**Abstract**

**AIM:** To describe protease serine 1 (PRSS1) gene mutations in patients with autoimmune pancreatitis (AIP) and the clinical features of AIP.

**METHODS:** Fourteen patients with AIP, 56 with other chronic pancreatitis, 254 with pancreatic cancer and 120 normal controls were studied. The mutations and polymorphisms of four genes involved with pancreatitis or pancreatic cancer, PRSS1, SPINK1, CFTR and MEN1, were sequenced. The pathogenic mechanism of AIP was investigated by comparing the wild-type expression system with the p.81Leu→Met mutant expression system.

**RESULTS:** Two novel mutations (p.81Leu→Met and p.91Ala→Ala) were found in PRSS1 gene from four patients with AIP. PRSS1_p.81Leu→Met mutation led to a trypsin display reduction (76.2%) combined with phenyl agarose (Ca²⁺ induced failure). Moreover, the ratio of trypsin/amylase in patients with AIP was higher than in the patients with pancreatic cancer and other pancreatitis. A large number of lymphocytes and plasma cells were found in the bile ducts accompanied by hyperplasia of myofibroblasts.

**CONCLUSION:** Autoimmune pancreatitis may be related to PRSS1 gene mutations.

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**Key words:** Autoimmune pancreatitis; Molecular mechanism; p.81Leu→Met; PRSS1

**Core tip:** Novel mutations (p.81Leu→Met and p.91Ala→Ala) were found in protease serine 1 gene from the patients with autoimmune pancreatitis. Trypsinogen abnormal activation resulted in multiple organ injuries. And this offers direct evidence in support of the trypsinogen gene mutation and abnormal immune system.
INTRODUCTION

Most of the earlier literature about autoimmune pancreatitis (AIP) came from Japan[1-3]. AIP has been referred to a variety of names including sclerosing pancreatitis, tumefactive pancreatitis, and nonalcoholic destructive pancreatitis, depending in part on the specific pathologic findings and the presence of extrapancreatic manifestations. However, it is generally believed that the pathologic heterogeneity may reflect different stages or manifestations of the same disease. Immunoglobulin G4 (IgG4) positive plasma cells infiltration is considered a marker for the disease and can be detected in the pancreas and a variety of other tissues[4-7]. Unfortunately, serum IgG4 increase was not found in all patients with AIP and more than half of the patients with AIP had normal serum IgG4[8-10]. It is urgent to find some more specific diagnosis technology (including the molecular markers). Genetic mutation is often involved in immune system disorders, and protease serine 1 (PRSS1), cystic fibrosis conductance regulator (CFTR), serine protease inhibitor Kazal type 1 (SPINK1) and multiple endocrine neoplasia 1 (MEN1) mutation is followed by pancreatitis or pancreatic cancer[11-13]. We are keen on identifying these genes targeted by the inflammatory process in AIP. Although trypsin was historically believed to be immunologically active, it is now continued to be verified that abnormal activation of trypsin can be recognized by the immune system. This study aimed to determine whether PRSS1 gene p.T81M mutation contributes to the functions of calcium-induced trypsinogen activation and to explore its role in autoimmune pancreatitis.

MATERIALS AND METHODS

Patients

This study was approved by the Fujian Medical University Ethics Committee and all study participants gave informed consent for DNA analyses. Clinical information for the survey was obtained by personal interviews using a structured questionnaire and/or clinical trials. AIP diagnostic criteria were as follows: (I) pancreatic imaging studies show diffuse narrowing of the main pancreatic duct with irregular wall (more than 1/3 of length of the entire pancreas); (II) laboratory data demonstrate abnormally elevated levels of serum gamma globulin and/or IgG, or the presence of autoantibodies; and (III) histopathologic examination of the pancreas shows fibrotic changes with lymphocyte and plasma cell infiltration. For diagnosis, criterion I (pancreatic imaging) must be presented with criterion II (laboratory data) and/or III (histopathologic findings)[14-16]. Total one pedigree (Figure 1) and 12 unrelated patients with AIP, 56 with chronic pancreatitis, 254 with pancreatic cancer and 120 normal controls seen in the past six years were studied.

