Comprehensive Screening for PRSS1, SPINK1, CFTR, CTRC and CLDN2 Gene Mutations in Chinese Pediatric Patients with Idiopathic Chronic Pancreatitis

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Comprehensive Screening for *PRSS1*, *SPINK1*, *CFTR, CTRC* and *CLDN2* Gene Mutations in Chinese Pediatric Patients with Idiopathic Chronic Pancreatitis

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Article summary

Article Focus

- Genetic alterations may contribute to chronic pancreatitis in Chinese young patients.
- China is different from the Western countries in regards to ethnic and cultural backgrounds, ocioeconomic status, climatic conditions, and dietary habits.

Key Messages

- SPINK1 IVS3+2T>C mutation was more commonly associated with Chinese ICP children.
- Pediatric patients with SPINK1 IVS3+2T>C mutation had a higher rate of pancreatic duct stones, pancreatic pseudocyst, and pancreatic calcification than those without.
- SPINK1 IVS3+2T>C mutation may play an important role in the pathogenesis of Chinese pediatric ICP, which may be show some insights into a novel target for therapy.

Strengths and Limitations

- To the best of our knowledge, this is the first study to determine the spectrum and frequency of *PRSS1, SPINK1, CFTR, CTRC* and *CLDN2* gene mutations and *PRSS1* CNVs in unrelated CP children in China.
- Further study is needed to confirm and to investigate the role of these genes in the development of Chinese ICP.

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Abstract

Objective: Genetic alterations may contribute to chronic pancreatitis (CP) in Chinese young patients. This study was designed to investigate mutations of cationic trypsinogen (*PRSS1*), pancreatic secretory trypsin inhibitor or serine protease inhibitor Kazal type 1 (*SPINK1*), cystic fibrosis transmembrane conductance regulator *(CFTR)*, chymotrypsin C *(CTRC)*, and *CLDN2* genes and the copy number variations (CNVs) of *PRSS1* and assess associations with the development of idiopathic CP (ICP) in Chinese children. **Methods:** Seventy-five ICP children (40 boys and 35 girls) were recruited for the assessment of *PRSS1, SPINK1, CFTR, CTRC* and *CLDN2* gene mutations and CNVs using DNA sequencing and TaqMan® Probe-Based Gene Expression Analysis, respectively. **Results:** Seven patients had heterozygous mutations in *PRSS1*, i.e. N29I (n=1), R122H or R122C (n=6). The CNVs of PRSS1 in five patients had unmoral copies [1 copy (n=4), five copies (n=1)]. 43 patients had IVS3+2T>C (rs148954387) (10 homozygous and 33 heterozygous) in *SPINK1*. None of the *PRSS1* mutation patients carried a *SPINK1* mutation. Frequency of *PRSS1* and *SPINK1* mutations was 9.3% and 57.3%, respectively, with an overall frequency of 66.6% (50/75). In addition, one patient had a novel deletion of *CFTR* (GCTTCCTA from c.500 to c.508 leading to the shortened polypeptide molecule via a stop codon). Another patient had a novel missense in *CLDN2* exon 2 (c.592A>C mutation). Clinically, patients with *SPINK1* mutations had a higher rate of pancreatic duct stones, pancreatic pseudocyst, and pancreatic calcification than those without *SPINK1* mutations (P<0.05). **Conclusions:** *SPINK1* mutations were more commonly associated with Chinese ICP children. *SPINK1* IVS3+2T>C mutation may play an important role in the pathogenesis of Chinese pediatric ICP. However, further study is needed to confirm and to investigate the role of these genes in the development of Chinese ICP.

Key words: idiopathic chronic pancreatitis, *PRSS1*, *SPINK1*, *CFTR, CTRC*, *CLDN2*, CNVs, children

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Introduction

Chronic pancreatitis is an inflammatory disease characterized by irreversible destruction of the pancreatic normal structure and function and is associated with persistent abdominal pain or steatorrhea. In adults, alcohol abuse is an important cause of chronic pancreatitis, while other factors (such as anatomical changes, metabolic disease, trauma and heredity) may also cause or associate with chronic pancreatitis. However, up to 10-25% of patients with chronic pancreatitis have no clear risk factors and these patients are classified as having idiopathic chronic pancreatitis (ICP) [1]. In children, it is estimated that ICP accounts for approximately 40-60% of all children with chronic pancreatitis in Western countries, but the reported rate was as high as 73.8% in China [2]. The pathogenesis of ICP, especially in children, remains poorly understood. Since the conventional risk factors such as alcohol abuse are uncommon in children, it has reported that environmental risk factors may play a limited role in the pathogenesis of ICP in children; thus, patients with ICP at these ages are thought to be suitable for studies of genetic defects [3].

Over the last two decades, genetic factors have been shown in patients with chronic pancreatitis and these factors are believed to play an important role in the pathogenesis of chronic pancreatitis [4]. For example, previous studies reported identification of mutations in genes encoding cationic trypsinogen (UniGene name: protease serine 1: *PRSS1*) (OMIM 276000) [5], cystic fibrosis transmembrane conductance regulator (*CFTR*) (OMIM 602421) [6], the pancreatic secretory trypsin inhibitor or serine protease inhibitor Kazal type 1 (PSTI or *SPINK1*) (OMIM 167790) [3], and chymotrypsin C (*CTRC*) (OMIM 601405) [7], all of which reveal that hereditary pancreatitis is a more common form of chronic pancreatitis than once thought [1]. More recently, a newly detected candidate gene known as *CLDN2* has been shown to be associated with sporadic and alcohol-related chronic pancreatitis [8]. Thus, genetic study may reveal a genetic basis for significant percentage of patients with so-called “idiopathic” chronic pancreatitis. It becomes acceptable that development of chronic pancreatitis requires a combination of genetic predisposition and environmental, structural, or toxic insult [9].

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Therefore, identification of genetic and environmental risk factors could offer the potential tool for risk assessment, early diagnosis, and earlier intervention of chronic pancreatitis [10].

Many studies have discovered that mutations plus the copy number variations (CNVs) in *PRSS1* (trypsinogen), *SPINK1* (serine protease inhibitor Kazal-type 1), *CFTR* (cystic fibrosis transmembrane conductance regulator), and CTRC (chymotrypsin C, also known as caldecrin) were causally linked to the pathogenesis of ICP [11-13], while patients with *PRSS1* or *SPINK1* mutations may be at a higher risk of developing pancreatic cancer [14]. *CFTR* variants were associated with idiopathic and alcoholic chronic pancreatitis. Furthermore, *CLDN2* was shown to be strongly associated with chronic pancreatitis, suggesting that it probably acts as a disease modifier to accelerate the development and progression of chronic pancreatitis through a non-trypsin–dependent process since *CLDN2* is a highly regulated tight junction protein to form ion and water channels between endothelial cells [8]. However, as the largest populated country in the world, the incidence of chronic pancreatitis, including pediatric chronic pancreatitis, has risen rapidly [2, 15], whereas there have been only a few studies reporting that genetic factors contributed the pathogenesis of chronic pancreatitis in China, but none of these studies focused on ICP in children [13, 16]. In addition, China is different from the Western countries in regards to ethnic and cultural backgrounds, socioeconomic status, climatic conditions, and dietary habits. In this pilot study, we identified mutations in *PRSS1, SPINK1, CFTR, CTRC* and *CLDN2* genes and CNVs of *PRSS1* to determine the spectrum and frequency of the mutations and CNVs in unrelated children with ICP in the mainland of China. The data from this study will provide information into the genetic basis of pediatric ICP in China.

Patients and Methods

Study population and diagnosis criteria

We recruited 75 ICP patients under 18 years old from Changhai Hospital between January 1997 and December 2008. There was no history of tobacco smoking or alcohol

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consumption in these patients. All patients originated from the Han ethnicity in the mainland of China. The diagnose criteria of CP and ICP was defined as a condition characterized by typical history (abdominal pain, diabetes mellitus and/or steatorrhea) and the presence of any one of the following findings: i) ductal changes on ERCP; ii) pancreatic calcification on imaging examination; or iii) histological evidence of CP [17-18]. Furthermore, affected individuals were classified as having ICP if precipitating risk factors (such as alcohol abuse, trauma, previous medication, infection, metabolic disorders and/or a positive family history) were absent [19]. This study was approved by the Ethics Committee of Changhai Hospital, Shanghai and a written informed consent was given by their parents or legal guardians according to the ethical guidelines of the Declaration of Helsinki.

DNA isolation

Peripheral blood samples were collected from each patient in a ethylene diamine tetra acetic acid (EDTA)-anticoagulated tube and frozen at -20^{o}C for subsequent DNA extraction. Genomic DNA was isolated from 500 µl samples using the Lab-aid 800 automatic nucleic acid extraction machine following a standard protocol and quantified using a NanoDrop machine (Thermo, Wilmington, USA).

PCR analyses of gene mutations

DNA samples from patients were subjected to polymerase chain reaction (PCR) analyses of gene mutations. Specifically, primers flanking the targeted regions of *PRSS1, SPINK1, CFTR, CTRC* and *CLDN2* genes were designed and synthesized according to the nucleotide sequence published by NCBI as shown in supplemental Table 1. The data on mutation analyses were confirmed by DNA sequence and repeated using PCR. PCR was performed in the GeneAmp 9700 System (Applied Biosystems, Foster city, CA) using a 15-µL reaction mixture containing 7.5 µl 2×Taq Mix (Vivantis, USA), 5.7 µl ddH_2O, 1.5 µl DNA templates (10 ng/µl), and 0.15 µl of each primer (20 mM). PCR amplification was set at an initial 6 min

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denaturation at 94°C, followed by 35 cycles of 30 s at 94°C, 30 s at 58°C (*PRSS1*, *SPINK1*) or 56℃ (CFTR, *CTRC, CLDN2*), 45 s at 72℃, and a final extension at 72°C for 7 min. PCR products were then incubated with 0.1 U shrimp alkaline phosphatase at 37°C for 1 h, followed by heat inactivation at 85°C for 20 min and then sequenced using an ABI Prism BigDye Terminator Cycle Sequencing Kit, version 3.1 on an ABI Prism 3730 sequencer (Applied Biosystems). Data passed a duplicate quality-control test using four samples and showed 100% concordance.

Vector NT1, Chromas and Bioedit software were applied to analyze the results.

Detection of gene copy number variations (CNVs)

A pre-designed and validated CNV assay kit to assess PRSS1 CNVs was obtained from AB Life Technologies (Hs03184214_cn) (see details in supplemental Table 2). RT-PCR was performed using 2×Taqman Genotyping Master Mix 5 μl (Vivantis), ddH_2O 0.5 μl, DNA templates (10 ng/μl) 4 μl, 0.25 μl Taqman Copy Number Assay (Vivantis) and 0.25 Taqman Copy Number Assay (Vivantis) in a total volume of 10 μl. Cycle conditions were as follows: initial denaturation for 10 minutes at 95°C, followed by 40 cycles of (15 s at 95°C,and 60s at 60°C) in an 7900 RT-PCR thermal cycler (Applied Biosystems). The assays were performed in triplicate and repeated at least once for each sample. PRSS1 gene copy number was calculated compared to the proportion to RNAseP reference assay (AB Life Technologies cat#4403326). Data were analyzed using Copy Caller Software (version 1.0 from AB Life Technologies).

Statistical analyses

Continuous data were reported as mean ± standard deviation (SD) analyzed using a Student's t-test and/or u-test. Fisher's exact test or chi-square test was used to analyze the categorical data. The gene mutations or CNVs were associated with age at diagnosis,

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pancreatic calcification, changes in pancreatic duct (stenosis or dilatation), pancreatic calcification, and pancreatic pseudocyst for clinical significance of these mutations [19].

The age onset was divided into two subgroups according to the mean value. A *P* value of less than 0.05 was considered statistically significant and all reported *P* values were two-sided.

Results

Baseline clinical characteristics of study participants

A total of 75 unrelated children were included in this study, i.e., 40 boys and 35 girls and age at first onset was 11.91 ± 3.79 years (ranged between 3 and 18 years). Clinically, 81.3% (61/75) of the patients showed acute pancreatitis, 13 patients began with pure abdominal pain, 3 patients with weight loss, 1 patient with diarrhea, and 1 patient with high blood glucose.

Imaging examinations, including CT, MRCP or ERCP, detected pancreatic duct stones in 45 patients, changes in pancreatic duct (stenosis or dilatation) in 57 patients, pancreatic calcification in 15 patients, pancreatic pseudocyst in 15 patients, and pancreatic divisum in 4 patients (Table 1). Laboratory tests showed that six patients had increased levels of CA199, a biomarker for pancreatic cancer, six patients had high blood cholesterol, two had increased blood glucose, and two patients had low blood calcium levels.

Analyses of PRSS1, SPINK1, CFTR and CTRC gene mutations in these patients

We then analyzed mutations of *PRSS1, SPINK1, CFTR* and *CTRC* gene in these 75 patients and found three types of heterozygous *PRSS1* mutations in seven patients, including N29I (n=1) in exon 2, and R122H (n=6) with c.365G>A (n=5) and c.364C> T (n=1) in exon 3. A single gene mutation of *SPINK1* occurred in 43 patients, including 10 homozygous and 33 heterozygous *SPINK1* mutation (IVS3+2T>C). However, there was no single patient with *PRSS1* mutation who had a *SPINK1* mutation, making 9.3% (7/75) and 57.3% (43/75) of,

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PRSS1 and *SPINK1* mutation rates, respectively and an overall rate of 66.6% (50/75) of patients who at least had one *PRSS1* or *SPINK1* mutation (Table 2).

Furthermore, one patient had *CFTR* gene deletions of GCTTCCTA sequences between c.500 to c.508 at exon4, leading to an early stop codon (Figure 1). *CFTR* gene C.2562 T>G polymorphism was also detected in 51 patients, 14 of whom were homozygous. Heterozygous *CFTR* gene c.4389G>A mutation was identified in another patient. Moreover, there were four types of TG-repeats and poly-T tract including (TG)10-T7(n=2), (TG)11-T7(n=55), (TG)12-T5(n=5) and (TG)12-T7(n=13) in the junction of intron 8 and exon 9 and (TG)11-T7 of *CFTR* gene found in 73.3% of these patients (supplemental Table 3). In addition, *CFTR* gene V allele was slightly more frequent than the M allele at codon 470 (59.3% and 40.7%, respectively). The dominant genotype was M/V followed by V/V and M/M (supplemental Table 4).

However, we screened six types of CTRC mutations, including c.143A>G, c.217G>A, c.180C>T in exon 3, c.703G>A, c.760C>T, and p.K247_R254del in exon 7 in these 75 patients, but did not find any mutations. In addition, we screened both exons of *CLDN2* and found four types of heterozygous mutations in a total of 26 patients (Table 2), i.e., c.22G>A at exon 1, c.327A>T, c.592A>C and c.768T>C at exon 2. C.592A>C is a missense mutation, while the other three types are nonsense. However, none of these patients had more than one type of *CLDN2* mutation.

Analyses of PRSS1 gene copy number variations in these patients

PRSS1 gene copy numbers were normal in most patients. Specifically, *PRSS1* gene copy number in 70 patients had two copies detected using the probe Hs03184214_cn, whereas four patients had only one copy and another patient had five copies (Figure 2).

Association of mutations with clinicopathological data

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We associated these genetic alterations with clinicopathological data from the 75 patients. Our data showed that three mutations of IVS3+2T>C in *SPINK1* gene, M470V and c.2562 T>G in *CFTR* gene had relatively higher frequencies than other genetic alterations and were associated with clinicopathological data. Briefly, the rates of pancreatic duct stones, pancreatic pseudocyst and pancreatic calcification were significantly higher in patients with a *SPINK1* gene IVS3+2T>C mutation than that of patients without IVS3+2T>C (69.8% vs., 46.9% *P*=0.045; 11.6% vs. 31.25% *P*=0.036; 27.9% vs. 9.4% *P*=0.047, respectively). The rate of pancreatic pseudocyst was significantly lower in patients with the *SPINK1* gene IVS3+2T>C mutation than that of patients without IVS3+2T>C (11.6% vs. 31.25% *P*=0.036) (Table 3). However, there was no statistical significance between age at diagnosis of patients with and without IVS3+2T>C mutation. M470V and c.2562 T>G were not significantly associated with these clinical characteristics.

