Phenotypic Variability of the Homozygous IVS3+2T>C Mutation in the Serine Protease Inhibitor Kazal Type 1 (SPINK1) Gene in Patients with Chronic Pancreatitis

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Chronic pancreatitis (CP) is a progressive inflammatory disease that eventually results in the impairment of exocrine and endocrine functions of the pancreas. Recent studies have shown an association between mutations in the serine protease inhibitor Kazal type 1 (SPINK1) gene and CP. SPINK1 provides the first line of defense against prematurely activated trypsinogen by physically blocking the active site of trypsin. The IVS3+2T>C (c.194+2T>C) mutation is a loss-of-function splicing mutation; it affects the consensus splicing donor site in intron 3 and may cause the skipping of the entire exon 3, where the trypsin-binding site is located. We report here three CP patients carrying this mutation in a homozygous form, with no noticeable family history of pancreatitis. The first patient is a 25-year-old male with juvenile-onset idiopathic CP. He suffered from repeated attacks of pancreatitis since 5 years old and underwent pancreatico-jejunostomy. He complained of epigastralgia, and was diagnosed as obstructive pancreatitis in the area of the accessory pancreatic duct. The second patient is a 75-year-old male with alcoholic CP. He did not have apparent attacks of pancreatitis, but had numerous calcifications throughout the pancreas and confirmed exocrine failure and diabetes mellitus. The last patient is a 44-year-old female with late-onset idiopathic CP. She suffered from repeated attacks of pancreatitis since 32 years old. She had numerous stones in the main pancreatic duct in the pancreas head and confirmed exocrine failure. The clinical courses of these patients are apparently different, indicating the phenotypic variability of the SPINK1 IVS3+2T>C mutation-associated CP.

Keywords: pancreatitis; trypsin; trypsinogen; mutation; polymorphism


Chronic pancreatitis (CP) is a progressive inflammatory disease that eventually results in the impairment of exocrine and endocrine functions of the pancreas (Steer et al. 1995; Etemad and Whitcomb 2001). It has been suggested that pancreatitis results from an imbalance of proteases and their inhibitors within the pancreatic parenchyma, and recent human genetic studies have supported this concept. The gain-of-function mutations in the cationic trypsinogen (protease, serine, 1; PRSS1) gene are causes of hereditary pancreatitis (Whitcomb et al. 1996; Masson et al. 2008). The loss-of-function alterations in the chymotrypsin C (CTRC) gene, whose product specifically degrades all human trypsin/trypsinogen isoforms, predispose to pancreatitis by diminishing its protective trypsin-degrading activity (Rosendahl et al. 2008). Serum protease inhibitor Kazal type 1 (SPINK1) provides the first line of defense against prematurely activated trypsinogen by physically blocking the active site of trypsin (Rinderknecht 1986; Witt et al. 2000). Mutations in the SPINK1 gene are thought to diminish the protection against prematurely activated trypsin, and are thereby linked to trypsin-related pancreatic injury (Pfützer et al. 2000; Witt et al. 2000; Chen et al. 2001; Bhatia et al. 2002; Kume et al. 2005, 2006; Aoun et al. 2008).

Previous studies have shown that mutations in the SPINK1 gene are associated with CP of various forms including idiopathic, familial, and tropical (Pfützer et al. 2000; Witt et al. 2000; Chen et al. 2001; Bhatia et al. 2002; Kume et al. 2005, 2006; Aoun et al. 2008). The p.N34S (c.101A>G) mutation has been found worldwide in CP patients and healthy controls, with an average allele frequency of 9.7% and 1%, respectively (Pfützer et al. 2000;
The haplotype associated with the p.N34S mutation increases the risk of CP 11-fold on average, with higher risk observed in idiopathic and tropical cases than in alcoholic CP (Aoun et al. 2008 and references therein). The second most common haplotype contains the c.-215G>A promoter polymorphism and the IVS3+2T>C (c.194+2T>C) mutation that affects the consensus splicing donor site in intron 3 (Burset et al. 2000; Faustino and Cooper 2003). It is therefore conceivable that this haplotype may cause the skipping of the whole exon 3, where the trypsin-binding site is located. Indeed, gastric biopsies from carriers of the IVS3+2T>C mutation-associated haplotype revealed the predominant expression of a shorter SPINK1 mRNA lacking exon 3 (Kume et al. 2006). This aberrant mRNA was predicted to code for a non-functional trypsin inhibitor, and trypsin inhibitor production was diminished in the minigene analysis (Kereszturi et al. 2009). Thus, the IVS3+2T>C mutation results in functional loss of SPINK1 and disturbs the protease/antiprotease balance within the pancreas, leading to the development of pancreatitis. This haplotype has been reported in patients with idiopathic, familial, and alcoholic CP as well as those with tropical pancreatitis (Pfützer et al. 2000; Witt et al. 2000; Rossi et al. 2001; Kume et al. 2005; Kalinin et al. 2006; Keiles and Kammesheidt 2006; Snabboon et al. 2006). While the phenotypes of CP patients carrying the p.N34S mutation have been rather well described, very few data are available in the literature about the clinical courses of the patients carrying the IVS3+2T>C mutation (Pfützer et al. 2000; Snabboon et al. 2006; Masamune et al. 2007). We here report three patients with CP carrying this mutation in a homozygous form.