DNA extraction and molecular genetic analysis

Genomic DNA was extracted from peripheral blood and other tissue specimens using a QIAamp DNA mini kit (Qiagen, Hilden, Germany). Primer pairs and experimental condition were used to generate specific fragments according to the references[12,15]. The polymerase chain reaction (PCR) products were purified for sequencing after electrophoresis on an agarose gel. For sequencing, a Perkin Elmer Big Dye Sequencing kit (Perkin-Elmer, Shelton, CT, United States) and an ABI PRISM7700 sequencer (Perkin-Elmer ABI, Foster, CA, United States) were used.

Pancreatitis/pancreatic cancer-associated gene detection

Four genes involved in pancreatitis/pancreatic cancer, PRSS1, SPINK1, CFTR and MEN1, were sequenced according to references[12,15]. A 20 µL mixture was prepared for each reaction and contained 1 × HotStarTaq buffer, 2.0 mmol/L MgCl2, 0.2 mmol/L dNTP, 0.2 µmol/L of each primer, 1 U HotStarTaq polymerase (Qiagen Inc., Valencia, CA, United States) and 1 µL template DNA. The cycling program was 95 ℃ for 15 min; 11 cycles of 94 ℃ for 15 s, 62 ± 0.5 ℃ per cycle for 40 s, 72 ℃ for 1 min; 24 cycles of 94 ℃ for 15 s, 57 ℃ for 30 s, 72 ℃ for 1 min, and 72 ℃ for 2 min. PCR purification was completed using SAP and ExoSap I 1 U SAP, and 6 U ExoSap I was added into 8 µL PCR products. The mixture was incubat-
ed at 37 °C for 60 min, followed by incubation at 70 °C for 10 min.

**Pancreatic tissue pathology**
Pancreatic tissues were stained with haematoxylin and eosin, modified gomori trichrome, periodic acid-Schiff stain and IgG4 special staining.

**Detection of serum trypsin and amylase**
The serum trypsin was tested with ELISA kits (R and D Systems, Minneapolis, MN, United States) and amylase with latex-enhanced nephelometric immunoassay (Dade Behring Marburg GmbH, Germany).

### Functional experiments on mutants
The complete mutated (p.T81M) and wild-type PRSS1 cDNA were introduced into plasmid pMD18-T (TaKaRa, China) and transformed into Escherichia coli DH5 competent cells. Primers were designed for PCR amplification. The forward primer was 5'-TGCAATTGTATGGCACATTCGACGATGATGACAAGAT-3' and the reverse primer was 5'-GAGTCGACTCAGCTAATTAAGCTε-3'. The expression products underwent isolation, purification and renaturation. Benzoyl L-arginine ethyl ester served as a substrate, and absorbance (A253) was measured at 253 nm within 30 min. The specific enzyme activity was calculated as follows: specific activity = enzyme activity/mg of protein = ΔA253/t × 1000/(ε × t × 0.001), where t refers to time (min) and ε refers to the amount of proteinase (μg) during the detection.

### $^{45}$Ca$^{2+}$ binding assay
Binding of $^{45}$Ca$^{2+}$ to wild-type recoverin and the L81M mutant was investigated as described previously[20]. In brief, 100 mol/L protein was dissolved in 20 mmol/L HEPES-KOH, pH 7.5, 100 mmol/L NaCl and 1 mmol/L DTT, and then it was transferred to centricron 10 devices. $^{45}$Ca$^{2+}$ was added, the samples were centrifuged for 1 min, and radioactivity of the filtrate was counted. Next, non-radioactive Ca$^{2+}$ was added and the centrifugation procedure was repeated. Protein-bound Ca$^{2+}$ versus free Ca$^{2+}$ was determined from the excess Ca$^{2+}$ in the protein sample in the ultrafiltration.

### Treatment and follow-up
Informed consent was obtained before treatment. Glucocorticoids were administered empirically [oral prednisone (40 mg) once daily with a 5-mg taper every 2 wk]. At the same time, oral acid-suppressing agents and calcium were also given.