Discussion

In the present study, we revealed *PRSS1, SPINK1, CFTR, CTRC* and *CLDN2* gene mutations in ICP patients, especially *PRSS1* and *SPINK1* gene mutations, occurred in almost 70% of Chinese ICP children. To the best of our knowledge, this is the first study to determine the spectrum and frequency of *PRSS1, SPINK1, CFTR, CTRC* and *CLDN2* gene mutations and *PRSS1* CNVs in unrelated CP children in China. The data indicate that genetic changes occurring in Chinese ICP patients could associate with ICP development. Further study will investigate how these gene alterations contribute to ICP development.

Our current data on the frequency of *PRSS1* mutations were similar to those (9.3% *vs.* 9-23%) of a previous study on children with CP or ICP [20], however, our data on the frequency of *SPINK1* mutations (57.3%) appeared higher than those (19-40%) reported in the previous study [3]. Moreover, Witt et al. [20] first showed *PRSS1* gene mutations (3/30 cases at 3×A16V) in German pediatric CP patients. Thereafter, they showed that in a study of 96 unrelated CP children, *PRSS1* gene mutations (5×A16V, 1×N29I, and 5×R122H) occurred in 11 (11.5%)

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patients [3]. In a review of 164 unrelated children with CP, the frequency was reported to be 9.1% (n=15, 8×A16V, 5×R122H, and 2×N29I) [21]. *PRSS1* gene mutations were detected in two (12.5%, 1×R122H and 1×A16V) of 16 patients classified as having early-onset ICP in a Swiss study [22] and in 11 (23.1%) of 52 children with CP (6×R122H, 4×R122C, 1×N29I) in a Polish study [23]. In our current study, *PRSS1* mutations were found in 9.3% of 75 Chinese pediatric ICP patients, which included N29I and R122H mutations. Heterozygous mutations of the *PRSS1* gene commonly occurred in CP patients in the Western populations [20,23] and was the only form of mutation in our patients, indicating that the main spectrum and frequency of *PRSS1* gene mutations in Chinese ICP children are similar to those reported in pediatric patients in Western countries [20-23]. The *PRSS1* mutations seem to be one of the predisposing factors for ICP, irrespective of race, although the third most common *PRSS1* mutation (i.e., A16V) is not found in our study. However, these spectrum and frequency are different from most previous Asian studies, in which *PRSS1* mutations were at a low frequency or even absent [13, 24-25]. In a previous study on 129 Chinese ICP patients (34 early-onset and 95 late-onset using a cut-off age of 35 years), Chang et al. [13] showed *PRSS1* mutations in six (4.6%) patients; in two (5.9%) patient with early-onset (1×R116C and 1×C139S) and four (4.2%) patients with late-onset (1×L104P, 1×R116C, 1×T137M and 1×C139S). These mutations are all considered relatively uncommon mutations in Western countries [26]. The potential reason for this discrepancy may be due to the sampling bias, i.e., Chang's study included both children and adults, whereas our current study only included children.

One of the most significant findings in our current study was the high frequency of *SPINK1* IVS3+2T>C mutation, which was first reported by Kume K et al. They showed a *SPINK1* IVS3+2T>C mutation in 13-16% of unrelated Japanese ICP patients [27]. However, two additional studies from Western countries reported IVS 3+2T>C mutation only in one (1.0%) of 96 and in 3 (2.7%) of 112 pediatric ICP patients [3, 12]. the IVS 3+2T>C mutation has been found in three (1.7%) of 172 German CP patients, but was thought to be a rare polymorphism and not a mutation [28]. However, a recent Chinese study on 129 ICP patients

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revealed a IVS 3+2T>C mutation in 8.5% of patients and the mutation was predominantly responsible for early-onset ICP (29.4% in early-onset vs. 1.1% in late-onset) [13]. Alternatively, Pfutzer et al. [12] showed a frequency of N34S mutation in *SPINK1* in 40.4% (23/57) of American ICP children, whereas Truninger et al. [11] reported a frequency of the mutation at 43% (6/14) German patients with early-onset ICP. In patients with tropical calcific pancreatitis, which is an idiopathic, juvenile, non-alcoholic form of CP widely prevalent in several tropical countries such as India, N34S mutation can reach 46%. In the present study, IVS 3+2T>C mutations were found in 57.3% (43/75) of unrelated Chinese ICP children, but we did not find any N34S mutations in the current study. These data suggest that the spectrum and frequency of *SPINK1* mutations vary geographically among different populations; IVS 3+2T>C mutations are more common in Chinese ICP children, whereas N34S mutations are more frequent in Western populations. The underlying role and molecular mechanisms of the IVS3+2T>C mutation in CP development are being explored. For example, this mutation was in complete linkage disequilibrium with -215G>A mutation, which might alter the efficiency of the *SPINK1* gene transcription. IVS3+2T>C mutation affects the splicing donor site that is highly conserved in eukaryotes [3]. IVS3+2T>C mutations can cause skipping of the whole of exon 3, where the trypsin binding site is located, leading to the loss of the trypsin binding site [27], altered expression of *SPINK1* protein in CP patients with the IVS3+2T>C mutation, affecting the protease/antiprotease balance within the pancreas. However, further studies are necessary to elucidate the underlying molecular mechanisms.

In addition, the second significant finding of our current study is *CFTR* gene polymorphisms, such as M470V (n=51), c.2562T>G (n=51), TG repeats, and poly T tract in Chinese ICP children. We found that 68% (51/75) patients had both c.2562 T>G and M470V polymorphisms, including heterozygous and homozygous alleles and one patient with heterozygous c.4389G>A mutation. Both c.2562T>G and c.4389G>A mutations are nonsense, while an obstructive tubulopathy of the pancreas due to the *CFTR* dysfunction is thought to play a primary role in CP development, although the exact pathogenic process of pancreatitis

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associated with *CFTR* mutations is still under investigation. The function of *CFTR* in the pancreas is to dilute and alkalinize the protein-rich acinar secretions, so that the formation of protein plugs that lead to pancreatic injury may be prevented. A M470V polymorphism on exon 10 affects the intrinsic chloride activity, and thereby affects CFTR protein function. The TG repeats and poly-T tract can influence *CFTR* at transcription levels because these intronic variants could lead to reductions in protein synthesis and expression, or altered splicing to compromise the intracellular transport and/or activity. Huang et al conducted the first comprehensive study on the functional polymorphisms of CFTR in Chinese healthy subjects and found that T7 was the most common haplotype (93.6%) and (TG)11 and (TG)12 were the dominant haplotypes in the junction of intron 8 and exon 9 [29]. Our current data also validated (TG)11-T7 as the most common type. The poly-T, TG-repeats and M470V distributions were similar to those studies on other East Asians [30-31]. In addition, a diverse range of *CFTR* loss-of-function variants have been reported to be associated with ICP and alcoholic CP, whereas their functional effects remain to be defined. Recently, Whitcomb et al reported that the coinheritance of *CFTR* R75Q and *SPINK1* variants is significantly higher in patients with ICP than in controls (8.75% vs. 0.38%). Using patch-clamp techniques, they also found that the *CFTR* genotype caused a selective defect in biocarbonate conductance [32]. Another study from Australia showed that symptomatic pancreatitis occurs in 20% of pancreatic sufficient cystic fibrosis patients. To evaluate genotype–phenotype interactions, they developed the Pancreatic Insufficiency Prevalence (PIP) score to determine severity in a large number of *CFTR* mutations and found that specific *CFTR* genotypes were associated with pancreatitis, i.e., patients carrying genotypes with mild phenotypic effects may have a greater risk of developing pancreatitis than patients carrying genotypes with moderate-severe phenotypic consequences at any given time [33]. In addition, common *CFTR* haplotypes seem to modulate susceptibility to CP.

Although multiple rare *CTRC* gene mutations have been associated with CP in European and Asian populations, our current study did not find any *CTRC* mutations in Chinese ICP children, indicating that *CTRC* mutation varies geographically or ethnically. According to the

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biochemical activities and the functional properties of *CTRC* variants, Zhou and Sahin-Tóth hypothesized three mutually nonexclusive models to demonstrate the possible role of *CTRC* variants in predisposing to CP: i). Impaired trypsinogen and/or trypsin degradation; ii). Impaired activation of A-type carboxylpeptidases; and iii). Induction of endoplasmic reticulum stress [34]. We infer that *CTRC* might play a limited role, if any, in the pathogenesis of CP in China.

However, until 2012, genetic variation in *CLDN2* has not previously been associated with disease in humans. A two-stage (discovery and replication) genome-wide association study (GWAS) showed that *CLDN2* genotype confers the greatest risk for CP, and its alleles via interacting with alcohol consumption, can amplify the risk. These data could partially explain the higher frequency of alcohol-related pancreatitis in men than women while the real causal relationship between CP and *CLDN2* has been ambiguously defined. Our current data are the first study on *CLDN2* SNPs in Chinese ICP patients to report four novel SNPs. Only one of these is a missense mutation known as c.592A>C in CDS of *CLDN2* gene, making the amino acid change from Met to Leu. This patient was a girl who was diagnosed as ICP after several episodes of acute pancreatitis since she was 13 years old. All of these clinical characteristics showed no deviances from other patients. Claudin-2 encoded by CLDN2 is normally expressed at low levels in the tight junction between cells of the pancreatic ducts and in pancreatic islets. But when stressed, acinar cells can also express claudin-2, proved by porcine models of acute pancreatitis [35]. Besides, the *CLDN2* promoter includes a nuclear factor (NF)-κB-binding site [36], and *CLDN2* expression is enhanced in other cells under conditions associated with injury and stress.

In an attempt to associate these mutations with clinical parameters from these ICP patients, we did not observe any association between the gene mutations and an earlier age of CP diagnosis, which is contrary to a previous study [37]. However, showed that patients with IVS3+2T>C mutation were more likely to have pancreatic duct stones or pancreatic calcification than those without such a mutation. It has been well known that patients with pancreatic calcification are more severe than those without pancreatic calcification.

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Therefore, our observations, along with previous findings [27] suggest that a IVS3+2T>C mutation in *SPINK1* predisposes to more severity of CP. Moreover, CP patients have a markedly increased risk in developing pancreatic cancer compared to the general population [14, 15] and *PRSS1* or *SPINK1* mutation may be a predictor for pancreatic cancer development in CP patients. The IVS3+2T>C mutation was present in 0.6% of the sporadic pancreatic cancer patients. Thus, CP patients with *PRSS1* or *SPINK1* mutation should avoid any risk factors, including alcohol and tobacco, be monitored for any signs or symptoms (pain, weight loss, jaundice, and/or abdominal mass) or with serum markers and imaging examination for pancreatic cancer.

CNVs often occur in human cancers and the compositions of CNVs may contain deletion, amplification, deletion plus amplification, multiple alleles and complicated locus. Lafrate and Sebat were the first to respectively report CNVs in human genome in 2004 [38]. To date, many studies proved that both CNVs and SNP can affect gene expressions. However, the effect of *PRSS1* gene CNVs on ICP has not been fully studied.

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Contributorship statement

Prof. Zhuan Liao and Zhao-Shen Li conceived the project. Dr. Xiao-Tian Sun, Wei Wang, Xiao-Ling Weng, and Dai-Zhan Zhou completed the DNA isolation, PCR analyses of gene mutations, and detection of gene copy number variations (CNVs). Dr. Chang Sun, Tian Xia, Liang-Hao Hu, Xiao-Wei Lai, Bo Ye, Mu-Yun Liu , Fei Jiang, and Jun Gao collected the peripheral blood samples and clinical data. Lu-Min Bo and Yun Liu completed the statistical analyses.

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Competing Interests

None

Data sharing

No additional data available.

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Table 1 Characteristics of the ICP study participants

Age, median (range), years	11.91 (3-18)
Sex, n (%)	
Female	35
Male	40
Clinical symptoms, n (%)	
Acute pancreatitis	61
Pure abdominal pain	13
Weight loss	3
Diarrhea	1
High blood glucose	1
Other	0
Imaging examination (CT, MRCP or ERCP)	
Pancreatic duct stenosis or dilation	57
Pancreatic duct stones	45
Pancreatic pseudocyst	15
Pancreatic calcification	15
Other	4*
Laboratory tests	
Increased CA199	6
Increased blood cholesterol	6
Increased blood glucose	2
Decreased blood calcium	2

* Pancreatic Divisum

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Table 2 *PRSS1, SPINK1, CFTR* and *CTR*C gene mutations in the ICP patients

Gene mutations	Region	Functional class	Positive, n (%)
PRSS1			
A16V	Exon 2	Missense	0
N29I	Exon 2	Missense	1 (1.3)
E79K	Exon 3	Missense	0
R116C	Exon 3	Missense	0
A121T	Exon 3	Missense	0
R122H or R122C	Exon 3	Missense	6 (8.0)*
SPINK1			
N34S	Exon 3	Missense	0
IVS3+2T>C	IVS 3	Splicing	43 (57.3)**
CFTR			
R117H	Exon 4	Missense	0***
F508del	Exon 11	Del	0
c.2562T>G	Exon 15	Nonsense	51 (68.0)****
c.4389G>A	Exon 27	Nonsense	1 (1.3)
CTRC			
c.143A>G	Exon 3	Missense	0
c.180C>T	Exon 3	Nonsense	0
c.217G>A	Exon 3	Missense	0

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c.703G>A	Exon 7	Missense	0
c.760C>T	Exon 7	Missense	0
p.K247_R254del	Exon 7	Del	0
CLDN2			
c.22G>A	Exon 1	Nonsense	2
c.327A>T	Exon 2	Nonsense	1
c.592A>C	Exon 2	Missense	1
c.768T>C	Exon 2	Nonsense	22

* one was c.364 C>T and other five were c.365 G>A

** 33 were heterozygous and 10 were homozygous

*** one patient has the deletions of GCTTCCTA from c.500 to c.508

**** 37 were heterozygous and 14 were homozygous

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Table 3 Association of gene mutation (IVS3+2T>C, M470V and c.2562T>G) with clinicopathological data from the 75 patients

Mutations		Age at diagnosis				Pancreatic duct stenosis or dilation				Pancreatic duct stones				Pancreatic pseudocyst				Pancreatic calcification			
		<12	≥12	X^2	*P* value	Yes	No	X^2	*P* value	Yes	No	X^2	*P* value	Yes	No	X^2	*P* value	Yes	No	X^2	*P* value
IVS3+2T>C	Yes	17	26	0.815	0.367	34	9	0.521	0.471	30	13	4.006	0.045	5	38	4.415	0.036	12	31	3.938	0.047
	No	16	16			23	9			15	17			10	22			3	29		
M470V	MM	18	23	0.199	0.905	32	9	0.305	0.859	25	16	0.773	0.679	9	32	1.566	0.457	11	30	2.664	0.264
	MV	5	5			7	3			7	3			3	7			1	9		
	VV	10	14			18	6			13	11			3	21			3	21		
c.2562T>G	Yes	23	28	0.078	0.780	39	12	0.019	0.899	32	19	0.500	0.479	12	39	1.241	0.265	12	39	1.241	0.265
	No	10	14			18	6			13	11			3	21			3	21		

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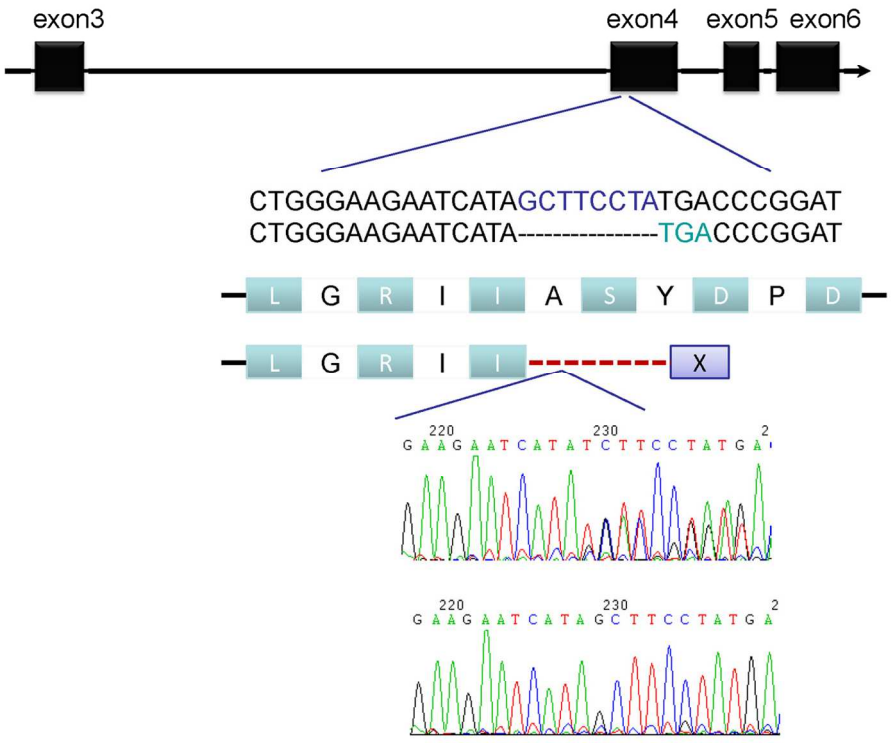

