

## ***PRSS1*\_p.Leu81Met mutation results in autoimmune pancreatitis**

Feng Gao, Yue-Ming Li, Guo-Lin Hong, Zhi-Feng Xu, Qi-Cai Liu, Qing-Liang He, Li-Qing Lin, Shao-Huang Weng

Feng Gao, Department of Pathology, the First Affiliated Hospital of Fujian Medical University, Fuzhou 350005, Fujian Province, China

Yue-Ming Li, Department of Radiology, the First Affiliated Hospital of Fujian Medical University, Fuzhou 350005, Fujian Province, China

Guo-Lin Hong, Department of Laboratory Medicine, the Second Hospital of Fuzhou, Fuzhou 350007, Fujian Province, China

Zhi-Feng Xu, Department of Surgery, the 95 Hospital of PLA, Putian 351100, Fujian Province, China

Qi-Cai Liu, Department of Laboratory Medicine, the First Affiliated Hospital of Fujian Medical University, Fuzhou 350005, Fujian Province, China

Qi-Cai Liu, Department of Laboratory Medicine, Medical Technology and Engineering College of Fujian Medical University, Fuzhou 350005, Fujian Province, China

Qing-Liang He, Department of Surgery, the First Affiliated Hospital of Fujian Medical University, Fuzhou 350005, Fujian Province, China

Li-Qing Lin, Shao-Huang Weng, Department of Pharmaceutical Analysis, Fujian Medical University, Fuzhou 350005, Fujian Province, China

**Author contributions:** Gao F and Li YM contributed equally to this work; Li YM, Hong GL and Liu QC defined the research theme; Gao F and Xu ZF designed methods and experiments, carried out the laboratory experiments, analyzed the data, interpreted the results and wrote the paper; He QL and Lin LQ co-designed the dispersal and colonization experiments, and co-worked on data collection and interpretation; Weng SH co-designed experiments and discussed analyses, interpretation, and presentation; all authors have read and approved the manuscript to be published.

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**Correspondence to:** Qi-Cai Liu, MD, Department of Laboratory Medicine, the First Affiliated Hospital of Fujian Medical University, 20 Chazhong Road, Fuzhou 350005, Fujian Province, China. [lqc673673673@163.com](mailto:lqc673673673@163.com)

Telephone: +86-591-87981972 Fax: +86-591-2263520

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### **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 (Ca<sup>2+</sup> 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.

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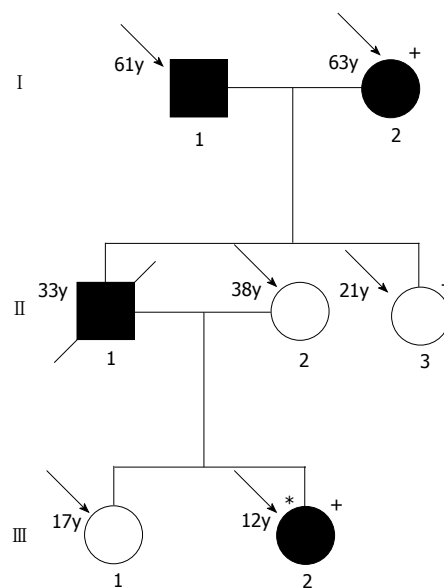
## INTRODUCTION

Most of the earlier literature about autoimmune pancreatitis (AIP) came from Japan<sup>[1-3]</sup>. 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<sup>[3-7]</sup>. 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<sup>[8-10]</sup>. 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<sup>[11-15]</sup>. 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



**Figure 1** p.L81M mutation of the protease serine 1 gene in autoimmune pancreatitis family. Hatched symbols: Patients with chronic pancreatitis; Striped symbols: Individuals with suspected chronic pancreatitis; Arrows: Subjects who were available for genetic analysis; Plus: Presence of heterozygous mutation; Asterisk: Index patient.

