Clinical Features and Expressions of Foxp3 and IL-17 in Type 1 Autoimmune Pancreatitis in China

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Background: Autoimmune pancreatitis (AIP) is a distinct type of pancreatitis associated with a presumed autoimmune mechanism. The aim of this study was to analyze the clinical features and expressions of forkhead box P3 (Foxp3) and interleukin-17 (IL-17) in type 1 AIP in China and to identify factors for differentiation of AIP from non-AIP chronic pancreatitis (CP).

Material/Methods: We retrospectively reviewed pancreatic specimens with diagnosis of type 1 AIP and non-AIP CP at Sun Yat-Sen Memorial Hospital in China from January 2000 to December 2013. The clinical symptoms, serological data, imaging findings, histopathology, and immunohistochemical findings of Foxp3 and IL-17 in the 2 groups were analyzed.

Results: Twenty-nine patients with type 1 AIP and 20 patients with non-AIP CP were enrolled. Obstructive jaundice was more common in type 1 AIP than in non-AIP CP (62.1% vs. 30.0%, P=0.042). The diffuse or segmental enlargement of the pancreas was more frequent in type 1 AIP than in non-AIP CP (72.4% vs. 40.0%, P=0.038). Histopathology of type 1 AIP presented dense lymphoplasmacytic infiltration, “snowstorm-like” fibrosis and abundant immunoglobulin (Ig) G4+ cells. Foxp3+ cells were more frequently observed in type 1 AIP. IL-17+ cell infiltration was similar between the 2 groups. Furthermore, a positive correlation was found between Foxp3+ and IgG4+ cell counts in the pancreas of patients with type 1 AIP.

Conclusions: Type 1 AIP has distinctive symptoms, image, and pathological characteristics, which could be used for differentiation from non-AIP CP. Foxp3+ cells might be helpful to distinguish type 1 AIP from non-AIP CP.

MeSH Keywords: Forkhead Transcription Factors • Immunoglobulin G • Interleukin-17 • Pancreatitis • Pancreatitis, Chronic

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Background

Autoimmune pancreatitis (AIP) is a distinct type of pancreatitis with a presumed autoimmune etiology [1]. Its clinical features, treatment, and prognosis are significantly different from those of non-AIP chronic pancreatitis (CP). Presently, epidemiological information about AIP mainly comes from Japan, South Korea, the United States, and other countries. The morbidity of AIP is 2.2 per 100,000 population in Japan [2]. In North America, among CP cases, the prevalence of AIP is 5–6% [3]. According to the morbidity in these countries, it is estimated that there are thousands of patients with AIP in China, but only few cases are reported because most AIP patients in China have been misdiagnosed and consequently not correctly treated [4,5]. In a report of 36 AIP patients in China, 18 (50%) cases were misdiagnosed as pancreatic cancer and 10 (28%) as non-AIP pancreatitis. Thus, further studies are needed to analyze the characteristics of AIP in China and distinguish it from non-AIP CP.

The primary pathogenesis of type 1 AIP is still not clear. The participation of CD4+CD25+ regulatory T cells (Tregs) in the pathogenesis of the IgG4 reaction in AIP has been proposed. It was suggested that Tregs might be involved in AIP through in situ production of interleukin-10 (IL-10) and transformation growth factor β (TGF-β), which could be followed by IgG4 class switching and fibroplasia [6]. Therefore, forkhead box P3 (Foxp3), as a good marker of CD4+CD25+ Tregs, was analyzed to investigate the significance of CD4+CD25+ Tregs in type 1 AIP.

Interleukin-17 (IL-17) is a proinflammatory cytokine produced mainly by Th17 cells [7]. It has been reported that IL-17 plays a key role in the fibrosis of chronic inflammation [8]. Increasing IL-17 expression was also reported as being involved in the pathogenesis of IgG4-related sclerosing cholangitis [9]. Type 1 AIP is an IgG4-related systemic autoimmune disease with dense fibrosis in the pancreas, but IL-17 expression remains unclear in type 1 AIP.