Figure 1. A Novel CFTR gene deletion detected using DNA sequence of PCR products.
129x106mm (300 x 300 DPI)

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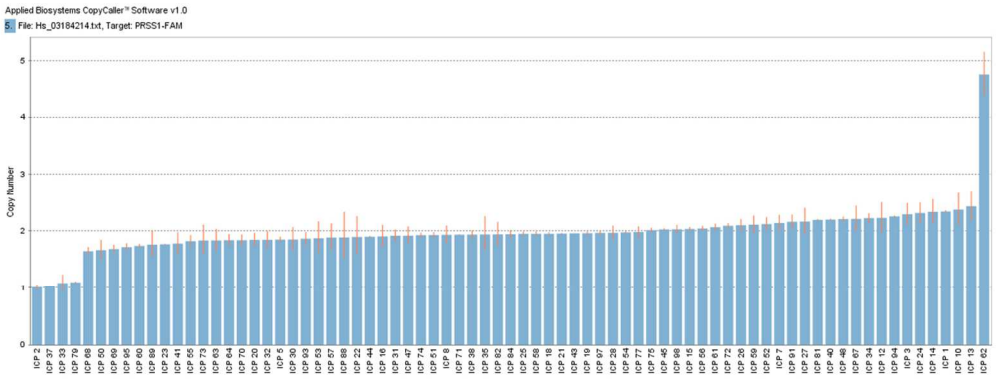

Figure 2. Copy number variations (Hs03184214_cn) of PRSS1 gene in 75 Chinese ICP patients.
119x45mm (300 x 300 DPI)

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Supplemental Table 1. PCP primers used to detect P*RSS1, SPINK1, CFTR, CTRC* and *CLDN2* gene mutations

Gene	Primers
PRSS1	
A16V、N29I	5’-cagagacttgggagccaca-3’
	5’-accacaacccttggtgtttc-3’
E79K, R116C, A121T, R122H	5’-accttcactgacccacatcc-3’
	5’-agccaagtccttgatagtttgc-3’
SPINK1	
N34S	5’-aaggttttctgtctccagatagtagg-3’
	5’-ccaagctatcgactattttgctg-3’
IVS3+2T>C	5’-agatgtggccaacctgagag-3’
	5’-gcttttctcggggtgagatt-3’
CFTR	
R117H	5’-aaacttgtctcccactgttgc-3’
	5’-caacagaggcagtttacagaaga-3’
1210TG/T	5’-ggccatgtgcttttcaaacta-3’
	5’-cgccaacaactgtcctcttt-3’

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M470V	5’-caagtgaatcctgagcgtga-3’
	5’-tgctttgatgacgcttctgt-3’
F508del	5’-cccttgtatcttttgtgcatagc-3’
	5’-gcttcttaaagcataggtcatgtg-3’
c.2562T>G	5’-acaatggtggcatgaaactg-3’
	5’-gccttctactttgagctttcg-3’
c.4389G>A	5’-cgacagggtgaagctctttc-3’
	5’-tctggcttgcaaaacacaag-3’
CTRC	
c.143A>G ,	5’-gtgtagggctgggaggtaca-3’
c.180C>T ,	
c.217G>A	
	5’-ttcccgagagcacagacttt-3’
c.703G>A ,	5’-cagttggagaacggttcctg-3’
c.760C>T ,	
c.738_761del24	
	5’-gtgcttgatgaaggcagtga-3’
CLDN2	
Exon 1	5’-ctgccaacacagtctcctca-3’
	5’-ggatttgttgcctagggtga-3’
Exon 2	5’-gtcagcctggcagagagact-3’
	5’-ctgtgtgtggcacattccat-3’

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5’-ttgtgacagcagttggcttc-3’
5’-caagaggttgggcttggtag-3’
5’-cctgggattcattcctgttg-3’
5’-tccagtggtagtgtccctca-3’

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Supplemental Table 2. Probe used to screen *PRSS-1* CNVs

Probe	Hs03184214_cn
Assay Location	Chr7:142460752 on NCBI build 37
Cytoband	7q34f
Species	H. sapiens
Variation Type	Copy Number

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Supplemental Table 3. TG-repeats and poly-T tract polymorphism in the junction of intron 8 (IVS-8) and exon 9 of *CFTR*

Total Number (n)	Number (frequencies) of individuals with genotypes			
	(TG)10-T7	(TG)11-T7	(TG)12-T5	(TG)12-T7
75 (100%)	2 (2.7%)	55 (73.3%)	5 (6.7%)	13 (17.3%)

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Supplemental Table 4. M470V polymorphism at exon 10 of *CFTR*

Total Number (n)	Number (frequencies) of individuals with genotypes			Number (frequencies) of individuals with alleles	
	MM	MV	VV	M	V
75(100%)	10(13.3%)	41(54.7%)	24(32%)	61(40.7%)	89(59.3%)

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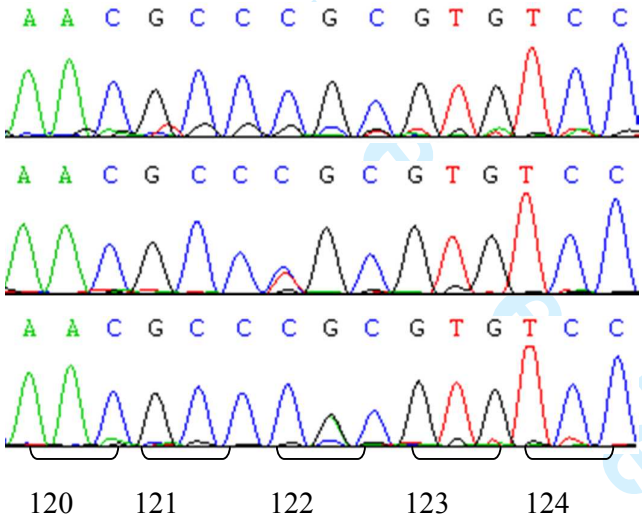
Supplemental Figure 1. Representative illustrations of mutations detected in 75 patients with idiopathic chronic pancreatitis.

A, Representatives of exon 3 in *PRSS1* gene in patients without mutation (top), patients with heterozygous R122C mutation (meddle) and patients with heterozygous p.R122H mutation (bottom). *R122C* and *R122H* mutations are reflected by C→T and G→A transitions, respectively, at codon 122 that result in an arginine to cysteine and histidine substitution at amino acid 122, respectively.



Arg: C G C

Cys: T G C

His: C A C

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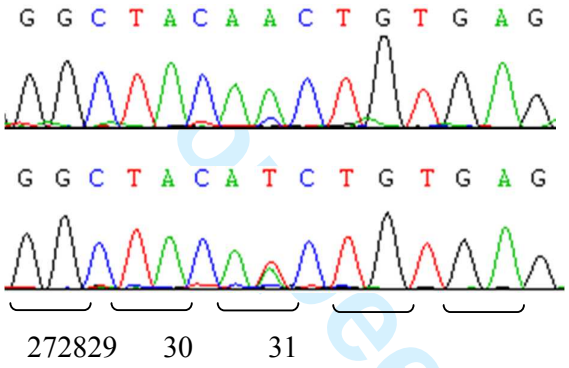
B, Representatives of *PRSS1* gene exon 2 in patients without mutation (top), patients with heterozygous N29I (bottom) mutations. N29I mutation is reflected by A→T transition at codon 29 that results in an asparagine to isoleucine substitution at amino acid 29.



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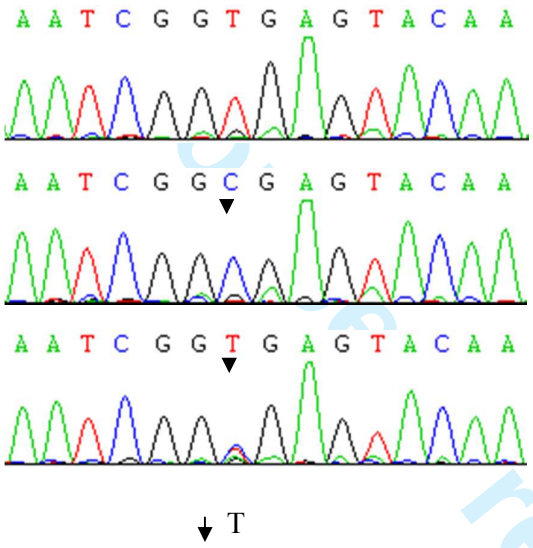
C, Representatives of the nucleotide sequences in *SPINK1* gene promoter region in patients without mutation (top), patients with homozygous IVS3+2T>C mutation (middle) and those with heterozygous IVS3+2T>C mutation (bottom). IVS3+2T>C mutation is reflected by C residue, instead of T, at nucleotide 2 downstream from the end point of exon 3 (right).



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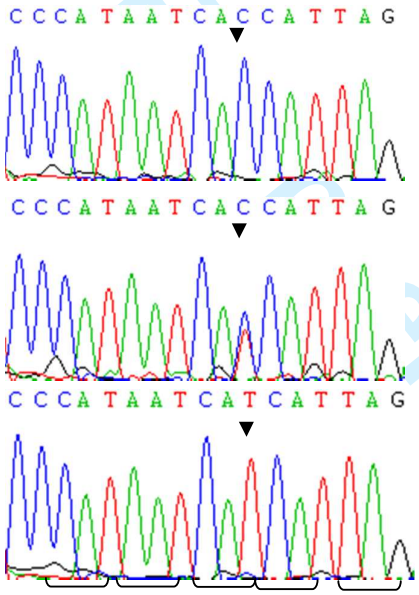
D, Representatives of *CFTR* gene exon 10 mutation in patients without mutation (top), patients with heterozygous M470V mutation (meddle) and patients with homozygousM470Vmutation (bottom). This mutation is reflected by G→A transition, at codon 470 that results in a valine to methionine substitution at amino acid 470 (an anti-sense sequence).



Val: GTG

↓

Met: ATG

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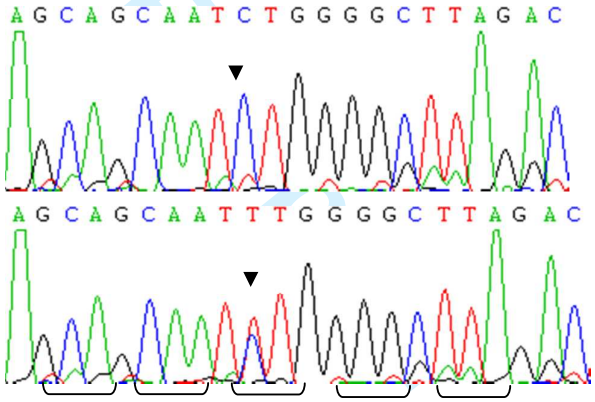
E, Representatives of *CFTR* gene exon 27 mutation in patients without mutation (top), patients with heterozygous c.4389G>A mutation (bottom). This mutation is reflected by G→A transition, at codon 1463 that is nonsense one (an anti-sense sequence).



Gln: CAG

↓

CAA

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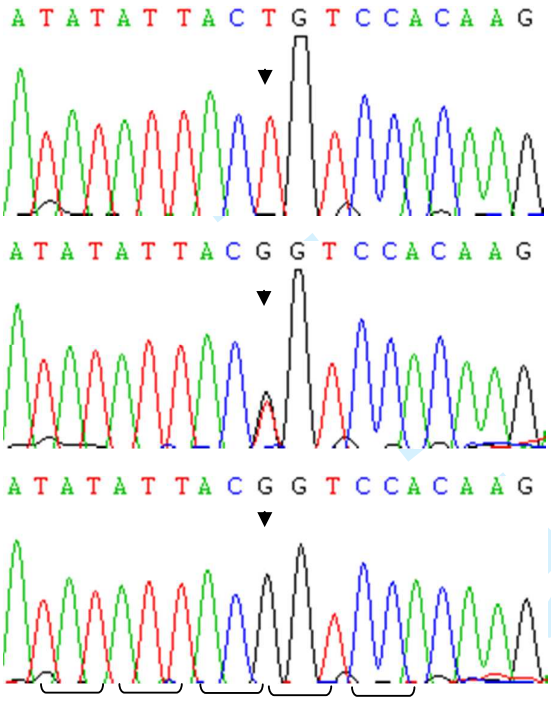
F, Representatives of *CFTR* gene exon 15 mutation in patients without mutation (top), patients with heterozygous c.2562T>G mutation (meddle) and patients with homozygousc.2562T>G mutation (bottom). This mutation is reflected by T→G transition, at codon 854 that is nonsense one.



Thr: ACT

↓

ACG

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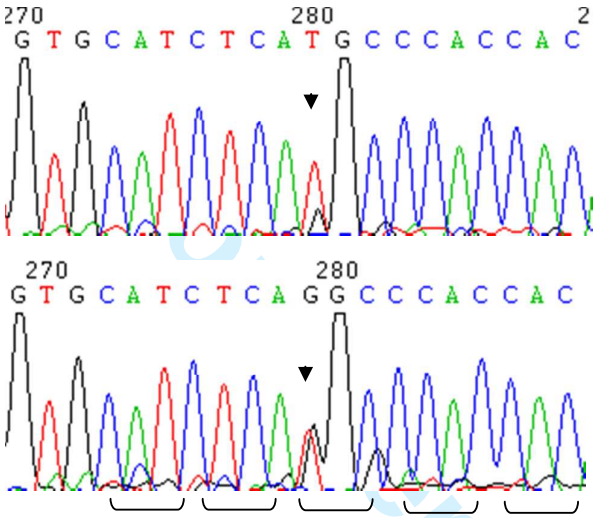
G, Representatives of *CLDN2* gene exon 2 mutation in patients without mutation (top), patients with heterozygous mutation (bottom). This mutation is reflected by A→C transition that is missense.



Met: ATG

↓

Leu: CTG

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Comprehensive Screening for PRSS1, SPINK1, CFTR, CTRC and CLDN2 Gene Mutations in Chinese Pediatric Patients with Idiopathic Chronic Pancreatitis

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Comprehensive Screening for *PRSS1*, *SPINK1*, *CFTR*, *CTRC* and *CLDN2* Gene Mutations in Chinese Pediatric Patients with Idiopathic Chronic Pancreatitis

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Abstract

Objective: Genetic alterations may contribute to chronic pancreatitis (CP) in Chinese young patients. This study was designed to investigate mutations of cationic trypsinogen (*PRSS1*), pancreatic secretory trypsin inhibitor or serine protease inhibitor Kazal type 1 (*SPINK1*), cystic fibrosis transmembrane conductance regulator *(CFTR)*, chymotrypsin C *(CTRC)*, and *CLDN2* genes and the copy number variations (CNVs) of *PRSS1* and assess associations with the development of idiopathic CP (ICP) in Chinese children.

Design： Retrospective.

Setting: A single center.

Participants: Seventy-five ICP Chinese children (40 boys and 35 girls).

Primary and secondary outcome measures: Mutations of *PRSS1, SPINK1, CFTR, CTRC* and *CLDN2* genes and CNVs.