findings)<sup>[16-19]</sup>. 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<sup>[12,15]</sup>. 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<sup>[12-15]</sup>. A 20  $\mu$ L mixture was prepared for each reaction and contained 1  $\times$  HotStarTaq buffer, 2.0 mmol/L  $Mg^{2+}$ , 0.2 mmol/L dNTP, 0.2  $\mu$ mol/L of each primer, 1 U HotStarTaq polymerase (Qiagen Inc., Valencia, CA, United States) and 1  $\mu$ L template DNA. The cycling program was 95  $^{\circ}$ C for 15 min; 11 cycles of 94  $^{\circ}$ C for 15 s, 62  $\pm$  0.5  $^{\circ}$ C per cycle for 40 s, 72  $^{\circ}$ C for 1 min; 24 cycles of 94  $^{\circ}$ C for 15 s, 57  $^{\circ}$ C for 30 s, 72  $^{\circ}$ C for 1 min, and 72  $^{\circ}$ C for 2 min. PCR purification was completed using SAP and *Exo* I 1 U SAP, and 6 U *Exo* I was added into 8  $\mu$ L PCR products. The mixture was incubat-

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

Number	1 (I 1)	2 (II 2)	3	4	5	6	7	8	9	10	11	12	13	14
Type	1	1	2	2	1	1	2	2	2	1	1	2	2	1
Sex	M	F	M	M	F	F	M	M	F	M	F	F	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 (0-16)	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.

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'-TGCAATTGTATGGCAC-CATTCGACGATGATGACAAGAT-3' and the reverse primer was 5'-GAGTCGACTCAGCTAATTAAGCT-TAGTG-3'. In addition, *Mun*I and *Sal*I digestion sites were designed in the forward and reverse primers, respectively. The expression products underwent isolation, purification and renaturation. Benzoyl L-arginine ethyl ester served as a substrate, and absorbance ( $A_{253}$ ) was measured at 253 nm within 30 min. The specific enzyme activity was calculated as follows: specific activity = enzyme activity/mg of protein =  $\Delta A_{253}/t \times 1000/(\varepsilon \times t \times 0.001)$ , where  $t$  refers to time (min) and  $\varepsilon$  refers to the amount of proteinase ( $\mu$ g) during the detection.

### $^{45}\text{Ca}^{2+}$ binding assay

Binding of  $^{45}\text{Ca}^{2+}$  to wild-type recoverin and the L81M mutant was investigated as described previously<sup>[20]</sup>. 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 centricon 10 devices.  $^{45}\text{Ca}^{2+}$  was added, the samples were centrifuged for 1 min, and radioactivity of the filtrate was counted. Next, non-radioactive  $\text{Ca}^{2+}$  was added and the centrifugation procedure was repeated. Protein-bound  $\text{Ca}^{2+}$  versus free  $\text{Ca}^{2+}$  was determined from the excess  $\text{Ca}^{2+}$  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 (+).

### Molecular genetic analysis

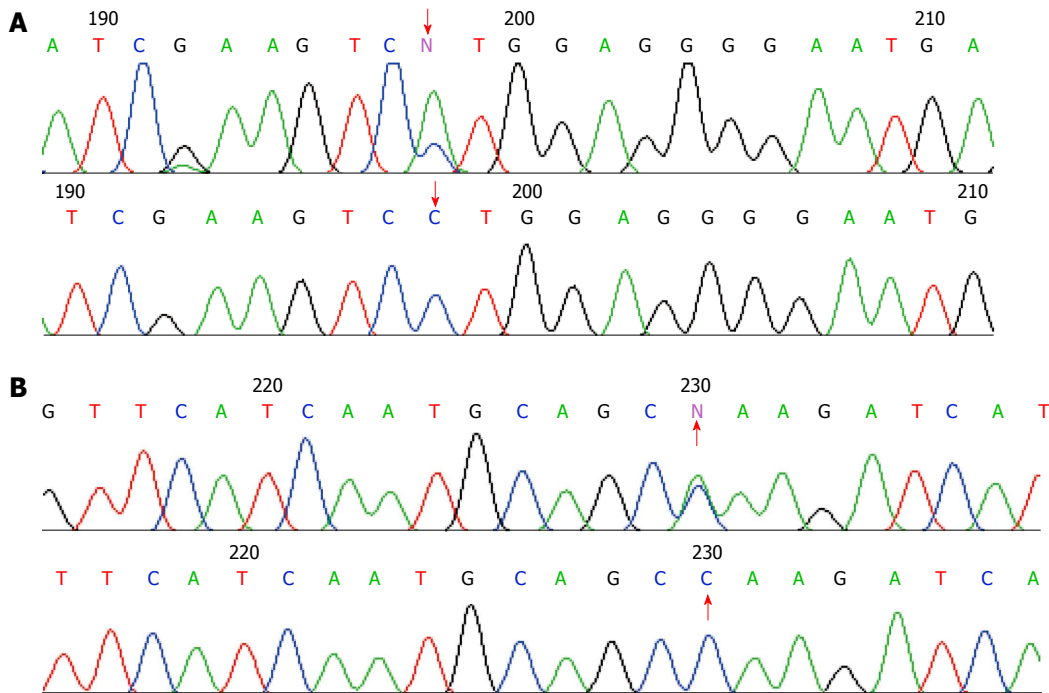
In the affected patients, novel mutations were found in the genes coding for *PRSS1* (Figure 2). They were *PRSS1*\_c.247 C > A (p.81Leu→Met) (No. 1, 2, 6 and 7) and *PRSS1*\_c.279 C > A (p.91Ala→Ala) (No. 7). None of these mutations were found in the normal controls and other patients with chronic pancreatitis and pancreatic cancer.