In this study, we analyzed the clinical features of type 1 AIP, detected the immunohistochemical expressions of Foxp3 and IL-17 in type 1 AIP, and compared them with non-AIP CP to improve the understanding of AIP and identify factors for differentiation of the 2 diseases.

Material and Methods

Case collection

Because diagnosis of AIP is primarily based on pathological features, clinically suspected type 1 AIP and non-AIP CP cases with pancreatic specimens were all reviewed at Sun Yat-Sen Memorial Hospital from January 2000 to December 2013. The diagnosis of type 1 AIP was according to ICDC [detailed description in ref. 10]. The diagnosis of non-AIP CP followed the diagnostic criteria in China and Italy: (1) clinical manifestations: recurrent abdominal pain or acute pancreatitis; (2) histopathologic examination: pancreatic gland bubble destruction, pancreatic fibrosis, duct dilation, and cyst formation; (3) imaging findings: pancreatic calcification or calculus pancreas growth or reduction, contour irregularity, irregular dilation of pancreatic duct, and pancreatic pseudocyst; (4) laboratory tests: pancreatic exocrine insufficiency. A definitive diagnosis of CP could be made with (2) or (3) and a diagnosis of suspected CP was made by (1) and (4). Only cases with a definitive diagnosis of CP were included [11,12]. Cases that were in accordance with the inclusion standard of the AIP group were excluded from the non-AIP CP group.

The following data of the 2 groups were collected and compared: (1) age and sex; (2) symptoms like abdominal pain, obstructive jaundice, abnormal stool, weight loss, diabetes mellitus, and combination with other autoimmune diseases; (3) serological data: γ-glutamyl transferase (γ-GT), alkaline phosphatase (ALP), total bilirubin (TBIL), alanine aminotransferase (ALT), serum amylase (SAMy), lipase (LPS), carbohydrate antigen 19-9 (CA19-9), serum globulin, and autoantibodies; (4) examination results of computed tomography (CT), magnetic resonance imaging (MRI), and magnetic resonance cholangiopancreatography (MRCP); and (5) histopathological features in the pancreas. Informed consent was obtained from the patients or the patients’ families. This study was approved by the Ethics Committee of Sun Yat-Sen Memorial Hospital.

Immunohistochemical staining

One paraffin block from each case was selected for immunohistochemical (IHC) staining for IgG4, Foxp3, and IL-17. The IHC staining was performed as follows: serial sections of each sample were cut at 5 μm, baked in an oven at 60°C for at least 60 min, deparaffinized, rehydrated, and pretreated with citric acid at pH 6.0. Endogenous peroxidase activity was quenched with 3% H2O2 for 10 min. All sections were incubated with normal non-immune goat serum for 15 min at room temperature. Sections were incubated overnight with the primary antibodies directly against IgG4 (rabbit polyclonal, diluted 1:500, Abcam, Cambridge, UK), Foxp3 (rabbit polyclonal, diluted 1:500, Abcam, Cambridge, UK), and IL-17 (rabbit polyclonal, diluted 1:500, Santa Cruz, USA). Incubations with biotin-labeled goat secondary antibody (Abcam, Cambridge, UK) and streptavidin-horseradish peroxidase, both at room temperature for 15 min, were performed successively. Positive controls that were known to express the antigens were included. Diaminobenzidine (DAB substrate kit, Abcam, Cambridge, UK) was used to visualize the immunoreaction. Sections were counterstained with hematoxylin. Intervening washing by phosphate buffered saline (PBS, 0.1 mol/L, pH 7.4) was necessary between each step.
The IHC stains were examined to determine whether any IgG4+, Foxp3+, and IL-17+ cells were present and, if so, in what numbers. The number of IgG4+ cells was counted in 3 high-power (40×) fields (HPFS) in the highest-density area of IgG4 staining, and these 3 numbers were averaged to determine the greatest average density of IgG4+ cells/hpf for each case. The cells staining positive for Foxp3 and IL-17 were counted in 5 HPFS in the area of maximum density and averaged to 1 HPF.