Results: Seven patients had heterozygous mutations in *PRSS1*, i.e. N29I (n=1), R122H or R122C (n=6). The CNVs of PRSS1 in five patients had abnormal copies [1 copy (n=4), five copies (n=1)]. 43 patients had IVS3+2T>C (rs148954387) (10 homozygous and 33 heterozygous) in *SPINK1*. None of the *PRSS1* mutation patients carried a *SPINK1* mutation. Frequency of *PRSS1* and *SPINK1* mutations was 9.3% and 57.3%, respectively, with an overall frequency of 66.6% (50/75). In addition, one patient had a novel deletion of *CFTR* (GCTTCCTA from c.500 to c.508 leading to the shortened polypeptide molecule via a stop codon). Another patient had a novel missense in *CLDN2* exon 2 (c.592A>C mutation). Clinically, patients with *SPINK1* mutations had a higher rate of pancreatic duct stones, pancreatic pseudocyst, and pancreatic calcification than those without *SPINK1* mutations ($P<0.05$).

Conclusions: *SPINK1* mutations were more commonly associated with Chinese ICP children. *SPINK1* IVS3+2T>C mutation may play an important role in the pathogenesis of Chinese pediatric ICP. However, further study is needed to confirm and to investigate the role of these genes in the development of Chinese ICP.

Key words: idiopathic chronic pancreatitis, *PRSS1*, *SPINK1*, *CFTR, CTRC*, *CLDN2*, CNVs,

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children

Summary Box

Article focus

- Genetic alterations may contribute to chronic pancreatitis in Chinese young patients.
- China is different from the Western countries in regards to ethnic and cultural backgrounds, ocioeconomic status, climatic conditions, and dietary habits.

Key messages

- SPINK1 IVS3+2T>C mutation was more commonly associated with Chinese ICP children.
- Pediatric patients with SPINK1 IVS3+2T>C mutation had a higher rate of pancreatic duct stones, pancreatic pseudocyst, and pancreatic calcification than those without.
- SPINK1 IVS3+2T>C mutation may play an important role in the pathogenesis of Chinese pediatric ICP, which may be show some insights into a novel target for therapy.

Strengths and limitations

- To the best of our knowledge, this is the first study to determine the spectrum and frequency of PRSS1, SPINK1, CFTR, CTRC and CLDN2 gene mutations and PRSS1 CNVs in unrelated CP children in China.
- Further study is needed to confirm and to investigate the role of these genes in the development of Chinese ICP..

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Introduction

Chronic pancreatitis is an inflammatory disease characterized by irreversible destruction of the pancreatic normal structure and function and is associated with persistent abdominal pain or steatorrhea. In adults, alcohol abuse is an important cause of chronic pancreatitis, while other factors (such as anatomical changes, metabolic disease, trauma and heredity) may also cause or associate with chronic pancreatitis. However, up to 10-25% of patients with chronic pancreatitis have no clear risk factors and these patients are classified as having idiopathic chronic pancreatitis (ICP) [1]. In children, it is estimated that ICP accounts for approximately 40-60% of all children with chronic pancreatitis in Western countries, but the reported rate was as high as 73.8% in China [2]. The pathogenesis of ICP, especially in children, remains poorly understood. Since the conventional risk factors such as alcohol abuse are uncommon in children, it has reported that environmental risk factors may play a limited role in the pathogenesis of ICP in children; thus, patients with ICP at these ages are thought to be suitable for studies of genetic defects [3].

Over the last two decades, genetic factors have been shown in patients with chronic pancreatitis and these factors are believed to play an important role in the pathogenesis of chronic pancreatitis [4]. For example, previous studies reported identification of mutations in genes encoding cationic trypsinogen (UniGene name: protease serine 1: *PRSS1*) (OMIM 276000) [5], cystic fibrosis transmembrane conductance regulator (*CFTR*) (OMIM 602421) [6], the pancreatic secretory trypsin inhibitor or serine protease inhibitor Kazal type 1 (PSTI or *SPINK1*) (OMIM 167790) [3], and chymotrypsin C (*CTRC*) (OMIM 601405) [7], all of which reveal that hereditary pancreatitis is a more common form of chronic pancreatitis than once thought [1]. More recently, a newly detected candidate gene known as *CLDN2* has been shown to be associated with sporadic and alcohol-related chronic pancreatitis [8]. Thus, genetic study may reveal a genetic basis for significant percentage of patients with so-called “idiopathic” chronic pancreatitis. It becomes acceptable that development of chronic pancreatitis requires a combination of genetic predisposition and environmental, structural, or toxic insult [9].

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Therefore, identification of genetic and environmental risk factors could offer the potential tool for risk assessment, early diagnosis, and earlier intervention of chronic pancreatitis [10].

Many studies have discovered that mutations plus the copy number variations (CNVs) in *PRSS1* (trypsinogen), *SPINK1* (serine protease inhibitor Kazal-type 1), *CFTR* (cystic fibrosis transmembrane conductance regulator), and CTRC (chymotrypsin C, also known as caldecrin) were causally linked to the pathogenesis of ICP [11-13], while patients with *PRSS1* or *SPINK1* mutations may be at a higher risk of developing pancreatic cancer [14]. *CFTR* variants were associated with idiopathic and alcoholic chronic pancreatitis. Furthermore, *CLDN2* was shown to be strongly associated with chronic pancreatitis, suggesting that it probably acts as a disease modifier to accelerate the development and progression of chronic pancreatitis through a non-trypsin–dependent process since *CLDN2* is a highly regulated tight junction protein to form ion and water channels between endothelial cells [8]. However, as the largest populated country in the world, the incidence of chronic pancreatitis, including pediatric chronic pancreatitis, has risen rapidly [2, 15], whereas there have been only a few studies reporting that genetic factors contributed the pathogenesis of chronic pancreatitis in China, but none of these studies focused on ICP in children [13, 16]. In addition, China is different from the Western countries in regards to ethnic and cultural backgrounds, socioeconomic status, climatic conditions, and dietary habits. In this pilot study, we identified mutations in *PRSS1, SPINK1, CFTR, CTRC* and *CLDN2* genes and CNVs of *PRSS1* to determine the spectrum and frequency of the mutations and CNVs in unrelated children with ICP in the mainland of China. The data from this study will provide information into the genetic basis of pediatric ICP in China.

Patients and Methods

Study population and diagnosis criteria

We recruited 75 ICP patients under 18 years old from Changhai Hospital between January 1997 and December 2008. There was no history of tobacco smoking or alcohol

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consumption in these patients. All patients originated from the Han ethnicity in the mainland of China. The diagnose criteria of CP and ICP was defined as a condition characterized by typical history (abdominal pain, diabetes mellitus and/or steatorrhea) and the presence of any one of the following findings: i) ductal changes on ERCP; ii) pancreatic calcification on imaging examination; or iii) histological evidence of CP [17-18]. Furthermore, affected individuals were classified as having ICP if precipitating risk factors (such as alcohol abuse, trauma, previous medication, infection, metabolic disorders and/or a positive family history) were absent [19]. This study was approved by the Ethics Committee of Changhai Hospital, Shanghai and a written informed consent was given by their parents or legal guardians according to the ethical guidelines of the Declaration of Helsinki.

DNA isolation

Peripheral blood samples were collected from each patient in a ethylene diamine tetra acetic acid (EDTA)-anticoagulated tube and frozen at -20^{o}C for subsequent DNA extraction. Genomic DNA was isolated from 500 µl samples using the Lab-aid 800 automatic nucleic acid extraction machine following a standard protocol and quantified using a NanoDrop machine (Thermo, Wilmington, USA).

PCR analyses of gene mutations

DNA samples from patients were subjected to polymerase chain reaction (PCR) analyses of gene mutations. Specifically, primers flanking the targeted regions of *PRSS1, SPINK1, CFTR, CTRC* and *CLDN2* genes were designed and synthesized according to the nucleotide sequence published by NCBI as shown in Supplemental Table 1. The data on mutation analyses were confirmed by DNA sequence and repeated using PCR. PCR was performed in the GeneAmp 9700 System (Applied Biosystems, Foster city, CA) using a 15-µL reaction mixture containing 7.5 µl 2×Taq Mix (Vivantis, USA), 5.7 µl ddH_2O, 1.5 µl DNA templates (10 ng/µl), and 0.15 µl of each primer (20 mM). PCR amplification was set at an initial 6 min

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denaturation at 94°C, followed by 35 cycles of 30 s at 94°C, 30 s at 58°C (*PRSS1*, *SPINK1*) or 56℃ (CFTR, *CTRC, CLDN2*), 45 s at 72°C, and a final extension at 72°C for 7 min. PCR products were then incubated with 0.1 U shrimp alkaline phosphatase at 37°C for 1 h, followed by heat inactivation at 85°C for 20 min and then sequenced using an ABI Prism BigDye Terminator Cycle Sequencing Kit, version 3.1 on an ABI Prism 3730 sequencer (Applied Biosystems). Data passed a duplicate quality-control test using four samples and showed 100% concordance.

Vector NT1, Chromas and Bioedit software were applied to analyze the results.

Detection of gene copy number variations (CNVs)

A pre-designed and validated CNV assay kit to assess PRSS1 CNVs was obtained from AB Life Technologies (Hs03184214_cn) (see details in Supplemental Table 2). RT-PCR was performed using 2×Taqman Genotyping Master Mix 5 μl (Vivantis), ddH_2O 0.5 μl, DNA templates (10 ng/μl) 4 μl, 0.25 μl Taqman Copy Number Assay (Vivantis) and 0.25 Taqman Copy Number Assay (Vivantis) in a total volume of 10 μl. Cycle conditions were as follows: initial denaturation for 10 minutes at 95°C, followed by 40 cycles of (15 s at 95°C,and 60s at 60°C) in an 7900 RT-PCR thermal cycler (Applied Biosystems). The assays were performed in triplicate and repeated at least once for each sample. PRSS1 gene copy number was calculated compared to the proportion to RNAseP reference assay (AB Life Technologies cat#4403326). Data were analyzed using Copy Caller Software (version 1.0 from AB Life Technologies).

Statistical analyses

Continuous data were reported as mean ± standard deviation (SD) analyzed using a Student's t-test and/or u-test. Fisher's exact test or chi-square test was used to analyze the categorical data. The gene mutations or CNVs were associated with age at diagnosis,

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pancreatic calcification, changes in pancreatic duct (stenosis or dilatation), pancreatic calcification, and pancreatic pseudocyst for clinical significance of these mutations [19].

The age onset was divided into two subgroups according to the mean value. A *P* value of less than 0.05 was considered statistically significant and all reported *P* values were two-sided.

Results

Baseline clinical characteristics of study participants

A total of 75 unrelated children were included in this study, i.e., 40 boys and 35 girls and age at first onset was 11.91 ± 3.79 years (ranged between 3 and 18 years). Clinically, 81.3% (61/75) of the patients showed acute pancreatitis, 13 patients began with pure abdominal pain, 3 patients with weight loss, 1 patient with diarrhea, and 1 patient with high blood glucose.

Imaging examinations, including CT, MRCP or ERCP, detected pancreatic duct stones in 45 patients, changes in pancreatic duct (stenosis or dilatation) in 57 patients, pancreatic calcification in 15 patients, pancreatic pseudocyst in 15 patients, and pancreatic divisum in 4 patients (Table 1). Laboratory tests showed that six patients had increased levels of CA199, a biomarker for pancreatic cancer, six patients had high blood cholesterol, two had increased blood glucose, and two patients had low blood calcium levels.

Analyses of PRSS1, SPINK1, CFTR and CTRC gene mutations in these patients

We then analyzed mutations of *PRSS1, SPINK1, CFTR* and *CTRC* gene in these 75 patients and found three types of heterozygous *PRSS1* mutations in seven patients, including N29I (n=1) in exon 2, and R122H (n=6) with c.365G>A (n=5) and c.364C> T (n=1) in exon 3(Supplemental Figure 1). A single gene mutation of *SPINK1* occurred in 43 patients, including 10 homozygous and 33 heterozygous *SPINK1* mutation (IVS3+2T>C). However, there was no single patient with *PRSS1* mutation who had a *SPINK1* mutation, making 9.3%

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(7/75) and 57.3% (43/75) of, *PRSS1* and *SPINK1* mutation rates, respectively and an overall rate of 66.6% (50/75) of patients who at least had one *PRSS1* or *SPINK1* mutation (Table 2).

Furthermore, one patient had *CFTR* gene deletions of GCTTCCTA sequences between c.500 to c.508 at exon4, leading to an early stop codon (Figure 1). *CFTR* gene C.2562 T>G polymorphism was also detected in 51 patients, 14 of whom were homozygous. Heterozygous *CFTR* gene c.4389G>A mutation was identified in another patient. Moreover, there were four types of TG-repeats and poly-T tract including (TG)10-T7(n=2), (TG)11-T7(n=55), (TG)12-T5(n=5) and (TG)12-T7(n=13) in the junction of intron 8 and exon 9 and (TG)11-T7 of *CFTR* gene found in 73.3% of these patients (Supplemental Table 3). In addition, *CFTR* gene V allele was slightly more frequent than the M allele at codon 470 (59.3% and 40.7%, respectively). The dominant genotype was M/V followed by V/V and M/M (Supplemental Table 4).

However, we screened six types of CTRC mutations, including c.143A>G, c.217G>A, c.180C>T in exon 3, c.703G>A, c.760C>T, and p.K247_R254del in exon 7 in these 75 patients, but did not find any mutations. In addition, we screened both exons of *CLDN2* and found four types of heterozygous mutations in a total of 26 patients (Table 2), i.e., c.22G>A at exon 1, c.327A>T, c.592A>C and c.768T>C at exon 2. C.592A>C is a missense mutation, while the other three types are nonsense. However, none of these patients had more than one type of *CLDN2* mutation.

Analyses of PRSS1 gene copy number variations in these patients

PRSS1 gene copy numbers were normal in most patients. Specifically, *PRSS1* gene copy number in 70 patients had two copies detected using the probe Hs03184214_cn, whereas four patients had only one copy and another patient had five copies (Figure 2).

Association of mutations with clinicopathological data

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We associated these genetic alterations with clinicopathological data from the 75 patients. Our data showed that three mutations of IVS3+2T>C in *SPINK1* gene, M470V and c.2562 T>G in *CFTR* gene had relatively higher frequencies than other genetic alterations and were associated with clinicopathological data. Briefly, the rates of pancreatic duct stones, pancreatic pseudocyst and pancreatic calcification were higher in patients with a *SPINK1* gene IVS3+2T>C mutation than that of patients without IVS3+2T>C (69.8% vs., 46.9% *P*=0.045; 11.6% vs. 31.25% *P*=0.036; 27.9% vs. 9.4% *P*=0.047, respectively). The rate of pancreatic pseudocyst was lower in patients with the *SPINK1* gene IVS3+2T>C mutation than that of patients without IVS3+2T>C (11.6% vs. 31.25% *P*=0.036) (Table 3). However, there was no statistical significance between age at diagnosis of patients with and without IVS3+2T>C mutation. M470V and c.2562 T>G were not significantly associated with these clinical characteristics.

Discussion

In the present study, we revealed *PRSS1, SPINK1, CFTR, CTRC* and *CLDN2* gene mutations in ICP patients, especially *PRSS1* and *SPINK1* gene mutations, occurred in almost 70% of Chinese ICP children. To the best of our knowledge, this is the first study to determine the spectrum and frequency of *PRSS1, SPINK1, CFTR, CTRC* and *CLDN2* gene mutations and *PRSS1* CNVs in unrelated CP children in China. The data indicate that genetic changes occurring in Chinese ICP patients could associate with ICP development. Further study will investigate how these gene alterations contribute to ICP development.

