroid diagnostic trial is not generally recommended and it should only be performed by pancreatologists with extreme caution in limited cases after a negative investigation for pancreatic cancer, including endoscopic ultrasound-guided fine needle aspiration (EUS-FNA; box 1).

In conclusion, oral steroid is a standard treatment for AIP. Indications for steroid treatment in patients with AIP are symptoms such as obstructive jaundice due to sclerosing cholangitis, abdominal pain, and hydronephrosis due to associated retroperitoneal fibrosis. We recommend that the initial dose of prednisolone be 0.6 mg/kg/day, and that it be reduced to a maintenance dose over a period of 3–6 months. To prevent relapses, continued maintenance treatment with low-dose prednisolone for 6 months to 3 years is recommended, but it dose not eliminate relapse entirely.

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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).

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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

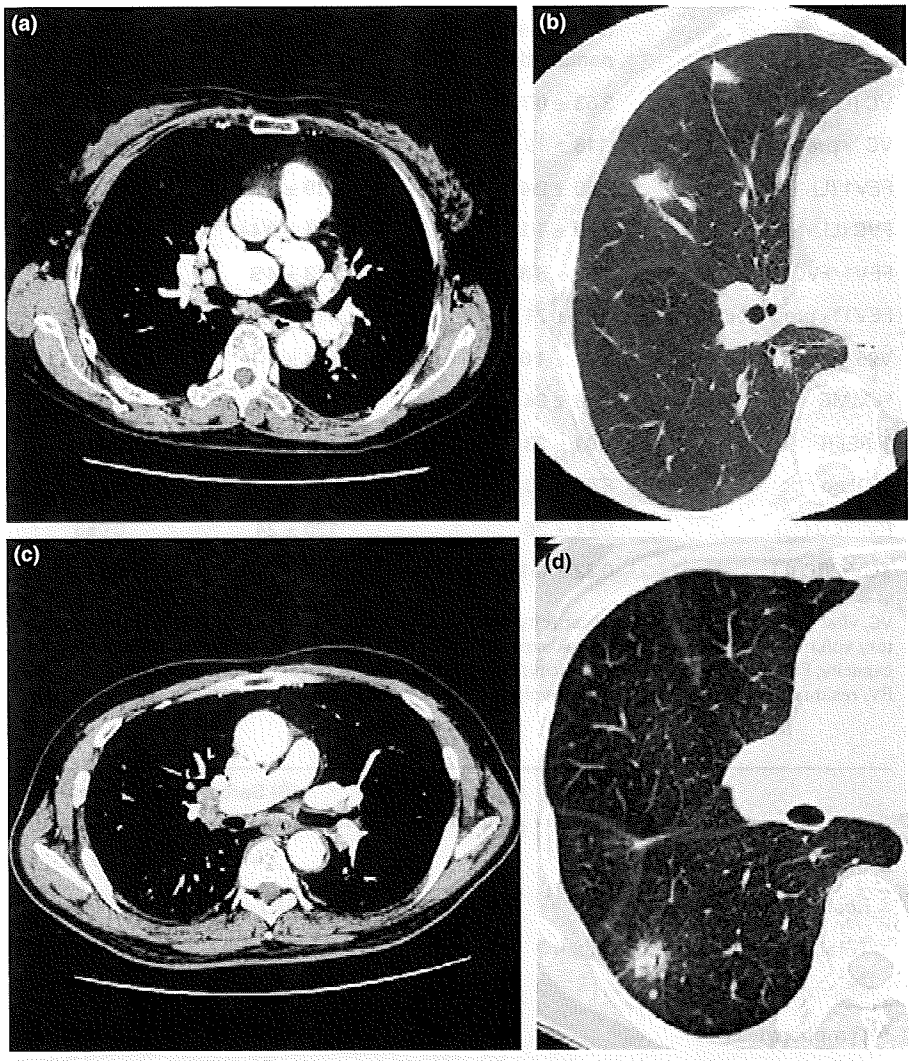

Figure 1 (a) A 78-year-old female with autoimmune pancreatitis showed bilateral hilar lymphadenopathy and an enlargement of the mediastinal lymph nodes on chest CT scans. (b) The same patient showed consolidated nodules with speculation in the right lower lobe and the inguinal lobe. (c) A 65-year-old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

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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 (μg 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

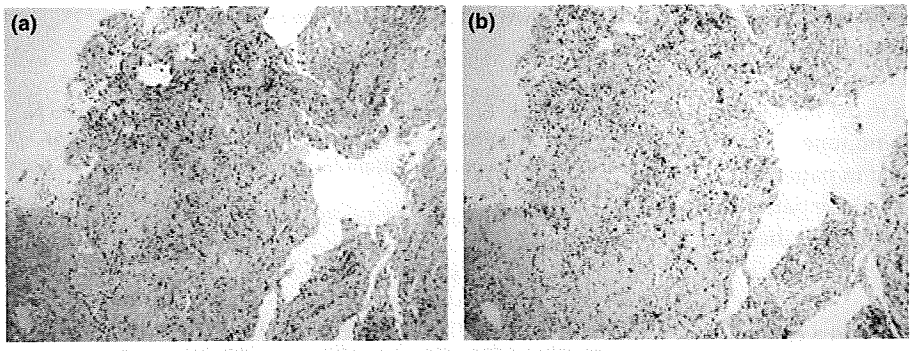

Figure 2 (a) A 71-year-old male with autoimmune pancreatitis showed granulation tissue and severe infiltration of lymphocytes and plasma cells (H–E stain ×10). (b) The same patient showed IgG4-positive plasma cells (IgG4 antibody immunostaining ×10)

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

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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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