### 2 Statistical analysis

Variables are reported in terms of means (standard deviations) and simple proportions. Univariate analyses were performed using 2-tailed t test and χ² test (or Fisher’s exact test when appropriate). Multivariate logistic regression analysis adjusted for age and sex was performed to evaluate an independent association among clinical features of type 1 AIP vs. non-AIP CP. The cutoff for inclusion in the multivariate analysis was P<0.1 in the univariate analyses. Correlation of IgG4 with Foxp3 and IL-17 was determined by Spearman correlation coefficient. The data were analyzed using SPSS software version 13.0 (SPSS Inc, Chicago, IL). A 2-tailed P value of less than 0.05 was considered statistically significant.

### Results

#### Clinical symptoms

Twenty-nine cases of type 1 AIP and 20 cases of non-AIP CP (6 alcoholic, 3 hyperlipidemia, 5 obstructive, and 6 idiopathic) were enrolled at our institution from January 2000 to December
The clinical symptoms of type 1 AIP and non-AIP CP are shown in Table 1. In the type 1 AIP group, 18 cases were male and 11 female, with the ratio of 1.64:1. The average age of type 1 AIP was 54.8 years, older than that of non-AIP CP (45.9 years). Abdominal pain was the most frequent symptom in type 1 AIP (69.0%), followed by obstructive jaundice (62.1%) and weight loss (34.5%). Obstructive jaundice was more common in type 1 AIP than in non-AIP CP (62.1% vs. 30.0%, \( P = 0.042 \)). Three cases complicated with other autoimmune diseases were found in the type 1 AIP group, 2 with Sjögren syndrome and 1 with systemic lupus erythematosus (SLE). By contrast, none of autoimmune disease was observed in the non-AIP CP group.

The 2 groups did not show statistically significant differences in the frequencies of abdominal pain, abnormal stool, weight loss, and diabetes mellitus. Obstructive jaundice was associated with type 1 AIP after multivariate analysis (\( P = 0.036 \); Table 1).

Serological data

The serological data of the type 1 AIP group compared with the non-AIP CP group are shown in Table 2. The frequencies of elevated \( \gamma \)-GT, TBIL, LPS, and CA19-9 levels in the type 1 AIP group were higher than those in the non-AIP CP group (\( P = 0.046, 0.042, 0.007, \) and 0.019, respectively). There were
no statistically significant differences in the frequencies of elevated ALP, ALT, and SAMY between type 1 AIP and non-AIP CP groups. After multivariate analysis, all variables lost their significance.

**Immunological tests**

Nineteen patients in the type 1 AIP group were tested for IgG, antinuclear antibody (ANA), anti-double-stranded DNA (Anti-dsDNA) antibody, rheumatoid factor (RF), and anti-Sjögren’s syndrome antigen-A/B (Anti-SSA/SSB) antibodies. IgG was increased >17 g/L in 15 (78.9%) cases (range, 17.2–25.6 g/L; average, 20.4 g/L). ANA was positive in 5 (26.3%) patients, anti-dsDNA antibody in 3 (15.8%) patients, and RF in 1 (5.2%) patient. Anti-SSA/SSB antibodies were negative in all 19 AIP patients. The tests of IgG, ANA, anti-dsDNA antibody, RF, and anti-SSA/SSB antibodies were performed on only 3 patients in the non-AIP CP group and all were in the normal range.

**Imaging examination**

CT, MRI, and MRCP were performed on all the type 1 AIP and non-AIP CP patients (Figures 1 and 2). The imaging examination results are shown in Table 3. Diffuse or segmental enlargement of the pancreas with delayed enhancement was more frequent in type 1 AIP than in non-AIP CP (72.4% vs. 40.0%, P=0.038). By contrast, pancreatic atrophy, pancreatic pseudocyst, and pancreatic duct dilation were more common in non-AIP CP than in type 1 AIP (P=0.050, <0.001, and <0.001, respectively). Multivariate analysis showed that pancreatic atrophy and pancreatic duct dilation remained independent and significantly different associations between type 1 AIP and non-AIP CP (P=0.047 and P=0.005, respectively), but other variables lost their significance.