Our current data on the frequency of *PRSS1* mutations were similar to those (9.3% *vs.* 9-23%) of a previous study on children with CP or ICP [20], however, our data on the frequency of *SPINK1* mutations (57.3%) appeared higher than those (19-40%) reported in the previous study [3]. Moreover, Witt et al. [20] first showed *PRSS1* gene mutations (3/30 cases at 3×A16V) in German pediatric CP patients. Thereafter, they showed that in a study of 96 unrelated CP children, *PRSS1* gene mutations (5×A16V, 1×N29I, and 5×R122H) occurred in 11 (11.5%)

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patients [3]. In a review of 164 unrelated children with CP, the frequency was reported to be 9.1% (n=15, 8×A16V, 5×R122H, and 2×N29I) [21]. *PRSS1* gene mutations were detected in two (12.5%, 1×R122H and 1×A16V) of 16 patients classified as having early-onset ICP in a Swiss study [22] and in 11 (23.1%) of 52 children with CP (6×R122H, 4×R122C, 1×N29I) in a Polish study [23]. In our current study, *PRSS1* mutations were found in 9.3% of 75 Chinese pediatric ICP patients, which included N29I and R122H mutations. Heterozygous mutations of the *PRSS1* gene commonly occurred in CP patients in the Western populations [20,23] and was the only form of mutation in our patients, indicating that the main spectrum and frequency of *PRSS1* gene mutations in Chinese ICP children are similar to those reported in pediatric patients in Western countries [20-23]. The *PRSS1* mutations seem to be one of the predisposing factors for ICP, irrespective of race, although the third most common *PRSS1* mutation (i.e., A16V) is not found in our study. However, these spectrum and frequency are different from most previous Asian studies, in which *PRSS1* mutations were at a low frequency or even absent [13, 24-25]. In a previous study on 129 Chinese ICP patients (34 early-onset and 95 late-onset using a cut-off age of 35 years), Chang et al. [13] showed *PRSS1* mutations in six (4.6%) patients; in two (5.9%) patient with early-onset (1×R116C and 1×C139S) and four (4.2%) patients with late-onset (1×L104P, 1×R116C, 1×T137M and 1×C139S). These mutations are all considered relatively uncommon mutations in Western countries [26]. The potential reason for this discrepancy may be due to the sampling bias, i.e., Chang's study included both children and adults, whereas our current study only included children.

One of the most significant findings in our current study was the high frequency of *SPINK1* IVS3+2T>C mutation, which was first reported by Kume K et al. They showed a *SPINK1* IVS3+2T>C mutation in 13-16% of unrelated Japanese ICP patients [27]. However, two additional studies from Western countries reported IVS 3+2T>C mutation only in one (1.0%) of 96 and in 3 (2.7%) of 112 pediatric ICP patients [3, 12]. the IVS 3+2T>C mutation has been found in three (1.7%) of 172 German CP patients, but was thought to be a rare polymorphism and not a mutation [28]. However, a recent Chinese study on 129 ICP patients

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revealed a IVS 3+2T>C mutation in 8.5% of patients and the mutation was predominantly responsible for early-onset ICP (29.4% in early-onset vs. 1.1% in late-onset) [13]. Alternatively, Pfutzer et al. [12] showed a frequency of N34S mutation in *SPINK1* in 40.4% (23/57) of American ICP children, whereas Truninger et al. [11] reported a frequency of the mutation at 43% (6/14) German patients with early-onset ICP. In patients with tropical calcific pancreatitis, which is an idiopathic, juvenile, non-alcoholic form of CP widely prevalent in several tropical countries such as India, N34S mutation can reach 46%. In the present study, IVS 3+2T>C mutations were found in 57.3% (43/75) of unrelated Chinese ICP children, but we did not find any N34S mutations in the current study. These data suggest that the spectrum and frequency of *SPINK1* mutations vary geographically among different populations; IVS 3+2T>C mutations are more common in Chinese ICP children, whereas N34S mutations are more frequent in Western populations. The underlying role and molecular mechanisms of the IVS3+2T>C mutation in CP development are being explored. For example, this mutation was in complete linkage disequilibrium with -215G>A mutation, which might alter the efficiency of the *SPINK1* gene transcription. IVS3+2T>C mutation affects the splicing donor site that is highly conserved in eukaryotes [3]. IVS3+2T>C mutations can cause skipping of the whole of exon 3, where the trypsin binding site is located, leading to the loss of the trypsin binding site [27], altered expression of *SPINK1* protein in CP patients with the IVS3+2T>C mutation, affecting the protease/antiprotease balance within the pancreas. However, further studies are necessary to elucidate the underlying molecular mechanisms.

In addition, the second significant finding of our current study is *CFTR* gene polymorphisms, such as M470V (n=51), c.2562T>G (n=51), TG repeats, and poly T tract in Chinese ICP children. We found that 68% (51/75) patients had both c.2562 T>G and M470V polymorphisms, including heterozygous and homozygous alleles and one patient with heterozygous c.4389G>A mutation. Both c.2562T>G and c.4389G>A mutations are nonsense, while an obstructive tubulopathy of the pancreas due to the *CFTR* dysfunction is thought to play a primary role in CP development, although the exact pathogenic process of pancreatitis

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associated with *CFTR* mutations is still under investigation. The function of *CFTR* in the pancreas is to dilute and alkalinize the protein-rich acinar secretions, so that the formation of protein plugs that lead to pancreatic injury may be prevented. A M470V polymorphism on exon 10 affects the intrinsic chloride activity, and thereby affects CFTR protein function. The TG repeats and poly-T tract can influence *CFTR* at transcription levels because these intronic variants could lead to reductions in protein synthesis and expression, or altered splicing to compromise the intracellular transport and/or activity. Huang et al conducted the first comprehensive study on the functional polymorphisms of CFTR in Chinese healthy subjects and found that T7 was the most common haplotype (93.6%) and (TG)11 and (TG)12 were the dominant haplotypes in the junction of intron 8 and exon 9 [29]. Our current data also validated (TG)11-T7 as the most common type. The poly-T, TG-repeats and M470V distributions were similar to those studies on other East Asians [30-31]. In addition, a diverse range of *CFTR* loss-of-function variants have been reported to be associated with ICP and alcoholic CP, whereas their functional effects remain to be defined. Recently, Whitcomb et al reported that the coinheritance of *CFTR* R75Q and *SPINK1* variants is significantly higher in patients with ICP than in controls (8.75% vs. 0.38%). Using patch-clamp techniques, they also found that the *CFTR* genotype caused a selective defect in biocarbonate conductance [32]. Another study from Australia showed that symptomatic pancreatitis occurs in 20% of pancreatic sufficient cystic fibrosis patients. To evaluate genotype–phenotype interactions, they developed the Pancreatic Insufficiency Prevalence (PIP) score to determine severity in a large number of *CFTR* mutations and found that specific *CFTR* genotypes were associated with pancreatitis, i.e., patients carrying genotypes with mild phenotypic effects may have a greater risk of developing pancreatitis than patients carrying genotypes with moderate-severe phenotypic consequences at any given time [33]. In addition, common *CFTR* haplotypes seem to modulate susceptibility to CP.

Although multiple rare *CTRC* gene mutations have been associated with CP in European and Asian populations, our current study did not find any *CTRC* mutations in Chinese ICP children, indicating that *CTRC* mutation varies geographically or ethnically. According to the

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biochemical activities and the functional properties of *CTRC* variants, Zhou and Sahin-Tóth hypothesized three mutually nonexclusive models to demonstrate the possible role of *CTRC* variants in predisposing to CP: i). Impaired trypsinogen and/or trypsin degradation; ii). Impaired activation of A-type carboxylpeptidases; and iii). Induction of endoplasmic reticulum stress [34]. We infer that *CTRC* might play a limited role, if any, in the pathogenesis of CP in China.

However, until 2012, genetic variation in *CLDN2* has not previously been associated with disease in humans. A two-stage (discovery and replication) genome-wide association study (GWAS) showed that *CLDN2* genotype confers the greatest risk for CP, and its alleles via interacting with alcohol consumption, can amplify the risk. These data could partially explain the higher frequency of alcohol-related pancreatitis in men than women while the real causal relationship between CP and *CLDN2* has been ambiguously defined. Our current data are the first study on *CLDN2* SNPs in Chinese ICP patients to report four novel SNPs. Only one of these is a missense mutation known as c.592A>C in CDS of *CLDN2* gene, making the amino acid change from Met to Leu. This patient was a girl who was diagnosed as ICP after several episodes of acute pancreatitis since she was 13 years old. All of these clinical characteristics showed no deviances from other patients. Claudin-2 encoded by CLDN2 is normally expressed at low levels in the tight junction between cells of the pancreatic ducts and in pancreatic islets. But when stressed, acinar cells can also express claudin-2, proved by porcine models of acute pancreatitis [35]. Besides, the *CLDN2* promoter includes a nuclear factor (NF)-κB-binding site [36], and *CLDN2* expression is enhanced in other cells under conditions associated with injury and stress.

In an attempt to associate these mutations with clinical parameters from these ICP patients, we did not observe any association between the gene mutations and an earlier age of CP diagnosis, which is contrary to a previous study [37]. However, showed that patients with IVS3+2T>C mutation were more likely to have pancreatic duct stones or pancreatic calcification than those without such a mutation. It has been well known that patients with pancreatic calcification are more severe than those without pancreatic calcification.

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Therefore, our observations, along with previous findings [27] suggest that a IVS3+2T>C mutation in *SPINK1* predisposes to more severity of CP. Moreover, CP patients have a markedly increased risk in developing pancreatic cancer compared to the general population [14, 15] and *PRSS1* or *SPINK1* mutation may be a predictor for pancreatic cancer development in CP patients. The IVS3+2T>C mutation was present in 0.6% of the sporadic pancreatic cancer patients. Thus, CP patients with *PRSS1* or *SPINK1* mutation should avoid any risk factors, including alcohol and tobacco, be monitored for any signs or symptoms (pain, weight loss, jaundice, and/or abdominal mass) or with serum markers and imaging examination for pancreatic cancer.

CNVs often occur in human cancers and the compositions of CNVs may contain deletion, amplification, deletion plus amplification, multiple alleles and complicated locus. Lafrate and Sebat were the first to respectively report CNVs in human genome in 2004 [38]. To date, many studies proved that both CNVs and SNP can affect gene expressions. However, the effect of PRSS1 gene CNVs on ICP has not been fully studied. In our study, the 4 patients with 1 copy were found not to be complicated with any mutations screened above. So, reduced CNV can also be detected in patients with ICP, contradicting the hypothesis that reduced CNV may be a protect factor, which needs further studies.

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Contributorship statement

Prof. Zhuan Liao and Zhao-Shen Li conceived the project. Dr. Xiao-Tian Sun, Wei Wang, Xiao-Ling Weng, and Dai-Zhan Zhou completed the DNA isolation, PCR analyses of gene mutations, and detection of gene copy number variations (CNVs). Dr. Chang Sun, Tian Xia, Liang-Hao Hu, Xiao-Wei Lai, Bo Ye, Mu-Yun Liu, Fei Jiang, and Jun Gao collected the

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peripheral blood samples and clinical data. Lu-Min Bo and Yun Liu completed the statistical analyses.

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Table 1 Characteristics of the ICP study participants

Age, median (range), years	11.91 (3-18)
Sex, n (%)	
Female	35
Male	40
Clinical symptoms, n (%)	
Acute pancreatitis	61
Pure abdominal pain	13
Weight loss	3
Diarrhea	1
High blood glucose	1
Other	0
Imaging examination (CT, MRCP or ERCP)	
Pancreatic duct stenosis or dilation	57
Pancreatic duct stones	45
Pancreatic pseudocyst	15
Pancreatic calcification	15
Other	4*
Laboratory tests	
Increased CA199	6
Increased blood cholesterol	6
Increased blood glucose	2
Decreased blood calcium	2

* Pancreatic Divisum

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Table 2 *PRSS1, SPINK1, CFTR* and *CTR*C gene mutations in the ICP patients

Gene mutations	Region	Functional class	Positive, n (%)
PRSS1			
A16V	Exon 2	Missense	0
N29I	Exon 2	Missense	1 (1.3)
E79K	Exon 3	Missense	0
R116C	Exon 3	Missense	0
A121T	Exon 3	Missense	0
R122H or R122C	Exon 3	Missense	6 (8.0)*
SPINK1			
N34S	Exon 3	Missense	0
IVS3+2T>C	IVS 3	Splicing	43 (57.3)**
CFTR			
R117H	Exon 4	Missense	0***
F508del	Exon 11	Del	0
c.2562T>G	Exon 15	Nonsense	51 (68.0)****
c.4389G>A	Exon 27	Nonsense	1 (1.3)
CTRC			
c.143A>G	Exon 3	Missense	0
c.180C>T	Exon 3	Nonsense	0
c.217G>A	Exon 3	Missense	0

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c.703G>A	Exon 7	Missense	0
c.760C>T	Exon 7	Missense	0
p.K247_R254del	Exon 7	Del	0
CLDN2			
c.22G>A	Exon 1	Nonsense	2
c.327A>T	Exon 2	Nonsense	1
c.592A>C	Exon 2	Missense	1
c.768T>C	Exon 2	Nonsense	22

* one was c.364 C>T and other five were c.365 G>A

** 33 were heterozygous and 10 were homozygous

*** one patient has the deletions of GCTTCCTA from c.500 to c.508

**** 37 were heterozygous and 14 were homozygous

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Table 3 Association of gene mutation (IVS3+2T>C, M470V and c.2562T>G) with clinicopathological data from the 75 patients

Mutations		Age at diagnosis				Pancreatic duct stenosis or dilation				Pancreatic duct stones				Pancreatic pseudocyst				Pancreatic calcification			
		<12	≥12	X^2	*P* value	Yes	No	X^2	*P* value	Yes	No	X^2	*P* value	Yes	No	X^2	*P* value	Yes	No	X^2	*P* value
IVS3+2T>C	Yes	17	26	0.815	0.367	34	9	0.521	0.471	30	13	4.006	0.045	5	38	4.415	0.036	12	31	3.938	0.047
	No	16	16			23	9			15	17			10	22			3	29		
M470V	MM	18	23	0.199	0.905	32	9	0.305	0.859	25	16	0.773	0.679	9	32	1.566	0.457	11	30	2.664	0.264
	MV	5	5			7	3			7	3			3	7			1	9		
	VV	10	14			18	6			13	11			3	21			3	21		
c.2562T>G	Yes	23	28	0.078	0.780	39	12	0.019	0.899	32	19	0.500	0.479	12	39	1.241	0.265	12	39	1.241	0.265
	No	10	14			18	6			13	11			3	21			3	21		

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Comprehensive Screening for *PRSS1*, *SPINK1*, *CFTR*, *CTRC* and *CLDN2* Gene Mutations in Chinese Pediatric Patients with Idiopathic Chronic Pancreatitis

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Competing interest: There is no conflict of interest to declare.

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Abstract

Objective: Genetic alterations may contribute to chronic pancreatitis (CP) in Chinese young patients. This study was designed to investigate mutations of cationic trypsinogen (*PRSS1*), pancreatic secretory trypsin inhibitor or serine protease inhibitor Kazal type 1 (*SPINK1*), cystic fibrosis transmembrane conductance regulator *(CFTR)*, chymotrypsin C *(CTRC)*, and *CLDN2* genes and the copy number variations (CNVs) of *PRSS1* and assess associations with the development of idiopathic CP (ICP) in Chinese children.

Design： Retrospective.

Setting: A single center.

~~**Methods**~~**Participants:** Seventy-five ICP Chinese children (40 boys and 35 girls)~~, were recruited for the assessment of *PRSS1, SPINK1, CFTR, CTRC* and *CLDN2* gene mutations and CNVs using DNA sequencing and TaqMan® Probe Based Gene Expression Analysis, respectively.~~

Primary and secondary outcome measures: Mutations of *PRSS1, SPINK1, CFTR, CTRC* and *CLDN2* genes and CNVs.

Results: Seven patients had heterozygous mutations in *PRSS1*, i.e. N29I (n=1), R122H or R122C (n=6). The CNVs of PRSS1 in five patients had ~~unmoral~~ abnormal copies [1 copy (n=4), five copies (n=1)]. 43 patients had IVS3+2T>C (rs148954387) (10 homozygous and 33 heterozygous) in *SPINK1*. None of the *PRSS1* mutation patients carried a *SPINK1* mutation. Frequency of *PRSS1* and *SPINK1* mutations was 9.3% and 57.3%, respectively, with an overall frequency of 66.6% (50/75). In addition, one patient had a novel deletion of *CFTR* (GCTTCCTA from c.500 to c.508 leading to the shortened polypeptide molecule via a stop codon). Another patient had a novel missense in *CLDN2* exon 2 (c.592A>C mutation). Clinically, patients with *SPINK1* mutations had a higher rate of pancreatic duct stones, pancreatic pseudocyst, and pancreatic calcification than those without *SPINK1* mutations (P<0.05).