### RESULTS

#### Clinical data of patients with AIP
The auxiliary test results of the patients with AIP (male/female = 7:7) are shown in Table 1. There was significant weight loss (2-9 kg/12 mo) and abnormally increased serum IgG4 level: 5/14 (+).

**Table 1** Clinical data of the patients with autoimmune pancreatitis

| Number | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 |
|--------|---|---|---|---|---|---|---|---|---|----|----|----|----|----|
| Sex    | M | F | M | M | F | M | F | M | F | M | F | M | F | M |
| Age/onset | 61/59 | 63/52 | 70/58 | 59/52 | 68/62 | 70/62 | 53/46 | 46/46 | 60/48 | 59/52 | 62/60 | 62/55 | 48/42 | 33/32 |
| Weight loss (kg/12 mo) | 5 | 3 | 3 | 4 | 6 | 2 | 8 | 3 | 5 | 8 | 6 | 3 | 6 | 9 |
| Nausea/vomit | +/- | -/+ | +/- | +/- | +/- | +/- | +/- | +/- | +/- | +/- | +/- | +/- | +/- | +/- |
| IgG (0-36) | 12.5 | 6.9 | 11 | 9.6 | 8.5 | 6.3 | 23.5 | 26.9 | 21 | 15.2 | 11.3 | 26.3 | 18.5 | 6.9 |
| IgG4 (0.08-1.4 g/L) | 2.53 | 0.82 | 0.89 | 1.12 | 2.69 | 0.77 | 1.75 | 0.25 | 0.96 | 4.25 | 2.01 | 0.75 | 0.63 | 0.56 |
| Glucose (mmol/L) | 14.32 | 4.56 | 5.14 | 18.69 | 4.33 | 6.55 | 4.25 | 6.35 | 7.15 | 8.66 | 4.12 | 5.6 | 4.23 | 5.02 |
| Trypsin (ng/mL) | 28.65 | 63.55 | 52.45 | 33.65 | 56.55 | 32.12 | 23.15 | 56.99 | 87.02 | 74.52 | 63.05 | 56.23 | 78.06 | 12.66 |

F: Female; M: Male; IgG: Immunoglobulin G.
and the jaundice improved. Throughout the course of the disease, the trypsin (ng/mL)/amylase (U/L) ratio was higher in patients with AIP (0.658 ± 0.309) than in patients with pancreatic cancer (0.163 ± 0.087) or other types of chronic pancreatitis (0.133 ± 0.095) (Figure 4).

Radiologic features
Computed tomography found a diffusely enlarged hypodense pancreas or a focal mass and retroperitoneal lymph node enlargement that may be mistaken for a pancreatic cancer. Magnetic resonance imaging revealed diffusely decreased signal intensity and delayed enhancement on dynamic scanning. The characteristic endoscopic retrograde cholangiopancreatographic finding was segmental or diffuse irregular narrowing of the main pan-

Figure 2  Sequencing of gene mutations from the patients with autoimmune pancreatitis. A: The sequencing c.247 C > A of PRSS1 gene mutation (p.81Leu → Met); B: Sequencing c.279 C > A of PRSS1 gene silent mutation (p.91Ala → Ala). The red arrow indicates the base mutation.

Figure 3  Histopathologic examination of the pancreas. A: A large number of lymphocytes and plasma cells were found in the bile ducts accompanied by hyperplasia of myofibroblasts (hematoxylin-eosin, × 20); B: The number of pancreatic acinar cells was markedly decreased (immunohistochemistry staining of cytokeratin, × 20).

Figure 4  Ratio of trypsin/amylase among the three groups.
creatic duct. The characteristic magnetic resonance cholangiopancreatographic finding was partial intrahepatic bile duct dilatation or narrowing and multiple focal high signal intensity in the liver. Multiple cysts occurred in the liver, pancreas, spleen and other organs (Figure 5).

**Activity of the products of the mutated gene**
A UV spectrophotometer was used to measure the activity of trypsinogen before and after enterokinase activation at 253 nm (ΔOD 253). The activity of products of the mutated gene remained unchanged after enterokinase activation. Using the aforementioned formula, the calculated specific activity of renatured recombinant trypsin was 126-183 BAEE U/mg, and the calculated specific activity of wild-type trypsin was 123-165 BAEE U/mg after enterokinase activation, showing that p.T81M mutation did not affect the activity of trypsin.