**Histopathological examination**

Because pathology testing is essential to diagnose AIP, all the enrolled cases had pancreatic specimens taken: 19 resections and 10 biopsies in the type 1 AIP group and 8 resections and 12 biopsies in the non-AIP CP group. Pancreatic parenchymal atrophy and fibrosis were remarkable in the type 1 AIP group and non-AIP CP group (Figure 3). In the type 1 AIP group, extensive infiltration of lymphocytes was observed, distributed primarily along the medium- and large-sized pancreatic duct, and partially invading small veins within the lesion area (Figure 3C). Eosinophil infiltration was also observed in some cases of type 1 AIP (Figure 3C). By contrast, lymphocyte infiltration was rare in the non-AIP CP group.

**Immunohistochemical findings of IgG4, Foxp3, and IL-17**

IHC findings of IgG4, Foxp3, and IL-17 in type 1 AIP and non-AIP CP groups are shown in Figure 4 and in Tables 4 and 5. Compared with non-AIP CP, IgG4+ and Foxp3+ cells were more frequent in type 1 AIP (both P<0.001). In addition, the average of IL-17+ cells was 8.8±8.2 cells/HPF in type 1 AIP, which was similar to that in non-AIP CP (7.9±7.9 cells/HPF; P=0.696).

Furthermore, correlation of IgG4 with Foxp3 and IL-17 in the type 1 AIP group was analyzed and the results are shown in Figure 5. Foxp3+ cell counts correlated closely with IgG4+ cell counts in the pancreas of type 1 AIP (R=0.930, P<0.001). However, IL-17+ cells had no correlation with IgG4+ cells (P=0.650).

**Discussion**

Since Yoshida first proposed the concept of AIP in 1995, the awareness and understanding of AIP has been increasing.

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**Table 3. Imaging features in type 1 AIP and non-AIP CP.**

|                      | Type 1 AIP [n=29; n (%)] | Non-AIP CP [n=20; n (%)] | P univariate | P multivariate* |
|----------------------|--------------------------|--------------------------|--------------|-----------------|
| Enlargement of the pancreas | 21 (72.4) | 8 (40.0) | 0.038 | 0.430 |
| Diffuse | 10 (34.5) | 4 (20.0) | 0.344 | – |
| Segmental (head) | 9 (31.0) | 3 (15.0) | 0.313 | – |
| Segmental (body or tail) | 2 (6.9) | 1 (5.0) | 0.785 | – |
| Pancreatic atrophy | 2 (6.9) | 6 (30.0) | 0.050 | 0.047 |
| Pancreatic pseudocyst | 0 | 10 (50.0) | <0.001 | 0.998 |
| Pancreatic calcification | 0 | 3 (15.0) | 0.062 | 0.999 |
| Pancreatic duct dilation | 3 (10.3) | 14 (70.0) | <0.001 | 0.005 |
| Lower bile duct stenosis | 7 (24.1) | 1 (5.0) | 0.119 | – |

* Multivariate logistic regression analysis adjusted for age and gender.
According to recent reports, AIP is classified into 2 distinct types: type 1 – LPSP and type 2 – IDCP. Type 1 AIP accounts for most cases of AIP in Asia. In contrast, type 2 AIP is mainly found in Europe and America, and rarely in Asia. In this study, we focused on the clinical features and expressions of Foxp3 and IL-17 in type 1 AIP in China and compared them with non-AIP CP to identify factors for differentiation of the 2 diseases.