Conclusions: *SPINK1* mutations were more commonly associated with Chinese ICP children. *SPINK1* IVS3+2T>C mutation may play an important role in the pathogenesis of Chinese

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pediatric ICP. However, further study is needed to confirm and to investigate the role of these genes in the development of Chinese ICP.

Key words: idiopathic chronic pancreatitis, *PRSS1*, *SPINK1*, *CFTR, CTRC*, *CLDN2*, CNVs, children

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Summary Box

Article focus

- ~~What is already known about this subject?~~

- Genetic alterations may contribute to chronic pancreatitis in Chinese young patients.
- China is different from the Western countries in regards to ethnic and cultural backgrounds, ocioeconomic status, climatic conditions, and dietary habits.

Key messages~~**What are the new findings?**~~

- SPINK1 IVS3+2T>C mutation was more commonly associated with Chinese ICP children.
- Pediatric patients with SPINK1 IVS3+2T>C mutation had a higher rate of pancreatic duct stones, pancreatic pseudocyst, and pancreatic calcification than those without.

~~**How might it impact on clinical practice in the foreseeable future?**~~

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- SPINK1 IVS3+2T>C mutation may play an important role in the pathogenesis of Chinese pediatric ICP, which may be show some insights into a novel target for therapy.

Strengths and limitations

- To the best of our knowledge, this is the first study to determine the spectrum and frequency of PRSS1, SPINK1, CFTR, CTRC and CLDN2 gene mutations and PRSS1 CNVs in unrelated CP children in China.
- Further study is needed to confirm and to investigate the role of these genes in the development of Chinese ICP..

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Introduction

Chronic pancreatitis is an inflammatory disease characterized by irreversible destruction of the pancreatic normal structure and function and is associated with persistent abdominal pain or steatorrhea. In adults, alcohol abuse is an important cause of chronic pancreatitis, while other factors (such as anatomical changes, metabolic disease, trauma and heredity) may also cause or associate with chronic pancreatitis. However, up to 10-25% of patients with chronic pancreatitis have no clear risk factors and these patients are classified as having idiopathic chronic pancreatitis (ICP) [1]. In children, it is estimated that ICP accounts for approximately 40-60% of all children with chronic pancreatitis in Western countries, but the reported rate was as high as 73.8% in China [2]. The pathogenesis of ICP, especially in children, remains poorly understood. Since the conventional risk factors such as alcohol abuse are uncommon in children, it has reported that environmental risk factors may play a limited role in the pathogenesis of ICP in children; thus, patients with ICP at these ages are thought to be suitable for studies of genetic defects [3].

Over the last two decades, genetic factors have been shown in patients with chronic pancreatitis and these factors are believed to play an important role in the pathogenesis of chronic pancreatitis [4]. For example, previous studies reported identification of mutations in genes encoding cationic trypsinogen (UniGene name: protease serine 1: *PRSS1*) (OMIM 276000) [5], cystic fibrosis transmembrane conductance regulator (*CFTR*) (OMIM 602421) [6], the pancreatic secretory trypsin inhibitor or serine protease inhibitor Kazal type 1 (PSTI or *SPINK1*) (OMIM 167790) [3], and chymotrypsin C (*CTRC*) (OMIM 601405) [7], all of which reveal that hereditary pancreatitis is a more common form of chronic pancreatitis than once thought [1]. More recently, a newly detected candidate gene known as *CLDN2* has been shown to be associated with sporadic and alcohol-related chronic pancreatitis [8]. Thus, genetic study may reveal a genetic basis for significant percentage of patients with so-called “idiopathic” chronic pancreatitis. It becomes acceptable that development of chronic pancreatitis requires a combination of genetic predisposition and environmental, structural, or toxic insult [9].

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Therefore, identification of genetic and environmental risk factors could offer the potential tool for risk assessment, early diagnosis, and earlier intervention of chronic pancreatitis [10].

Many studies have discovered that mutations plus the copy number variations (CNVs) in *PRSS1* (trypsinogen), *SPINK1* (serine protease inhibitor Kazal-type 1), *CFTR* (cystic fibrosis transmembrane conductance regulator), and CTRC (chymotrypsin C, also known as caldecrin) were causally linked to the pathogenesis of ICP [11-13], while patients with *PRSS1* or *SPINK1* mutations may be at a higher risk of developing pancreatic cancer [14]. *CFTR* variants were associated with idiopathic and alcoholic chronic pancreatitis. Furthermore, *CLDN2* was shown to be strongly associated with chronic pancreatitis, suggesting that it probably acts as a disease modifier to accelerate the development and progression of chronic pancreatitis through a non-trypsin–dependent process since *CLDN2* is a highly regulated tight junction protein to form ion and water channels between endothelial cells [8]. However, as the largest populated country in the world, the incidence of chronic pancreatitis, including pediatric chronic pancreatitis, has risen rapidly [2, 15], whereas there have been only a few studies reporting that genetic factors contributed the pathogenesis of chronic pancreatitis in China, but none of these studies focused on ICP in children [13, 16]. In addition, China is different from the Western countries in regards to ethnic and cultural backgrounds, socioeconomic status, climatic conditions, and dietary habits. In this pilot study, we identified mutations in *PRSS1, SPINK1, CFTR, CTRC* and *CLDN2* genes and CNVs of *PRSS1* to determine the spectrum and frequency of the mutations and CNVs in unrelated children with ICP in the mainland of China. The data from this study will provide information into the genetic basis of pediatric ICP in China.

Patients and Methods

Study population and diagnosis criteria

We recruited 75 ICP patients under 18 years old from Changhai Hospital between January 1997 and December 2008. There was no history of tobacco smoking or alcohol

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consumption in these patients. All patients originated from the Han ethnicity in the mainland of China. The diagnose criteria of CP and ICP was defined as a condition characterized by typical history (abdominal pain, diabetes mellitus and/or steatorrhea) and the presence of any one of the following findings: i) ductal changes on ERCP; ii) pancreatic calcification on imaging examination; or iii) histological evidence of CP [17-18]. Furthermore, affected individuals were classified as having ICP if precipitating risk factors (such as alcohol abuse, trauma, previous medication, infection, metabolic disorders and/or a positive family history) were absent [19]. This study was approved by the Ethics Committee of Changhai Hospital, Shanghai and a written informed consent was given by their parents or legal guardians according to the ethical guidelines of the Declaration of Helsinki.

DNA isolation

Peripheral blood samples were collected from each patient in a ethylene diamine tetra acetic acid (EDTA)-anticoagulated tube and frozen at -20^{o}C for subsequent DNA extraction. Genomic DNA was isolated from 500 µl samples using the Lab-aid 800 automatic nucleic acid extraction machine following a standard protocol and quantified using a NanoDrop machine (Thermo, Wilmington, USA).

PCR analyses of gene mutations

DNA samples from patients were subjected to polymerase chain reaction (PCR) analyses of gene mutations. Specifically, primers flanking the targeted regions of *PRSS1, SPINK1, CFTR, CTRC* and *CLDN2* genes were designed and synthesized according to the nucleotide sequence published by NCBI as shown in Supplemental Table 1. The data on mutation analyses were confirmed by DNA sequence and repeated using PCR. PCR was performed in the GeneAmp 9700 System (Applied Biosystems, Foster city, CA) using a 15-µL reaction mixture containing 7.5 µl 2×Taq Mix (Vivantis, USA), 5.7 µl ddH_2O, 1.5 µl DNA templates (10 ng/µl), and 0.15 µl of each primer (20 mM). PCR amplification was set at an initial 6 min

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denaturation at 94°C, followed by 35 cycles of 30 s at 94°C, 30 s at 58°C (*PRSS1*, *SPINK1*) or 56℃ (CFTR, *CTRC, CLDN2*), 45 s at 72°C, and a final extension at 72°C for 7 min. PCR products were then incubated with 0.1 U shrimp alkaline phosphatase at 37°C for 1 h, followed by heat inactivation at 85°C for 20 min and then sequenced using an ABI Prism BigDye Terminator Cycle Sequencing Kit, version 3.1 on an ABI Prism 3730 sequencer (Applied Biosystems). Data passed a duplicate quality-control test using four samples and showed 100% concordance.

Vector NT1, Chromas and Bioedit software were applied to analyze the results.

Detection of gene copy number variations (CNVs)

A pre-designed and validated CNV assay kit to assess PRSS1 CNVs was obtained from AB Life Technologies (Hs03184214_cn) (see details in Supplemental Table 2). RT-PCR was performed using 2×Taqman Genotyping Master Mix 5 μl (Vivantis), ddH_2O 0.5 μl, DNA templates (10 ng/μl) 4 μl, 0.25 μl Taqman Copy Number Assay (Vivantis) and 0.25 Taqman Copy Number Assay (Vivantis) in a total volume of 10 μl. Cycle conditions were as follows: initial denaturation for 10 minutes at 95°C, followed by 40 cycles of (15 s at 95°C,and 60s at 60°C) in an 7900 RT-PCR thermal cycler (Applied Biosystems). The assays were performed in triplicate and repeated at least once for each sample. PRSS1 gene copy number was calculated compared to the proportion to RNAseP reference assay (AB Life Technologies cat#4403326). Data were analyzed using Copy Caller Software (version 1.0 from AB Life Technologies).

Statistical analyses

Continuous data were reported as mean ± standard deviation (SD) analyzed using a Student's t-test and/or u-test. Fisher's exact test or chi-square test was used to analyze the categorical data. The gene mutations or CNVs were associated with age at diagnosis,

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pancreatic calcification, changes in pancreatic duct (stenosis or dilatation), pancreatic calcification, and pancreatic pseudocyst for clinical significance of these mutations [19].

The age onset was divided into two subgroups according to the mean value. A *P* value of less than 0.05 was considered statistically significant and all reported *P* values were two-sided.

Results

Baseline clinical characteristics of study participants

A total of 75 unrelated children were included in this study, i.e., 40 boys and 35 girls and age at first onset was 11.91 ± 3.79 years (ranged between 3 and 18 years). Clinically, 81.3% (61/75) of the patients showed acute pancreatitis, 13 patients began with pure abdominal pain, 3 patients with weight loss, 1 patient with diarrhea, and 1 patient with high blood glucose.

Imaging examinations, including CT, MRCP or ERCP, detected pancreatic duct stones in 45 patients, changes in pancreatic duct (stenosis or dilatation) in 57 patients, pancreatic calcification in 15 patients, pancreatic pseudocyst in 15 patients, and pancreatic divisum in 4 patients (Table 1). Laboratory tests showed that six patients had increased levels of CA199, a biomarker for pancreatic cancer, six patients had high blood cholesterol, two had increased blood glucose, and two patients had low blood calcium levels.

Analyses of PRSS1, SPINK1, CFTR and CTRC gene mutations in these patients

We then analyzed mutations of *PRSS1, SPINK1, CFTR* and *CTRC* gene in these 75 patients and found three types of heterozygous *PRSS1* mutations in seven patients, including N29I (n=1) in exon 2, and R122H (n=6) with c.365G>A (n=5) and c.364C> T (n=1) in exon 3(Supplemental Figure 1). A single gene mutation of *SPINK1* occurred in 43 patients, including 10 homozygous and 33 heterozygous *SPINK1* mutation (IVS3+2T>C). However, there was no single patient with *PRSS1* mutation who had a *SPINK1* mutation, making 9.3%

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(7/75) and 57.3% (43/75) of, *PRSS1* and *SPINK1* mutation rates, respectively and an overall rate of 66.6% (50/75) of patients who at least had one *PRSS1* or *SPINK1* mutation (Table 2).

Furthermore, one patient had *CFTR* gene deletions of GCTTCCTA sequences between c.500 to c.508 at exon4, leading to an early stop codon (Figure 1). *CFTR* gene C.2562 T>G polymorphism was also detected in 51 patients, 14 of whom were homozygous. Heterozygous *CFTR* gene c.4389G>A mutation was identified in another patient. Moreover, there were four types of TG-repeats and poly-T tract including (TG)10-T7(n=2), (TG)11-T7(n=55), (TG)12-T5(n=5) and (TG)12-T7(n=13) in the junction of intron 8 and exon 9 and (TG)11-T7 of *CFTR* gene found in 73.3% of these patients (Supplemental Table 3). In addition, *CFTR* gene V allele was slightly more frequent than the M allele at codon 470 (59.3% and 40.7%, respectively). The dominant genotype was M/V followed by V/V and M/M (Supplemental Table 4).

However, we screened six types of CTRC mutations, including c.143A>G, c.217G>A, c.180C>T in exon 3, c.703G>A, c.760C>T, and p.K247_R254del in exon 7 in these 75 patients, but did not find any mutations. In addition, we screened both exons of *CLDN2* and found four types of heterozygous mutations in a total of 26 patients (Table 2), i.e., c.22G>A at exon 1, c.327A>T, c.592A>C and c.768T>C at exon 2. C.592A>C is a missense mutation, while the other three types are nonsense. However, none of these patients had more than one type of *CLDN2* mutation.

Analyses of PRSS1 gene copy number variations in these patients

PRSS1 gene copy numbers were normal in most patients. Specifically, *PRSS1* gene copy number in 70 patients had two copies detected using the probe Hs03184214_cn, whereas four patients had only one copy and another patient had five copies (Figure 2).

Association of mutations with clinicopathological data

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We associated these genetic alterations with clinicopathological data from the 75 patients. Our data showed that three mutations of IVS3+2T>C in *SPINK1* gene, M470V and c.2562 T>G in *CFTR* gene had relatively higher frequencies than other genetic alterations and were associated with clinicopathological data. Briefly, the rates of pancreatic duct stones, pancreatic pseudocyst and pancreatic calcification were ~~significantly~~ higher in patients with a *SPINK1* gene IVS3+2T>C mutation than that of patients without IVS3+2T>C (69.8% vs., 46.9% *P*=0.045; 11.6% vs. 31.25% *P*=0.036; 27.9% vs. 9.4% *P*=0.047, respectively). The rate of pancreatic pseudocyst was ~~significantly~~ lower in patients with the *SPINK1* gene IVS3+2T>C mutation than that of patients without IVS3+2T>C (11.6% vs. 31.25% *P*=0.036) (Table 3). However, there was no statistical significance between age at diagnosis of patients with and without IVS3+2T>C mutation. M470V and c.2562 T>G were not significantly associated with these clinical characteristics.

Discussion

In the present study, we revealed *PRSS1, SPINK1, CFTR, CTRC* and *CLDN2* gene mutations in ICP patients, especially *PRSS1* and *SPINK1* gene mutations, occurred in almost 70% of Chinese ICP children. To the best of our knowledge, this is the first study to determine the spectrum and frequency of *PRSS1, SPINK1, CFTR, CTRC* and *CLDN2* gene mutations and *PRSS1* CNVs in unrelated CP children in China. The data indicate that genetic changes occurring in Chinese ICP patients could associate with ICP development. Further study will investigate how these gene alterations contribute to ICP development.

Our current data on the frequency of *PRSS1* mutations were similar to those (9.3% *vs.* 9-23%) of a previous study on children with CP or ICP [20], however, our data on the frequency of *SPINK1* mutations (57.3%) appeared higher than those (19-40%) reported in the previous study [3]. Moreover, Witt et al. [20] first showed *PRSS1* gene mutations (3/30 cases at 3×A16V) in German pediatric CP patients. Thereafter, they showed that in a study of 96 unrelated CP children, *PRSS1* gene mutations (5×A16V, 1×N29I, and 5×R122H) occurred in 11 (11.5%)

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patients [3]. In a review of 164 unrelated children with CP, the frequency was reported to be 9.1% (n=15, 8×A16V, 5×R122H, and 2×N29I) [21]. *PRSS1* gene mutations were detected in two (12.5%, 1×R122H and 1×A16V) of 16 patients classified as having early-onset ICP in a Swiss study [22] and in 11 (23.1%) of 52 children with CP (6×R122H, 4×R122C, 1×N29I) in a Polish study [23]. In our current study, *PRSS1* mutations were found in 9.3% of 75 Chinese pediatric ICP patients, which included N29I and R122H mutations. Heterozygous mutations of the *PRSS1* gene commonly occurred in CP patients in the Western populations [20,23] and was the only form of mutation in our patients, indicating that the main spectrum and frequency of *PRSS1* gene mutations in Chinese ICP children are similar to those reported in pediatric patients in Western countries [20-23]. The *PRSS1* mutations seem to be one of the predisposing factors for ICP, irrespective of race, although the third most common *PRSS1* mutation (i.e., A16V) is not found in our study. However, these spectrum and frequency are different from most previous Asian studies, in which *PRSS1* mutations were at a low frequency or even absent [13, 24-25]. In a previous study on 129 Chinese ICP patients (34 early-onset and 95 late-onset using a cut-off age of 35 years), Chang et al. [13] showed *PRSS1* mutations in six (4.6%) patients; in two (5.9%) patient with early-onset (1×R116C and 1×C139S) and four (4.2%) patients with late-onset (1×L104P, 1×R116C, 1×T137M and 1×C139S). These mutations are all considered relatively uncommon mutations in Western countries [26]. The potential reason for this discrepancy may be due to the sampling bias, i.e., Chang's study included both children and adults, whereas our current study only included children.