### Pathological analysis

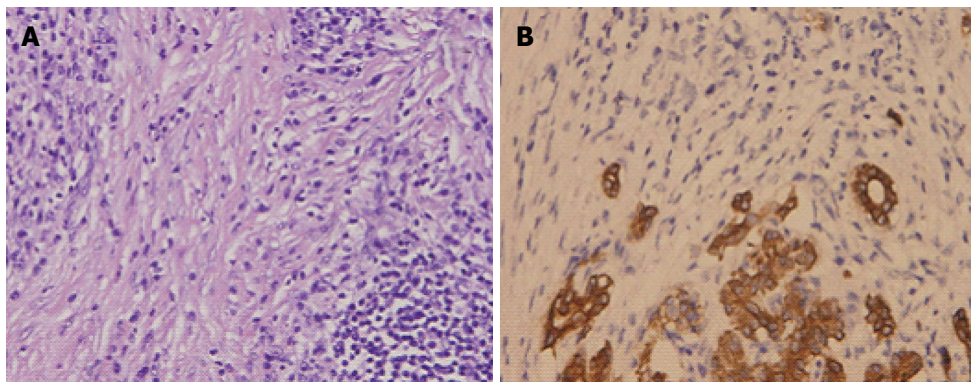
Histopathologic examination of the pancreas revealed a characteristic lymphoplasmacytic infiltrate of lymphocytes and IgG4 positive plasma cells, and interstitial fibrosis and acinar cell atrophy in later stages. However, localization and the degree of duct wall infiltration were variable. It has been proposed that a cytologic smear is rich in inflammatory cells. The sensitivity and the specificity of these criteria for differentiating AIP from neoplasia are unknown. A large number of lymphocytes and plasma cells were found in the bile ducts accompanied by hyperplasia of myofibroblasts (Figure 3A). The number of pancreatic acini was markedly reduced (Figure 3B) (immunohistochemistry by CK, CD3, CD20, CD38, CD68 and vimentin).

### Monitoring of serum enzyme

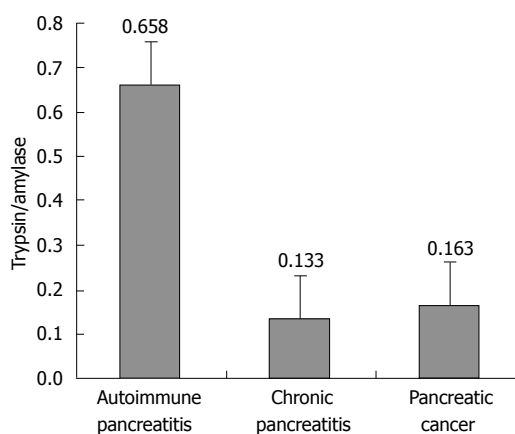
Patients were treated with glucocorticoids for 3-6 mo,