Currently, type 1 AIP is recognized as an IgG4-related systemic autoimmune disease involving the pancreas and extra-pancreatic organs (liver, biliary systems, salivary glands, retroperitoneum, kidneys, lymph nodes, or thyroid gland) [14,15]. Sclerosing cholangitis was the most frequent extra pancreatic lesion, presenting obstructive jaundice and lower or upper bile duct stenosis [15]. Moreover, hepatobiliary system involvement usually presented elevated TBIL, γ-GT, ALP, and CA19-9. In biochemical blood testing, 39–82% of cases were reported to be associated with an increase of TBIL, γ-GT, and ALP; and 35–59% of cases were associated with an increase of CA19-9 [1,14]. Elevated AMY and LPS were also reported in 36–64% of cases, indicating pancreatic injury in type 1 AIP. In our study, most AIP patients had obstructive jaundice and elevated γ-GT, ALP, TBIL, CA19-9, AMY, and LPS levels. Compared with non-AIP CP, elevated γ-GT, TBIL, CA19-9, and LPS levels were more frequent in type 1 AIP. These results demonstrated more cases with pancreatic injury and cholestasis in type 1 AIP. However, after multivariate analysis, all variables lost their significance, possibly because the sample was too small to have sufficient statistical power. Therefore, further studies with larger samples are needed to confirm these results.

Immunological testing in AIP often shows increased IgG/IgG4 and presence of autoantibodies [1]. IgG4 >1350 mg/L indicates the diagnosis of type 1 AIP, with sensitivity and specificity of 52–80% and 97%–98%, respectively [1]. The presence of autoantibodies also suggests the diagnosis of AIP. The reported positive ratios of ANA and RF are 60% and 20–30%, respectively [1]. In this study, 78.9% of type 1 AIP cases had increased serum IgG; 26.3% and 5.2% cases had ANA and RF. Because the clinical measurement of serum IgG4 levels was not available in our hospital during the study, it was
Table 4. Immunohistochemical findings of IgG4 in type 1 AIP and non-AIP CP.

|          | Mean of IgG4+ cells/hpf | Range of IgG4+ cells/hpf | No. of cases with >50 positive cells/hpf | No. of cases with 10–50 positive cells/hpf |
|----------|-------------------------|--------------------------|----------------------------------------|-------------------------------------|
| Type 1 AIP (n=29) | 46.5                    | 11–84                    | 11 (37.9%)                             | 18 (62.1%)                           |
| Non-AIP CP (n=20)  | 2.4                     | 0–9                      | 0                                      | 0                                    |

Table 5. Immunohistochemical findings of Foxp3 in type 1 AIP and non-AIP CP.

|          | Mean of Foxp3+ cells/hpf | Range of Foxp3+ cells/hpf | No. of cases with >20 positive cells/hpf | No. of cases with 5–20 positive cells/hpf |
|----------|--------------------------|---------------------------|----------------------------------------|-------------------------------------|
| Type 1 AIP (n=29) | 26.1                    | 8–42                      | 19 (65.5%)                             | 10 (34.5%)                           |
| Non-AIP CP (n=20)  | 5.0                     | 0–17                      | 0                                      | 11 (55.0%)                           |
impossible to provide detailed information on serum IgG4 levels. With increasing awareness of AIP, we are now strengthening the measurement of immunological indices for AIP. We have recently developed clinical serum IgG4 level detection.

In imaging examination, diffuse or segmental enlargement with delayed enhancement (sometimes associated with rim-like enhancement) of the pancreas without pancreatic duct dilatation/cutoff was the characteristic change in AIP [16,17]. Additionally, AIP often involves the bile duct and causes bile duct stenosis, especially in the pancreatic section [18]. Pancreatic calcification and pseudocyst occur less frequently in type 1 AIP cases [16]. Our study found 34.5% of AIP cases with diffuse pancreatic enlargement and 31.0% of cases with focal enlargement in the pancreatic head. Moreover, the diffuse or segmental enlargement of the pancreas was more frequent in type 1 AIP than in non-AIP CP (72.4% vs. 40.0%, P=0.038). In contrast, pancreatic atrophy, pancreatic pseudocyst, and pancreatic duct dilation were more common in non-AIP CP than in type 1 AIP. However, we did not detect the capsule-like rim, which is the unique imaging feature of AIP, reported in 25% cases [16]. The reason is not clear, probably because of small samples. Nevertheless, further studies with larger population are needed to confirm the results.