One of the most significant findings in our current study was the high frequency of *SPINK1* IVS3+2T>C mutation, which was first reported by Kume K et al. They showed a *SPINK1* IVS3+2T>C mutation in 13-16% of unrelated Japanese ICP patients [27]. However, two additional studies from Western countries reported IVS 3+2T>C mutation only in one (1.0%) of 96 and in 3 (2.7%) of 112 pediatric ICP patients [3, 12]. the IVS 3+2T>C mutation has been found in three (1.7%) of 172 German CP patients, but was thought to be a rare polymorphism and not a mutation [28]. However, a recent Chinese study on 129 ICP patients

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revealed a IVS 3+2T>C mutation in 8.5% of patients and the mutation was predominantly responsible for early-onset ICP (29.4% in early-onset vs. 1.1% in late-onset) [13]. Alternatively, Pfutzer et al. [12] showed a frequency of N34S mutation in *SPINK1* in 40.4% (23/57) of American ICP children, whereas Truninger et al. [11] reported a frequency of the mutation at 43% (6/14) German patients with early-onset ICP. In patients with tropical calcific pancreatitis, which is an idiopathic, juvenile, non-alcoholic form of CP widely prevalent in several tropical countries such as India, N34S mutation can reach 46%. In the present study, IVS 3+2T>C mutations were found in 57.3% (43/75) of unrelated Chinese ICP children, but we did not find any N34S mutations in the current study. These data suggest that the spectrum and frequency of *SPINK1* mutations vary geographically among different populations; IVS 3+2T>C mutations are more common in Chinese ICP children, whereas N34S mutations are more frequent in Western populations. The underlying role and molecular mechanisms of the IVS3+2T>C mutation in CP development are being explored. For example, this mutation was in complete linkage disequilibrium with -215G>A mutation, which might alter the efficiency of the *SPINK1* gene transcription. IVS3+2T>C mutation affects the splicing donor site that is highly conserved in eukaryotes [3]. IVS3+2T>C mutations can cause skipping of the whole of exon 3, where the trypsin binding site is located, leading to the loss of the trypsin binding site [27], altered expression of *SPINK1* protein in CP patients with the IVS3+2T>C mutation, affecting the protease/antiprotease balance within the pancreas. However, further studies are necessary to elucidate the underlying molecular mechanisms.

In addition, the second significant finding of our current study is *CFTR* gene polymorphisms, such as M470V (n=51), c.2562T>G (n=51), TG repeats, and poly T tract in Chinese ICP children. We found that 68% (51/75) patients had both c.2562 T>G and M470V polymorphisms, including heterozygous and homozygous alleles and one patient with heterozygous c.4389G>A mutation. Both c.2562T>G and c.4389G>A mutations are nonsense, while an obstructive tubulopathy of the pancreas due to the *CFTR* dysfunction is thought to play a primary role in CP development, although the exact pathogenic process of pancreatitis

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associated with *CFTR* mutations is still under investigation. The function of *CFTR* in the pancreas is to dilute and alkalinize the protein-rich acinar secretions, so that the formation of protein plugs that lead to pancreatic injury may be prevented. A M470V polymorphism on exon 10 affects the intrinsic chloride activity, and thereby affects CFTR protein function. The TG repeats and poly-T tract can influence *CFTR* at transcription levels because these intronic variants could lead to reductions in protein synthesis and expression, or altered splicing to compromise the intracellular transport and/or activity. Huang et al conducted the first comprehensive study on the functional polymorphisms of CFTR in Chinese healthy subjects and found that T7 was the most common haplotype (93.6%) and (TG)11 and (TG)12 were the dominant haplotypes in the junction of intron 8 and exon 9 [29]. Our current data also validated (TG)11-T7 as the most common type. The poly-T, TG-repeats and M470V distributions were similar to those studies on other East Asians [30-31]. In addition, a diverse range of *CFTR* loss-of-function variants have been reported to be associated with ICP and alcoholic CP, whereas their functional effects remain to be defined. Recently, Whitcomb et al reported that the coinheritance of *CFTR* R75Q and *SPINK1* variants is significantly higher in patients with ICP than in controls (8.75% vs. 0.38%). Using patch-clamp techniques, they also found that the *CFTR* genotype caused a selective defect in biocarbonate conductance [32]. Another study from Australia showed that symptomatic pancreatitis occurs in 20% of pancreatic sufficient cystic fibrosis patients. To evaluate genotype–phenotype interactions, they developed the Pancreatic Insufficiency Prevalence (PIP) score to determine severity in a large number of *CFTR* mutations and found that specific *CFTR* genotypes were associated with pancreatitis, i.e., patients carrying genotypes with mild phenotypic effects may have a greater risk of developing pancreatitis than patients carrying genotypes with moderate-severe phenotypic consequences at any given time [33]. In addition, common *CFTR* haplotypes seem to modulate susceptibility to CP.

Although multiple rare *CTRC* gene mutations have been associated with CP in European and Asian populations, our current study did not find any *CTRC* mutations in Chinese ICP children, indicating that *CTRC* mutation varies geographically or ethnically. According to the

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biochemical activities and the functional properties of *CTRC* variants, Zhou and Sahin-Tóth hypothesized three mutually nonexclusive models to demonstrate the possible role of *CTRC* variants in predisposing to CP: i). Impaired trypsinogen and/or trypsin degradation; ii). Impaired activation of A-type carboxylpeptidases; and iii). Induction of endoplasmic reticulum stress [34]. We infer that *CTRC* might play a limited role, if any, in the pathogenesis of CP in China.

However, until 2012, genetic variation in *CLDN2* has not previously been associated with disease in humans. A two-stage (discovery and replication) genome-wide association study (GWAS) showed that *CLDN2* genotype confers the greatest risk for CP, and its alleles via interacting with alcohol consumption, can amplify the risk. These data could partially explain the higher frequency of alcohol-related pancreatitis in men than women while the real causal relationship between CP and *CLDN2* has been ambiguously defined. Our current data are the first study on *CLDN2* SNPs in Chinese ICP patients to report four novel SNPs. Only one of these is a missense mutation known as c.592A>C in CDS of *CLDN2* gene, making the amino acid change from Met to Leu. This patient was a girl who was diagnosed as ICP after several episodes of acute pancreatitis since she was 13 years old. All of these clinical characteristics showed no deviances from other patients. Claudin-2 encoded by CLDN2 is normally expressed at low levels in the tight junction between cells of the pancreatic ducts and in pancreatic islets. But when stressed, acinar cells can also express claudin-2, proved by porcine models of acute pancreatitis [35]. Besides, the *CLDN2* promoter includes a nuclear factor (NF)-κB-binding site [36], and *CLDN2* expression is enhanced in other cells under conditions associated with injury and stress.

In an attempt to associate these mutations with clinical parameters from these ICP patients, we did not observe any association between the gene mutations and an earlier age of CP diagnosis, which is contrary to a previous study [37]. However, showed that patients with IVS3+2T>C mutation were more likely to have pancreatic duct stones or pancreatic calcification than those without such a mutation. It has been well known that patients with pancreatic calcification are more severe than those without pancreatic calcification.

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Therefore, our observations, along with previous findings [27] suggest that a IVS3+2T>C mutation in *SPINK1* predisposes to more severity of CP. Moreover, CP patients have a markedly increased risk in developing pancreatic cancer compared to the general population [14, 15] and *PRSS1* or *SPINK1* mutation may be a predictor for pancreatic cancer development in CP patients. The IVS3+2T>C mutation was present in 0.6% of the sporadic pancreatic cancer patients. Thus, CP patients with *PRSS1* or *SPINK1* mutation should avoid any risk factors, including alcohol and tobacco, be monitored for any signs or symptoms (pain, weight loss, jaundice, and/or abdominal mass) or with serum markers and imaging examination for pancreatic cancer.

CNVs often occur in human cancers and the compositions of CNVs may contain deletion, amplification, deletion plus amplification, multiple alleles and complicated locus. Lafrate and Sebat were the first to respectively report CNVs in human genome in 2004 [38]. To date, many studies proved that both CNVs and SNP can affect gene expressions. However, the effect of PRSS1 gene CNVs on ICP has not been fully studied. In our study, the 4 patients with 1 copy were found not to be complicated with any mutations screened above. So, reduced CNV can also be detected in patients with ICP, contradicting the hypothesis that reduced CNV may be a protect factor, which needs further studies.

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Contributorship statement

Prof. Zhuan Liao and Zhao-Shen Li conceived the project. Dr. Xiao-Tian Sun, Wei Wang, Xiao-Ling Weng, and Dai-Zhan Zhou completed the DNA isolation, PCR analyses of gene mutations, and detection of gene copy number variations (CNVs). Dr. Chang Sun, Tian Xia, Liang-Hao Hu, Xiao-Wei Lai, Bo Ye, Mu-Yun Liu, Fei Jiang, and Jun Gao collected the

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peripheral blood samples and clinical data. Lu-Min Bo and Yun Liu completed the statistical analyses.

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Table 1 Characteristics of the ICP study participants

Age, median (range), years	11.91 (3-18)
Sex, n (%)	
Female	35
Male	40
Clinical symptoms, n (%)	
Acute pancreatitis	61
Pure abdominal pain	13
Weight loss	3
Diarrhea	1
High blood glucose	1
Other	0
Imaging examination (CT, MRCP or ERCP)	
Pancreatic duct stenosis or dilation	57
Pancreatic duct stones	45
Pancreatic pseudocyst	15
Pancreatic calcification	15
Other	4*
Laboratory tests	
Increased CA199	6
Increased blood cholesterol	6
Increased blood glucose	2
Decreased blood calcium	2

* Pancreatic Divisum

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Table 2 *PRSS1, SPINK1, CFTR* and *CTR*C gene mutations in the ICP patients

Gene mutations	Region	Functional class	Positive, n (%)
PRSS1			
A16V	Exon 2	Missense	0
N29I	Exon 2	Missense	1 (1.3)
E79K	Exon 3	Missense	0
R116C	Exon 3	Missense	0
A121T	Exon 3	Missense	0
R122H or R122C	Exon 3	Missense	6 (8.0)*
SPINK1			
N34S	Exon 3	Missense	0
IVS3+2T>C	IVS 3	Splicing	43 (57.3)**
CFTR			
R117H	Exon 4	Missense	0***
F508del	Exon 11	Del	0
c.2562T>G	Exon 15	Nonsense	51 (68.0)****
c.4389G>A	Exon 27	Nonsense	1 (1.3)
CTRC			
c.143A>G	Exon 3	Missense	0
c.180C>T	Exon 3	Nonsense	0
c.217G>A	Exon 3	Missense	0

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c.703G>A	Exon 7	Missense	0
c.760C>T	Exon 7	Missense	0
p.K247_R254del	Exon 7	Del	0
CLDN2			
c.22G>A	Exon 1	Nonsense	2
c.327A>T	Exon 2	Nonsense	1
c.592A>C	Exon 2	Missense	1
c.768T>C	Exon 2	Nonsense	22

* one was c.364 C>T and other five were c.365 G>A

** 33 were heterozygous and 10 were homozygous

*** one patient has the deletions of GCTTCCTA from c.500 to c.508

**** 37 were heterozygous and 14 were homozygous

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Table 3 Association of gene mutation (IVS3+2T>C, M470V and c.2562T>G) with clinicopathological data from the 75 patients

Mutations		Age at diagnosis				Pancreatic duct stenosis or dilation				Pancreatic duct stones				Pancreatic pseudocyst				Pancreatic calcification			
		<12	≥12	X^2	*P* value	Yes	No	X^2	*P* value	Yes	No	X^2	*P* value	Yes	No	X^2	*P* value	Yes	No	X^2	*P* value
IVS3+2T>C	Yes	17	26	0.815	0.367	34	9	0.521	0.471	30	13	4.006	0.045	5	38	4.415	0.036	12	31	3.938	0.047
	No	16	16			23	9			15	17			10	22			3	29		
M470V	MM	18	23	0.199	0.905	32	9	0.305	0.859	25	16	0.773	0.679	9	32	1.566	0.457	11	30	2.664	0.264
	MV	5	5			7	3			7	3			3	7			1	9		
	VV	10	14			18	6			13	11			3	21			3	21		
c.2562T>G	Yes	23	28	0.078	0.780	39	12	0.019	0.899	32	19	0.500	0.479	12	39	1.241	0.265	12	39	1.241	0.265
	No	10	14			18	6			13	11			3	21			3	21		

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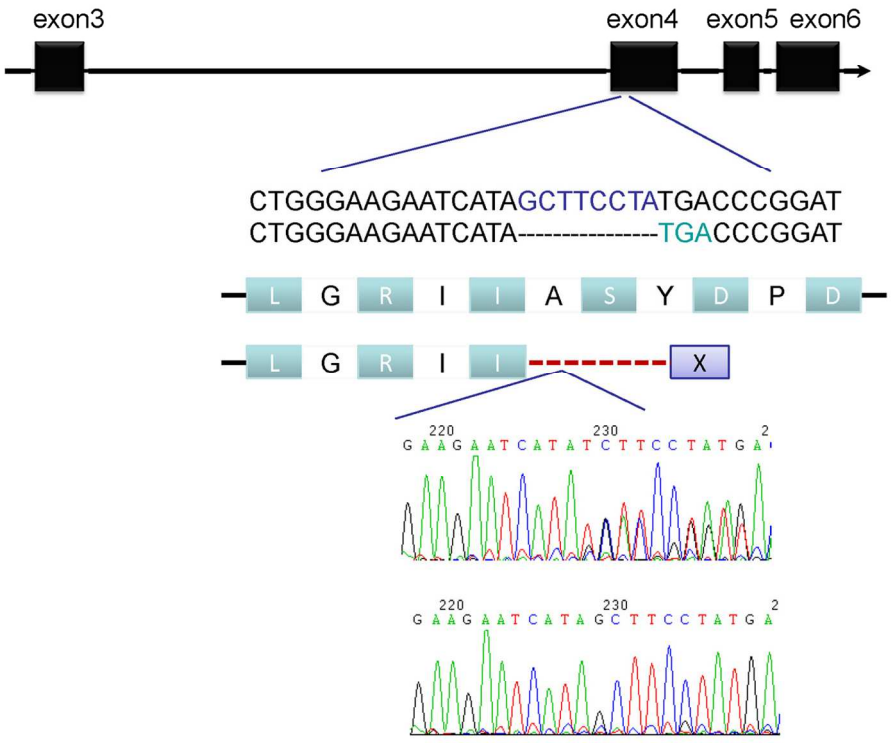

Figure 1. A Novel CFTR gene deletion detected using DNA sequence of PCR products.
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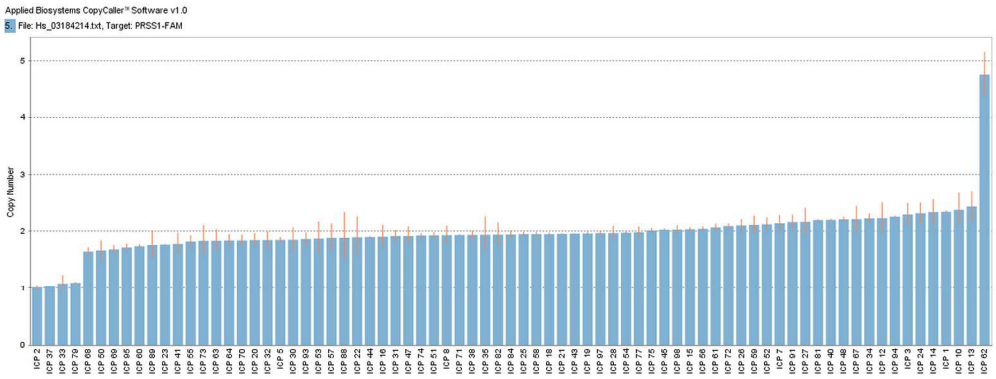

