Identification of a Novel Frame-Shift Mutation in PRSS1 Gene in Han Patients with Autoimmune Pancreatitis

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Abstract: Objective: To detect mutations of trypsinogen gene (PRSS1) in patients with autoimmune pancreatitis (AIP) and to determine the underlying pathogenesis.

Methods: DNA sequencing was used to detect full-length of PRSS1, cystic fibrosis transmembrane conductance regulator (CFTR), and pancreatic secretory trypsin inhibitor (SPINK1) genes mutations in an AIP family and a sporadic case and 520 normal controls. Furthermore, a mutant-expressing system was constructed for functional confirmation.

Results: For the first time, we report a deletion mutation at exon 2 of PRSS1 gene (IVS 2 +56_60 del CCCAG) which encoded a truncated PRSS1 protein without trypsinogen activation peptide (TAP). Vitro functional study suggested the identified mutation would result in loss of PRSS1 activity. Mutant trypsinogen activated at a faster rate than wild-type trypsinogen in the autoactivation experiment. Histopathologic examination revealed the ratio of IgG4/IgG-positive plasma cells exceeded 0.455 in pancreas, and the patients responded to glucocorticoids.

Conclusion: PRSS1: IVS 2 +56_60 del CCCAG is a novel mutant which may contribute to AIP pathogenesis.

Keywords: Autoimmune pancreatitis, IVS 2 +56_60 del CCCAG mutation, molecular mechanism, PRSS1 gene.

INTRODUCTION

Autoimmune pancreatitis (AIP) is an autoimmune chronic pancreatitis which is characterized by infiltration of lymphocytes and plasma cells, and pancreatic fibrosis and dysfunction [1-3]. AIP may also involve extrapancreatic organs, such as bile duct, gallbladder, and lungs. The clinical characteristics, treatment, and outcome of patients with AIP are different from patients with other types of chronic pancreatitis; specifically, patients with AIP usually respond to glucocorticoids [2, 3]. Moreover, AIP has similar clinical manifestations to pancreatic cancer in imaging, thus AIP is often misdiagnosed as pancreatic cancer, resulting in unnecessary surgical intervention. AIP not only poses a diagnostic challenge for clinicians, but also can lead to irreversible injury to patients [4-6]. IgG4 positive plasma cells infiltration is considered a marker for the disease and can be detected in the pancreas and other tissues [1, 2, 7]. Unfortunately, serum IgG4 increase is not found in all patients with AIP and more than half of the patients with AIP are normal [8-10]. There is an urgent need to find some more specific diagnosis technology.

During the last two decades, genetic factors have been identified in patients with chronic pancreatitis (CP) and these factors are believed to play an important role in the pathogenesis of CP. Mutations in protease serine 1 (PRSS1) (OMIM 276000), cystic fibrosis transmembrane conductance regulator (CFTR) (OMIM 602421), and pancreatic secretory trypsin inhibitor (SPINK1) (OMIM 167790) were causally linked to the pathogenesis of CP. However, as the largest populated country in the world, the incidence of AIP (a special kind of chronic pancreatitis) in China has risen rapidly. So far, there were only sporadic reports of individual genes and AIP susceptibility internationally. None of these studies focused on hereditary AIP cases. Gain-of-function PRSS1 missense mutations were first reported in individuals with CP in 1996 [11, 12]. Since then, the relationship between PRSS1 mutations and chronic pancreatitis has widely been studied [13]. It suggested an important role for prematurely activated trypsinogen within the pancreatic acini in initiating chronic pancreatitis. In the present study, we investigate the mutations of PRSS1 in patients with AIP and their potential roles in AIP pathogenesis.
MATERIALS AND METHODS

Study Population and Diagnosis Criteria

We recruited one hereditary AIP family (Fig. 1) and a sporadic case (Fig. 1A) from the first Affiliated Hospital, Fujian Medical University. A total of four patients were included in the study. There was no history of tobacco smoking or alcohol consumption in these patients. All patients originated from the Han ethnicity in the mainland of China. Patients with AIP were confirmed with HISORt criteria (histology, imaging, serology, involvement of extrapancreatic organs, and response to glucocorticoids) of the Mayo Clinic [14-16]. We randomly selected 520 unrelated healthy as normal controls, including 336 males and 184 females, with an age range from 17 to 68 years. This study was approved by the Fujian Medical University Ethics Committee which supervised the process of the experiment.