Pathological examination revealed that type 1 AIP was characterized by dense lymphocyte infiltration, “snowstorm-like” fibrosis, occlusive phlebitis, and numerous IgG4+ cell infiltrations [19–25]. Dhall et al. reported that using a cutoff of 50 IgG4-positive cells/HPF, the sensitivity and specificity for type 1 AIP vs. other types of pancreatitis was 84% and 100% [26]. Another recent study found high levels of IgG4 staining (>10 IgG4+ cells/HPF) in 17 of 20 (85%) AIP pancreatic and extra-pancreatic specimens compared with 1 of 175 (0.6%) control specimens, and positive IgG4 staining enabled a definitive diagnosis in 91% of AIP patients [27]. In our study, 37.9% cases had >50 IgG4+ cells/HPF and all the type 1 AIP patients had >10 IgG4+ cells/HPF. By contrast, in non-AIP CP group, 45% cases had no IgG+ cell infiltration, and others had <10 IgG+ cells/HPF, showing that numerous IgG4+ cell infiltration was helpful to differentiate the diagnosis of type 1 AIP and non-AIP CP.

Foxp3 is a specific marker of CD4+CD25+ Tregs, which play a critical role in immune tolerance. The activation of CD4+CD25+ Tregs has been proposed in the pathogenesis of AIP [28]. A higher expression level of Foxp3 mRNA in tissue was observed in IgG4-related sclerosing pancreatitis and cholangitis, as well as larger infiltrates of CD4+CD25+ Treg cells at involved organs and increased numbers of CD4+CD25(high) Treg cells in the blood [6,29]. Furthermore, Tregs can produce IL-10 and TGF-β, which could be followed by IgG4 class switching and fibrosis. Our results showed that Foxp3+ cells were significantly more frequent in type 1 AIP compared with non-AIP CP. A positive correlation of Foxp3+ with IgG4+ cells was also observed. These results suggest that Foxp3+ cells might be involved in the pathogenesis of type 1 AIP and can be helpful in distinguishing type 1 AIP from non-AIP CP.

IL-17 is a proinflammatory cytokine mainly produced by Th17 cells, which plays an important role in the fibrosis associated with chronic inflammation, multiple sclerosis, and rheumatoid arthritis [7]. IL-17 can induce the production of many cytokine (such as IL-16, TGF-β, and granulocyte colony-stimulating factor), attract neutrophils, and cause massive inflammation and fibrosis [30–32]. Increasing IL-17 expression was reported as being involved in the pathogenesis of IgG4-related sclerosing sialadenitis [9]. However, our study indicated that IL-17+ cells in type 1 AIP were similar to those in non-AIP CP, perhaps because both type 1 AIP and non-AIP CP have marked fibrosis and IL-17 is associated with the fibrosis of chronic inflammation. Moreover, IL-17+ cells had no correlation with IgG4+ cells. These results indicate that IL-17+ cells might not be useful for differentiation between type 1 AIP and non-AIP CP.
There are a few limitations of our study. Primarily, this was a single-center study with a small sample size. Furthermore, only cases with pathological specimens are included, which ensured the correct diagnosis, but could have excluded type 1 AIP in cases without pathological specimens and cause selection bias. Therefore, further prospective studies with larger sample size are needed to confirm the results of this study.

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Conclusions

Type 1 AIP is a unique type of chronic pancreatitis and has distinctive symptoms, serological, imaging, and pathological characteristics, which could be used for differentiation from non-AIP CP. Foxp3+ cells might be helpful to distinguish type 1 AIP from non-AIP CP.