**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).

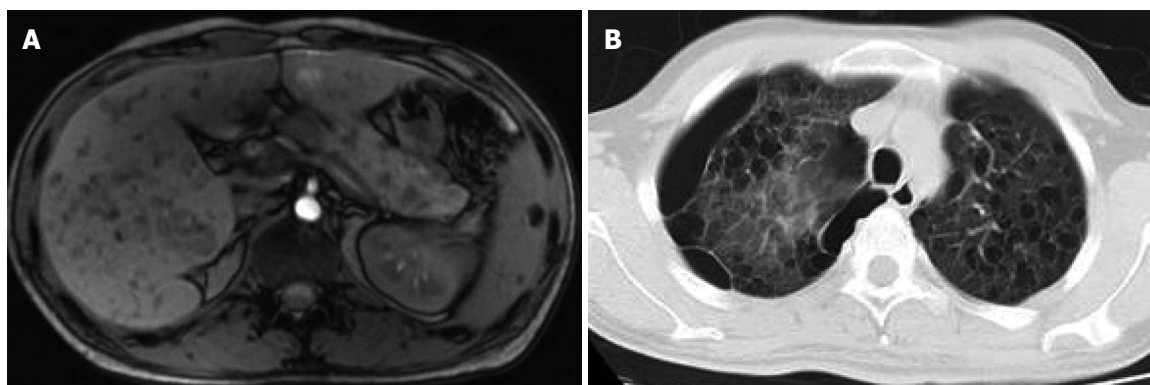


**Figure 4** Ratio of trypsin/amylase among the three groups.

and the jaundice improved. Throughout the course of the disease, the trypsin (ng/mL)/amylase (U/L) ratio was higher in patients with AIP ( $0.658 \pm 0.309$ ) than in patients with pancreatic cancer ( $0.163 \pm 0.087$ ) or other types of chronic pancreatitis ( $0.133 \pm 0.095$ ) (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 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.

creatic duct. The characteristic magnetic resonance cholangiopancreatographic finding was partial intrahepatic bile duct dilatation or narrowness 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 ( $\Delta OD_{253}$ ). 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<sup>[20]</sup>, the binding of recoverin to phenyl-agarose is thought to depend on the  $Ca^{2+}$ -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<sup>[6,9,21-24]</sup>, 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<sup>[10-12]</sup>. In Japan, a strong association with the HLA-DRB1\*0405/DQB1\*0401 haplotype has been identified<sup>[25]</sup>. However, the relationship has not been reported in other ethnic groups, which prompted us to search for AIP-related genes<sup>[25-30]</sup>.

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  $Ca^{2+}$ -binding sites)<sup>[20]</sup>. On the basis of this observation, a refined model of the role of the myristoyl group as an intrinsic allosteric modulator is proposed.  $Ca^{2+}$  stabilizes the hydrophobic pore structure of the trypsin molecule and increases folding to permit formation of two ionic bonds with  $\beta$  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<sup>[20,31-33]</sup>.

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 cholecystokinin stimulation. It is highly dependent on the release of calcium ions within subcellular structures<sup>[32-35]</sup> 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<sup>[20]</sup>. 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.

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.

## COMMENTS

### Background

Autoimmune pancreatitis (AIP) is an autoimmune chronic pancreatitis which is characterized by infiltration of lymphocytes and plasma cells, and pancreatic fibrosis and dysfunction. AIP and pancreatic cancer have similar clinical manifestations and findings on imaging, thus AIP is often misdiagnosed as pancreatic cancer, resulting in unnecessary surgical intervention. AIP not only poses a diagnostic challenge for clinicians, but can lead to severe and irreversible injury to patients.

### Research frontiers

The etiology of AIP is poorly understood. It has been reported that genetic factors play an important role in the pathogenesis of AIP. Parkdo *et al* reported that substitution of aspartic acid at the 57<sup>th</sup> position of haploid DQβ1 of the histocompatibility leukocyte antigen is closely related to the recurrence of AIP. The trypsinogen genes are inserted in the TCR vβ site; thus, the trypsinogen gene and TCR vβ gene share the same loci, and this arrangement is conserved in humans, mice, and chickens.

### Innovations and breakthroughs

*PRSS1*\_p.81Leu→Met mutation leads to a trypsin display reduction (76.2%) combined with phenyl agarose (Ca<sup>2+</sup> induced failure) which induced premature activation of trypsinogen and caused AIP-related polycystic lesions in the lungs, liver, gallbladder, pancreas, and spleen. Although trypsin was historically believed to be immunologically active, it is now continue to be verified that abnormal activation of trypsin can be recognized by the immune system.

### Applications

The authors' findings suggest that AIP can present with polycystic lesions in multiple organs, which can be found in patients with hereditary AIP, as well as those with sporadic AIP. In addition, functional studies have elucidated the molecular mechanisms underlying the pathogenesis of AIP due to the presence of mutations of the *PRSS1* gene. Additional clinical studies are required to investigate the clinical heterogeneity and molecular pathogenesis of AIP.

### Peer review

This paper reports the novel mutations (p.81Leu→Met and p.91Ala→Ala) found in *PRSS1* gene from patients with AIP. This finding is intriguing, but some of the cases described in the paper may not be AIP for the reasons written in major comments.

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