Figure 2. Copy number variations (Hs03184214_cn) of PRSS1 gene in 75 Chinese ICP patients.
319x119mm (300 x 300 DPI)

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Supplemental Table 1. PCP primers used to detect P*RSS1, SPINK1, CFTR, CTRC* and *CLDN2* gene mutations

Gene		Primers
PRSS1		
A16V、N29I	Forward	5’-cagagacttgggagccaca-3’
	Reverse	5’-accacaacccttggtgtttc-3’
E79K,R116C, A121T, R122H	Forward	5’-accttcactgacccacatcc-3’
	Reverse	5’-agccaagtccttgatagtttgc-3’
SPINK1		
N34S	Forward	5’-aaggttttctgtctccagatagtagg-3’
	Reverse	5’-ccaagctatcgactattttgctg-3’
IVS3+2T>C	Forward	5’-agatgtggccaacctgagag-3’
	Reverse	5’-gcttttctcggggtgagatt-3’
CFTR		
R117H	Forward	5’-aaacttgtctcccactgttgc-3’
	Reverse	5’-caacagaggcagtttacagaaga-3’
1210TG/T	Forward	5’-ggccatgtgcttttcaaacta-3’
	Reverse	5’-cgccaacaactgtcctcttt-3’
M470V	Forward	5’-caagtgaatcctgagcgtga-3’
	Reverse	5’-tgctttgatgacgcttctgt-3’

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F508del	Forward	5’-cccttgtatcttttgtgcatagc-3’
	Reverse	5’-gcttcttaaagcataggtcatgtg-3’
c.2562T>G	Forward	5’-acaatggtggcatgaaactg-3’
	Reverse	5’-gccttctactttgagctttcg-3’
c.4389G>A	Forward	5’-cgacagggtgaagctctttc-3’
	Reverse	5’-tctggcttgcaaaacacaag-3’
CTRC		
c.143A>G,c.180C>T，c.217G>A	Forward	5’-gtgtagggctgggaggtaca-3’
	Reverse	5’-ttcccgagagcacagacttt-3’
c.703G>A,c.760C>T, c.738_761del24	Forward	5’-cagttggagaacggttcctg-3’
	Reverse	5’-gtgcttgatgaaggcagtga-3’
CLDN2		
Exon 1	Forward	5’-ctgccaacacagtctcctca-3’
	Reverse	5’-ggatttgttgcctagggtga-3’
Exon 2*	Forward	5’-gtcagcctggcagagagact-3’
	Reverse	5’-ctgtgtgtggcacattccat-3’
	Forward	5’-ttgtgacagcagttggcttc-3’
	Reverse	5’-caagaggttgggcttggtag-3’
	Forward	5’-cctgggattcattcctgttg-3’
	Reverse	5’-tccagtggtagtgtccctca-3’

* Exon 2 was divided into 3 segments due to the limitation on the number of bases of sequencing.

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Supplemental Table 2. Probe used to screen *PRSS-1* CNVs

Probe	Hs03184214_cn
Assay Location	Chr7:142460752 on NCBI build 37
Cytoband	7q34f
Species	H. sapiens
Variation Type	Copy Number

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Supplemental Table 3. TG-repeats and poly-T tract polymorphism in the junction of intron 8 (IVS-8) and exon 9 of *CFTR*

Total Number (n)	Number (frequencies) of individuals with genotypes			
	(TG)10-T7	(TG)11-T7	(TG)12-T5	(TG)12-T7
75 (100%)	2 (2.7%)	55 (73.3%)	5 (6.7%)	13 (17.3%)

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Supplemental Table 4. M470V polymorphism at exon 10 of *CFTR*

Total Number (n)	Number (frequencies) of individuals with genotypes			Number (frequencies) of individuals with alleles	
	MM	MV	VV	M	V
75(100%)	10(13.3%)	41(54.7%)	24(32%)	61(40.7%)	89(59.3%)

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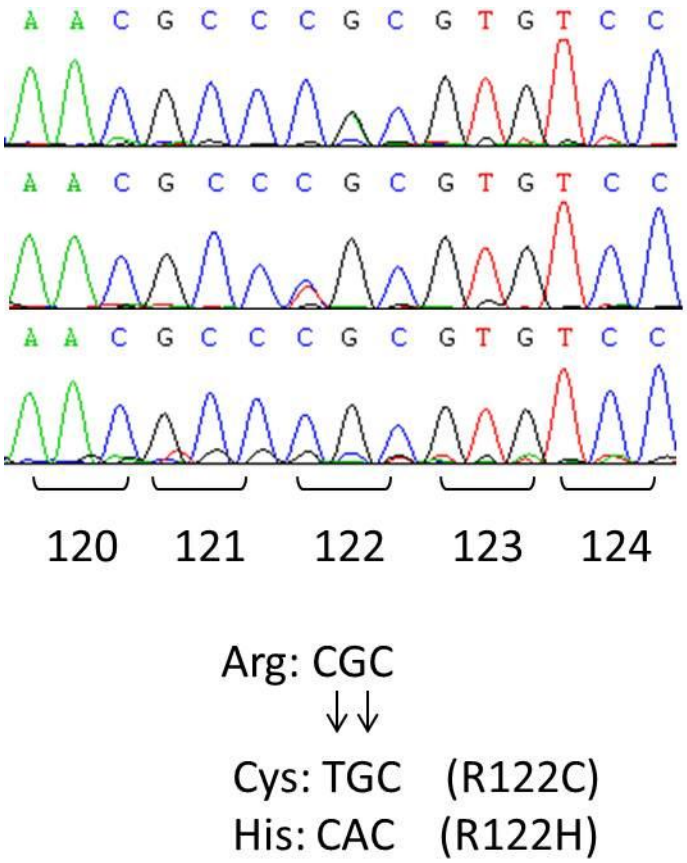
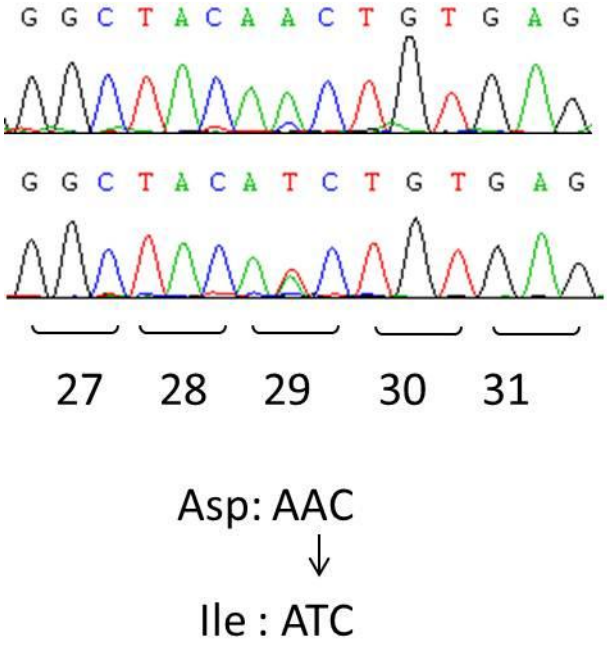
Supplemental Figure 1. Representative illustrations of mutations detected in 75 patients with idiopathic chronic pancreatitis.

A, Representatives of exon 3 in *PRSS1* gene in patients without mutation (top), patients with heterozygous R122C mutation (meddle) and patients with heterozygous p.R122H mutation (bottom). *R122C* and *R122H* mutations are reflected by C→T and G→A transitions, respectively, at codon 122 that result in an arginine to cysteine and histidine substitution at amino acid 122, respectively.



B, Representatives of *PRSS1* gene exon 2 in patients without mutation (top), patients with heterozygous N29I (bottom) mutations. N29I mutation is reflected by A→T transition at codon 29 that results in an asparagine to isoleucine substitution at amino acid 29.



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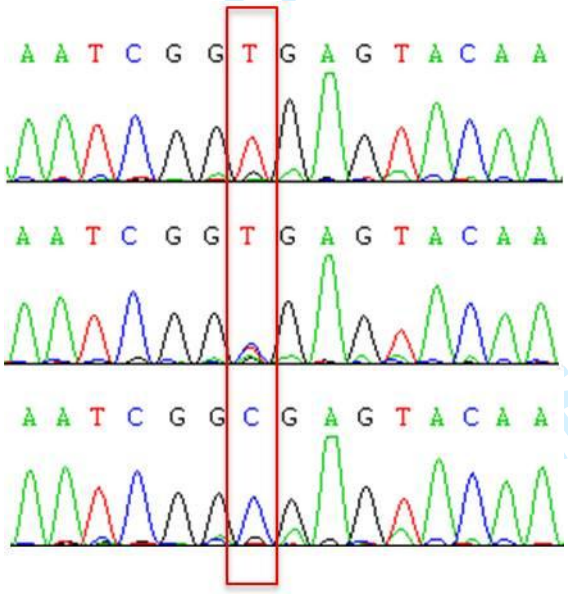
C, Representatives of the nucleotide sequences in *SPINK1* gene promoter region in patients without mutation (top), patients with homozygous IVS3+2T>C mutation (middle) and those with heterozygous IVS3+2T>C mutation (bottom). IVS3+2T>C mutation is reflected by C residue, instead of T, at nucleotide 2 downstream from the end point of exon 3 (right).



D, Representatives of *CFTR* gene exon 10 mutation in patients without mutation (top), patients with heterozygous M470V mutation (meddle) and patients with homozygousM470Vmutation (bottom). This mutation is reflected by G→A transition, at codon 470 that results in a valine to methionine substitution at amino acid 470 (anti-sense sequence).

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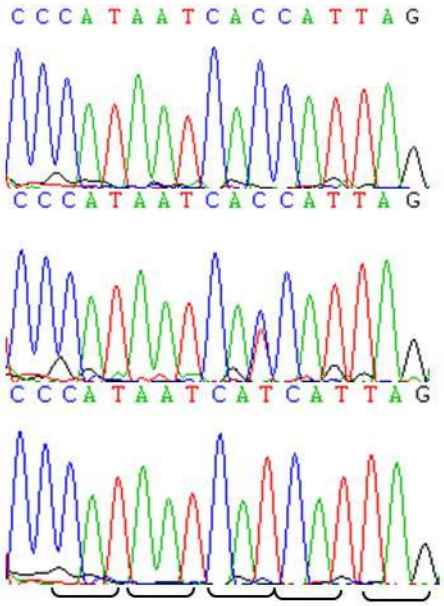

Val: GTG

↓

Met: ATG

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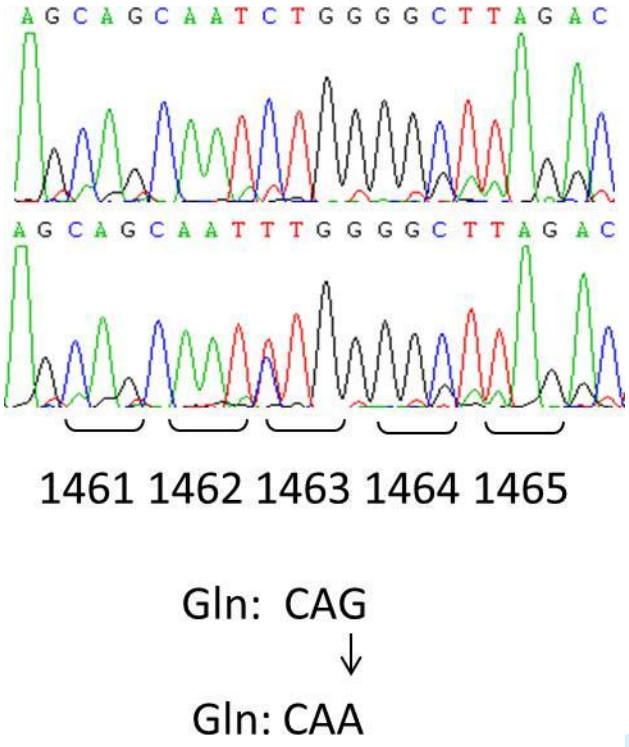
E, Representatives of *CFTR* gene exon 27 mutation in patients without mutation (top), patients with heterozygous c.4389G>A mutation (bottom). This mutation is reflected by G→A transition, at codon 1463 that is nonsense one (anti-sense sequence).



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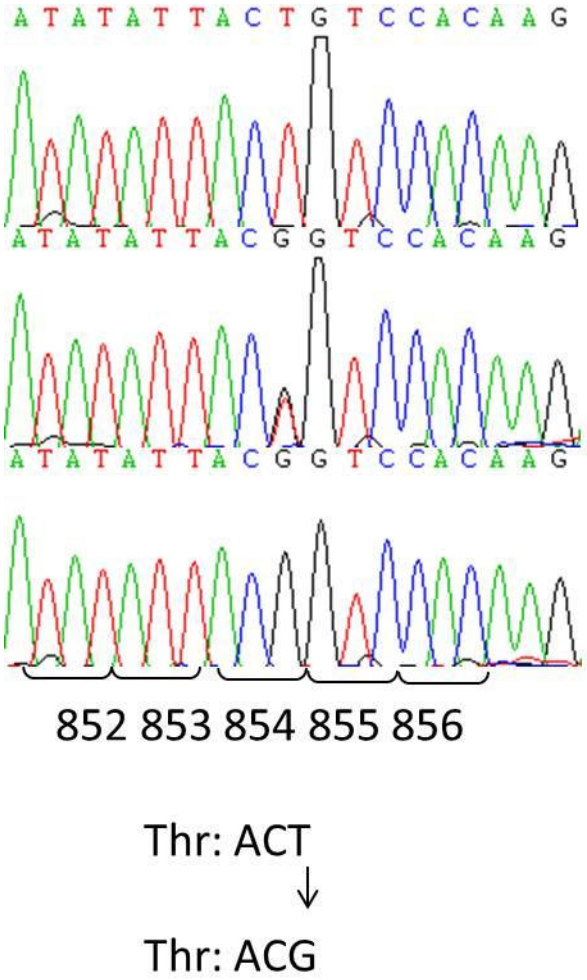
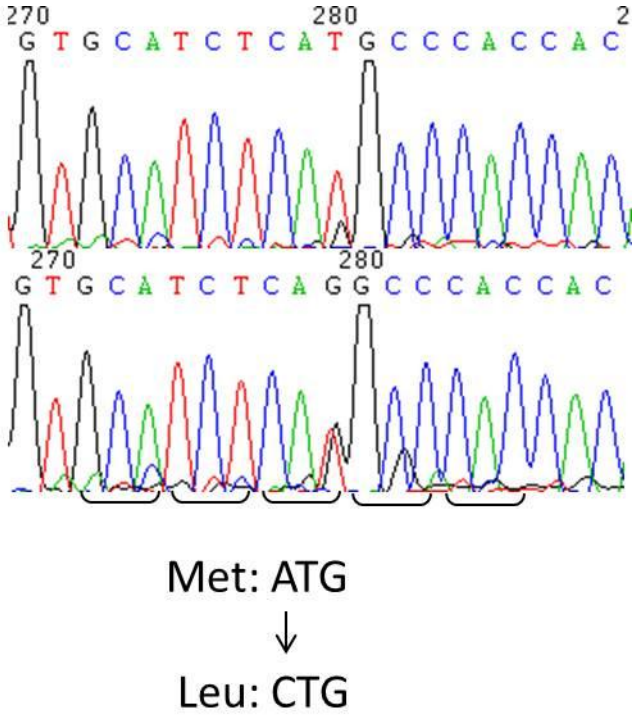
F, Representatives of *CFTR* gene exon 15 mutation in patients without mutation (top), patients with heterozygous c.2562T>G mutation (meddle) and patients with homozygousc.2562T>G mutation (bottom). This mutation is reflected by T→G transition, at codon 854 that is nonsense one.



G, Representatives of *CLDN2* gene exon 2 mutation in patients without mutation (top), patients with heterozygous mutation (bottom). This mutation is reflected by A→C transition that is missense. (anti-sense sequence)



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