**Interaction with phenyl-agarose**
According to the literature, the binding of recoverin to phenyl-agarose is thought to depend on the Ca²⁺-induced exposure of hydrophobic residues and not on the presence of the myristoyl group. Could the non-myristoylated L81M variant bind to phenyl-agarose in a fashion similar to the wild-type? In fact, the binding capacity of the mutant protein was lower (76.2% of that of the wild-type protein).

**Patient follow-up**
Six patients (No. 2, 3, 5, 6, 7 and 10) were treated with glucocorticoids for 3-6 mo, and the jaundice improved. The serum levels of total and direct bilirubin were reduced significantly. Wheezing was markedly improved and the body weight increased (2-5 kg/mo). The symptoms of the other 8 patients were improved to different degrees.

**DISCUSSION**
AIP shares many presenting symptoms with pancreatic carcinoma. Fortunately, AIP can be effectively treated and cured. Serum IgG4 levels are usually abnormally increased, but increased serum IgG4 levels are also found in patients with pancreatic cancer and some patients without AIP. Indeed, genetic analyses identified a specific gene, PRSS1, for hereditary pancreatitis and other types of chronic pancreatitis in 1996. Some PRSS1 mutations enhance trypsinogen autoactivation, explaining the young age of patients at onset of AIP. Other mutations may render some patients more susceptible to pancreatitis in the presence of other insults to the pancreas. In Japan, a strong association with the HLA-DRB1*0405/DQB1*0401 haplotype has been identified. However, the relationship has not been reported in other ethnic groups, which prompted us to search for AIP-related genes.

Although p.T81A mutant protein is not associated with functional activity, it binds to the sites that are quite dissimilar from 56Q-57W-58V-59V (i.e., classical Ca²⁺-binding sites). On the basis of this observation, a refined model of the role of the myristoyl group as an intrinsic allosteric modulator is proposed. Ca²⁺ stabilizes the hydrophobic pore structure of the trypsin molecule and increases folding to permit formation of two ionic bonds with β trypsin. Asp102, His57, Ser195 catalytic triad and Asp189, Gly216, Gly226 displayed by the substrate binding pocket of the trypsin are more stable, thereby improving the efficiency of the enzyme catalysis.

There is no controversy that trypsinogen activation plays a very important role in early pancreatitis. However, what activates trypsinogen is not entirely clear. Recent researches have focused on the relationship between the original concentration of intracellular calcium and trypsin activation within acinar cells. Trypsinogen activation starts in the apical part of the acinar cells after supramaximal interleukin stimulation. It is highly dependent on the release of calcium ions within subcellular structures and on repetitive calcium transients. Normally, trypsin cannot be activated by calcium. Mutation at the trypsin calcium binding point blocks such a conventional activation pathway. The gene mutation significantly increases the catalytic activity of trypsin but has no effect on the expression level of the mutant. Increased trypsin synthesis and secretion results in ectopic activation, leading to the occurrence of pancreatitis.

**Figure 5** Polycystic lesions in the liver, gallbladder, pancreas, and spleen, and retroperitoneal lymphadenopathy and bullae. A: Abdominal magnetic resonance imaging figure showed diffuse swelling; B: Computed tomography findings image of lung.
The abnormal increase in serum IgG4 is considered to be an indication of AIP. Unfortunately, serum IgG4 level is normal in more than half of the patients with AIP. In this study, most of our patients with AIP had normal serum IgG4 levels. Our findings suggest that patients with AIP can present with a variety of clinical phenotypes, and that genetic heterogeneity and clinical heterogeneity are features of AIP. In addition, studies of mutations in the PRSS1 gene have helped elucidate the molecular mechanism underlying the pathogenesis of AIP. Furthermore, high trypsin/amyase ratio contributes to the diagnosis of AIP.

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June 7, 2013 | Volume 19 | Issue 21 |

Gao F et al. PRSS1 mutation in autoimmune pancreatitis

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