Case 1 (Fig. 1A): Sporadic case, a 23-year-old male was admitted for the 4th time due to evaluation of jaundice in the skin and sclera, and shortness of breath for 20 days. A CT scan revealed enlargement of the pancreas, suspected lesions in the hepatic portal area, intrahepatic bile duct dilatation, emphysema, and multiple bullae in the lungs bilaterally. Four years earlier, in a physical examination he was diagnosed with liver dysfunction, but no discomfort was noted. Over the last three years, the patient lost approximately 20 kg. He was initially diagnosed with α1-antitrypsin (α1-AT) deficiency, but a subsequent blood test revealed that α1-AT was 2.0-fold higher than the normal upper limit. Further examination showed negative results for markers of hepatitis virus. He was suspected to have AIP and glucocorticoids were recommended for treatment. Two months later, the jaundice improved and the body weight increased by 5 kg/month.

Case 2 (Fig. 1B): A 21-year-old male (IV1) was admitted for evaluation of anorexia and abdominal pain for two weeks. He was a member of the AIP family. Six months earlier he had decreased appetite and episodic dull upper abdominal pain. Two months ago he developed yellow skin and sclera, pruritus, yellow urine, intermittent wheezing, and glucosuria. Magnetic resonance imaging (MRI) revealed enlargement of the head of the pancreas and extrahepatic bile duct dilatation. During the course of the disease, the patient was fatigued and lost 4 kg within one month. His father (II15) had a history of jaundice and underwent pulmonary bulla resection 10 years ago. His uncle (III1) also had a history of jaundice, cholecystitis, and chronic pancreatitis. The diagnosis of AIP was confirmed by histopathology.

METHODS

Analysis of Gene Mutations

Informed consent was obtained from patients and their relatives and controls. Blood was collected and DNA/RNA was extracted using a Tiangen Genomic extraction kit (Beijing, China). The full-length pancreatitis-related genes (PRSS1, CFTR, and SPINK1) were amplified. Following purification, sequencing was performed.

Functional Experiments on Mutant

The completely mutated and wild-type of PRSS1 cDNA was 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′-TGCAATTG TATG GCA CCA TTC GAC GAT GAT GAC AAG AT-3′ and the reverse primer was 5′-GA GTCGAC TC AGC TAA TTA AGC TTA GTG-3′. In addition, MunI and SalI

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Fig. (1). Pedigree of familial and sporadic AIP. ■●: patients with AIP; □: male; ○: female; ◇: abortion, unknown gender; ◆: carrier. *: no collection of DNA sample.
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 (D253) was measured at 253 nm within 60 min. The specific enzyme activity was calculated as follows: specific activity = enzyme activity/mg of protein = ΔD253/t ×1000/ (ε×t ×0.001), where t refers to time (min) and ε to the amount of proteinase (μg) during the detection.

Trypsinogen Autoactivation in the Presence of CTRC

Trypsinogen at 1 µM concentration was incubated with 5 nM human chymotrypsin C (CTRC) and 10 nM standard cationic trypsin in 0.1 M Tris-HCl (pH 8.0) and 1 mM CaCl₂ at 37°C. Trypsin activity was measured by adding N-CBZ-Gly-Pro-Arg-p-nitroanilide substrate and following the release of p-nitroanilin at 405 nm in a SpectraMax plus384 micro-plate reader.

Detection of Trypsin, α1-Antitrypsin, and TAP

Detection of serum elastase, trypsinogen activation peptides (TAP), and trypsin was done with ELISA kits (R&D Systems, Minneapolis, MN, USA). Serum levels of α1-AT, IgG and IgG4 were measured by latex-enhanced nephelometric immunoassay (Dade Behring Marburg GmbH, Germany).

Histopathology and Immunohistochemistry

Punctured samples (pancreas, liver, and lungs) were prepared and used for histopathological and immunohistochemical studies. The immunostaining of IgG4 was performed using a monoclonal antibody for human IgG4 (ZYMED Laboratories, San Francisco, CA) and that of IgG (Dade Behring Marburg GmbH, Germany). Immunohistochemistry (CK, CD3, CD20, CD38, CD68 and Vimentin) and H&E staining were done to observe the infiltration of lymphocytes and plasma cells.

Treatment and Follow-Up

Glucocorticoids were administered empirically (oral prednisone [40 mg] once daily with a 5-mg taper every 2 weeks). At the same time, oral acid-suppressing agents and calcium were also given. The therapeutic efficacy was evaluated based on manifestations and signs [14, 17-19].

RESULTS

Patient’s Profile and Laboratory Findings

In patients with AIP, the serum levels of trypsin and α1-AT were increased, and the body weight decreased significantly. The levels of IgG4, hyaluronic acid, type IV collagen, laminin, and type III procollagen N-terminal peptide were significantly increased (Table 1).

Molecular Genetic Analysis

Within the AIP family (II1, II1, II2, II5, II6, IV1) and the patient with sporadic AIP (case 1), a new mutation (IVS 2 +56 del CCCAG) of PRSS1 gene was noted (Fig. 2). The frame-shift mutation caused the deletion of a large fragment of exon 2 of the PRSS1 gene (14-54 aa) which contained trypsinogen activation peptide (TAP). This deletion mutation resulted in a truncated protein without TAP, while healthy controls

Table 1. The Clinical Data of the Patients with AIP and his Family Members

<table>
<thead>
<tr>
<th>Family No. 1 (Fig. 1A)</th>
<th>Family No. 2 (Fig. 1B)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Proband</td>
</tr>
<tr>
<td>AIP classify</td>
<td>Type 1</td>
</tr>
<tr>
<td>Age/ Age of onset</td>
<td>23/19</td>
</tr>
<tr>
<td>abdominal pain</td>
<td>Occasionally</td>
</tr>
<tr>
<td>weight loss (kg/12 months)</td>
<td>8</td>
</tr>
<tr>
<td>immunoglobulin G (lgG)(7-17 g/L)</td>
<td>26.8</td>
</tr>
<tr>
<td>IgG4(0.08-1.40g/L)</td>
<td>11.85</td>
</tr>
<tr>
<td>Fasting blood glucose (3.11-6.89) mmol/L</td>
<td>4.95</td>
</tr>
<tr>
<td>AMY (30-110) U/L</td>
<td>154</td>
</tr>
<tr>
<td>LIPA (&lt;330) U/L</td>
<td>493</td>
</tr>
<tr>
<td>El (52.3±13.4) ug/L</td>
<td>77.5</td>
</tr>
<tr>
<td>α1-AT(%) (0.90-2.00)</td>
<td>4.02</td>
</tr>
<tr>
<td>Trypsin (2-8) nmol/L</td>
<td>19.85</td>
</tr>
<tr>
<td>TAP (1.2±0.5) n mol/L</td>
<td>1.6</td>
</tr>
<tr>
<td>Hyaluronic acid (0-120) ng/mL</td>
<td>928.6</td>
</tr>
<tr>
<td>Laminin (0-130) ng/mL</td>
<td>191.0</td>
</tr>
<tr>
<td>Procollagen type III (&lt;12) ng/mL</td>
<td>17.3</td>
</tr>
<tr>
<td>Collagen type IV (0-140) ng/mL</td>
<td>201.7</td>
</tr>
</tbody>
</table>
and other healthy members of the AIP family were devoid of any such mutation.

Pathologic Analysis

As shown in Fig. (3), a large number of lymphocytes and plasma cells were found in the bile ducts accompanied by hyperplasia of myofibroblasts (Fig. 3A) and the number of pancreatic acinar was significantly reduced (Fig. 3B). The ratio of IgG4-positive plasma cells to infiltrated mononuclear cells (IgG4/IgG) was exceeded 0.455 in the pancreas (Fig. 3C, D). Diffuse swelling of hepatocytes and spotty necrosis were noted. An increase in fibrous tissues was noted in the portal area, and infiltration of lymphocytes and plasma cells was also observed (Fig. 4A). There were a lot of pulmonary bullae and fibrous tissue hyperplasia. Immunohistochemistry revealed the amounts of elastic fibers reduced and the reticular fibers were irregularly arranged, fractured, and reduced (Fig. 4B).

Diagnostic Imaging

Computed tomography (CT) findings include a diffusely enlarged hypodense pancreas or a focal mass and retroperitoneal lymph node enlargement that may be mistaken for pancreatic cancer. Magnetic resonance imaging (MRI) reveals diffusely decreased signal intensity and delayed enhancement on dynamic scanning (Fig. 5A). Polycystic lesions evidenced in the liver, gallbladder, pancreas, spleen, retroperitoneal lymphadenopathy and bullae are shown in Fig. (5B, C) and those evidenced in the lungs are shown bilaterally in Fig. (5D). After treatment, the pancreas was significantly reduced (Fig. 5E, F).

Activity of the PRSS1 Variants

A UV spectrophotometer was used to measure the activity of trypsinogen before and after enterokinase activation at 253 nm (ΔD253). The measurements were performed for 60 min. Based on the aforementioned formula, the specific activity of renatured recombinant trypsin before and after enterokinase activation was 65-75 and 60-78 BAEE U/mg, respectively. And the specific activity of wild-type trypsin was 123-165 BAEE U/mg after enterokinase activation. With this method, the activity of natural porcine trypsin, as a standard, was 1080 BAEE U/mg. The activity of products of the mutant gene remained unchanged after enterokinase activation which suggested the identified mutation would result in loss of PRSS1 activity (Fig. 6A).

Autoactivation of PRSS1 Variants in the Presence of CTRC

Trypsinogen at 1 µM concentration was incubated with 5 nM CTRC and 10 nM initial trypsin in 0.1 M Tris-HCl (pH 8.0), 1 mM CaCl2 and 0.05% Tween-20 at 37°C. When measured at pH 8.0 and in 1 mM Ca2+, PRSS1 variants exhibited increased activation in the presence of CTRC (Fig. 6B). Mutant type exhibited 1.5-fold increased autoactivation compared to that exhibited by wild type.

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**Fig. (2).** Deletion mutation of PRSS1 and the cDNA sequence. **A:** IVS 2 +56_60 del CCCAG of the PRSS1 gene, **B:** Sequence of PRSS1 gene mutated cDNA.
Patients underwent treatment with glucocorticoids for 3-6 months, and the jaundice improved. Imaging showed the size of the pancreas returned to near-normal. The serum levels of total and direct bilirubin and that of IgG4 reduced significantly. Wheezing was markedly improved and the body weight increased (5-8 kg/month).

**DISCUSSION**

AIP was the first time proposed by Yoshida in 1995 and the number of patients quickly increased [1, 3, 20, 21]. The clinical manifestations of AIP are diverse and can be characterized by pancreatic or extrapancreatic lesions. The most common symptoms in patients with AIP are jaundice and weight loss. These manifestations are an important reason for the misdiagnosis of AIP as
malignancies of the bile and/or pancreatic ducts [15-18]. In our study, all of the patients presented with typical jaundice and weight loss, and were initially diagnosed with pancreatic cancer. The large amount of lung bullae is a major cause of wheezing, and it was the novel and main clinical manifestation of AIP in this study. The onset age was 30 years earlier than the one previously reported, thus, it is prone to be misdiagnosed as a fibrocystic disease [22, 23].

It has been reported that genetic factors play an important role in the pathogenesis of AIP. In a Japanese population, the proportion of haploid DRB1*0405-DQB1*0401 in patients with AIP is higher than that in patients with other types of chronic pancreatitis [24]. Parkdo et al. reported that substitution of aspartic acid at the 57\textsuperscript{th} position of haploid DQ\textbeta1 of the histocompatibility leukocyte antigen is closely related to the recurrence of AIP [25]. PRSS1 gene mutations lead to increased cationic trypsinogen autoactivation or misfolding, intracellular retention and endoplasmic reticulum stress resulting into chronic pancreatitis. Until recently, more than 200 variants of PRSS1 have been reported, and pancreatitis-related mutations are mainly found in exons 2 and 3 [26-28]. The mechanism of action of pancreatitis-associated PRSS1 mutations involves increased autoactivation of mutant trypsinogens resulting in elevated intrapancreatic trypsin activity levels or activation in pancreatic internal ectopic. Recent studies uncovered that PRSS1 mutations alter the regulation of activation and degradation of cationic trypsinogen by chymotrypsin C (CTRC) [29]. Somewhat paradoxically, CTRC also promotes degradation of trypsinogen by cleaving the calcium binding loop. This cleavage in combination with a trypsin mediated autolytic cleavage results in inactivation of trypsinogen during

![Fig. (5). The images of examination and curative effect observation. A: T2WI: showed high signal, pancreatic uniform increase, obvious swelling of head of pancreas. B: T1WI: pancreatic morphology increase, local pancreatic duct dilatation. C: abdominal MRI, enlargement of the pancreas and cyst-like lessions; polycystic lesions in the liver, spleen. D: lung CT, polycystic lesions in the lung. E: pretherapy: pancrea enlargement, uniform density, pancreas body width is 38 mm. F: one month after treatment: pancreas significantly reduced (26mm) than before.](image-url)
autoactivation and lower trypsin levels are attained [30]. The common biochemical phenotype of PRSS1 mutations associated with chronic pancreatitis is the generation of greatly increased trypsin levels during autoactivation in the presence of CTRC. This phenomenon is due to increased sensitivity of trypsinogen mutants to CTRC-mediated stimulation of autoactivation and/or resistance to CTRC-dependent trypsinogen degradation. Löhr’s study showed that the expression of trypsinogens and other pancreatic enzymes was greatly reduced in the patients with AIP. Immunohistochemistry showed a near-loss of trypsin-positive acinar cells, which was also confirmed by western blotting. The serum of AIP patients contained high titers of autoantibodies against PRSS1 and PRSS2. It also supports that trypsin plays a great role in autoimmune pancreatitis [31].

In this study, we investigated IVS 2 +56_60 del CCCAG mutant autoactivated at a faster rate than wild-type trypsinogen. TAP regional variation may affect the trypsinogen folding and enhance its autoactivation. Under normal conditions (the presence of enterokinase), amino acids 16-23, also known as TAP, are removed from the N terminal of trypsinogen, resulting in trypsin. The active center consists of His40, Asp84, Trp193, and Ser177 and forms a “deep pocket” with a strict limitation on substrate. The substrates with positively charged Lys and Arg can bind to Asp-189 at the catalytic site of trypsin, which is negatively charged (Fig. 7). On deletion of TAP, trypsin appears due to a frame-shift mutation of PRSS1, and when the signal cleavage site is removed from trypsinogen in the endoplasmic reticulum, the formation of trypsin takes place. This directly results in ectopic activation of trypsinogen, which leads to mutant gene remained unchanged after enterokinase activation. The deletion mutation of trypsinogen results in the loss of protection by TAP and leads to direct activation, which in turn leads to activation of lysosomal enzymes and damage to organs.

Our findings suggest that AIP can present with polycystic lesions in multiple organs, which can be found in patients with hereditary AIP, as well as in patients with sporadic AIP. In addition, functional studies have elucidated the molecular mechanisms underlying the pathogenesis of AIP, which is due to the presence of deletion mutation of PRSS1 gene.

**Fig. (6).** Activity and autoactivation of the PRSS1 variants. A: Activity of the PRSS1 variants before and after enterokinase activation. B: Autoactivation of trypsinogen variant. The specific activity of renatured recombinant trypsin before and after enterokinase activation was 65-75 and 60-78 BAEE U/mg, respectively. Compared with the standard trypsin activity was about 7% and the specific activity of wild-type trypsin was 15%. Autoactivation of cationic trypsinogen variants in the presence of chymotrypsin C (CTRC). Trypsinogen was incubated at 1µM concentration with 5 nM CTRC and 10 nM initial trypsin in 0.1 M Tris-HCl (pH 8.0), 1 mM CaCl2 and 0.05% Tween-20 (final concentrations) at 37°C.

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**AUTHOR CONTRIBUTIONS**

Gao F and Li YM contributed equally to this manuscript; Liu QC, Hong GL and Li YM defined the research theme; Gao F and Xu ZF designed the methods and experiments, carried out the laboratory experiments, analyzed the data, interpreted the results and wrote the paper; Zhuang ZH and Wang CD co-designed the dispersal and colonization experiments, and co-worked on associated data collection and their interpretation; Chen JT co-designed experiments, discussed analyses, interpretation, and presentation; All the authors contributed to, went through and approved the manuscript.

**CONFLICT OF INTEREST STATEMENT**

It is declared that this article is devoid of any conflict of interest.

**ACKNOWLEDGEMENTS**

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Identification of a Novel Frame-Shift Mutation

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ETHICS STATEMENT
Informed consent to participate in the interviews and provide a blood sample was obtained from each participant. The study was approved by the Fujian Medical University Ethics Committee.

REFERENCES


Fig. (7). Gene structure and the activated form of trypsinogen. The active center of trypsin after activation of wild-type and mutated trypsinogen. Amino acids 16-23, also known as trypsinogen activation peptide, are removed from the N terminal of wild-type trypsinogen in the presence of enterokinase, resulting in the activation of trypsinogen (trypsin). The active center forms a “deep pocket” with strict limitation on the substrate. The mutated trypsinogen gene encodes a truncated protein, and the two walls of the active center cannot be formed. This pocket-like active center has poor selectivity for substrate and provides an opportunity for binding to small side chains, such as Ala of elastase.