**Patient 1**

A 25-years-old man was referred to our hospital for possible endoscopic treatment of pancreatic pain. Family history was unremarkable. He suffered from recurrent attacks of pancreatitis since he was 5 years old, and underwent pancreatico-jejunostomy at the age of 16. A few days later, he was admitted to our hospital complaining of severe epigastralgia. Laboratory data showed an elevation of the white blood cell count (15,100/mm$^3$). Serum amylase was 98 IU/L, which was within the normal range (43-116 IU/L) but increased compared to that before the episode (around 20 IU/L). Computed tomography showed swelling of the pancreas head, with scattered, tiny calcifications. The minor papilla of Vater was swollen and reddened, whereas the major papilla appeared normal. Endoscopic retrograde pancreatography (ERP) from the major papilla demonstrated the main pancreatic duct and the anastomosed jejunum without depiction of the accessory pancreatic duct (Fig. 1A). ERP from the minor papilla demonstrated that the accessory pancreatic duct was dilated with an irregular wall (Fig. 1B). Communication with the main pancreatic duct and the jejunum was absent. White pus-like pancreatic juice drained from the minor papilla after the catheter was pulled out (arrow in Fig. 1C). Based on the diagnosis of obstructive pancreatitis in the area of the accessory pancreatic duct, we placed an endoscopic naso-pancreatic drainage tube (5 French size) in the accessory pancreatic duct from the minor papilla. The next day, his symptom was relieved and the white blood cell count had decreased. Pancreatography from the endoscopic naso-pancreatic drainage tube showed communication with the anastomosed jejunum, and we removed the tube. He was discharged 9 days later. Impaired glucose tolerance has been recognized since he was 27 years old (2 years after the episode).

**Patient 2**

The patient was a 75-year-old male who was diagnosed as having alcoholic CP in 1980 at the age of 45. He had a history of excessive alcohol consumption (100 g per day for twenty five years). Although there was no definitive family history of pancreatitis, his mother and brother had diabetes mellitus, and his sister died of pancreatic cancer. Abdominal computed tomography revealed atrophy of the pancreatic parenchyma and numerous calcifications throughout the pancreas (Fig. 2). Since 1994, he had diabetes mellitus and was prescribed glibenclamide. In 1996 (at the age of 60), his exocrine function was evaluated by a secretin test and showed 3-factor abnormalities.

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![Fig. 1. ERP of the patient 1.](image-url)  
(A) ERP from the major papilla of patient 1 demonstrated the main pancreatic duct (arrow) and the anastomosed jejunum without depiction of the accessory pancreatic duct. (B) ERP from the minor papilla demonstrated a dilated accessory pancreatic duct with the irregular wall (arrow). Arrowhead denotes the catheter inserted from the minor papilla. (C) White pus-like pancreatic juice drained from the minor papilla after the catheter was pulled out (arrow).
Patient 3

A 44-year-old female was referred to our hospital for the treatment of pancreatic stones with extracorporeal shock wave lithotripsy. She had no history of alcohol consumption, and family history was unremarkable. She suffered from recurrent attacks of pancreatitis since she was 32 years old. Abdominal computed tomography revealed numerous stones in the dilated main pancreatic duct in the pancreas head. She had exocrine insufficiency confirmed by pancreatic function diagnostant test (54.4%; normal range ≥ 70%). The result of oral glucose tolerance test was within normal range.

Genetic Analysis

The patients gave written informed consent according to the ethical guidelines of the Declaration of Helsinki. Genetic analysis was performed under the approval of the Ethics Committee of Tohoku University School of Medicine (2009-403; Principal investigator: A. Masamune). Genomic DNA was extracted from peripheral blood leucocytes using a genomic DNA purification kit (Promega, Madison, WI).

The presence of IVS3+2T and/or IVS3+2C alleles was examined by primer-introduced restriction analysis. The sequences of the primers were 5’-CAAATGTTACATGA ACTTAATGGATG-3’ (forward) and 5’-GTTTAAAGA ACTCAAGTTGTACGC-3’ (reverse). The cycle condition was as follows: preheating at 95°C for 5 min, followed by 40 cycles of 95°C for 30 sec, 60°C for 30 sec, and 72°C for 30 sec; and then a final extension at 72°C for 5 min. The PCR products were digested with HhaI (New England Biolabs, Beverly, MA) overnight at 37°C, separated by 3% agarose gel electrophoresis and visualized under ultraviolet after staining with ethidium bromide. The unrestricted 134-bp product represents the T allele, whereas the C allele was cut into 107-bp and 27-bp fragments. The presence of the IVS3+2T>C mutation was confirmed by direct sequencing using a BigDye terminator cycle sequence ready kit and
alleles show varying penetrance, best
gene in a homozygous form in all of three
(Burset et al. 2000), indicating that the intron starting with
that 0.69% of introns were flanked by GC-AG dinucleotides
ones reported in previous studies.

difficult to compare the severity of phenotypes between the
creatitis at ages 10, 20, and 20, respectively (Pfützer et al.
three patients with familial pancreatitis in the United States (Keiles and
patients, eight patients carried the IVS3+2T>C mutation in
of these ten patients. Of these ten patients carrying the p.N34S mutation and in the
patients, four patients (data not shown). The 45-year-old daughter of patient 2 had
the IVS3+2T>C mutation in a heterozygous form (data not shown). She has no pancreatic symptoms, biochemical, or ultrasound pancreatic alterations so far.

Discussion

We here reported three patients of CP carrying the homozygous IVS3+2T>C mutation. The clinical courses of these patients are apparently different; the first patient was juvenile-onset and the third patient was late-onset idiopathic CP, whereas the second patient was alcoholic CP. Our report suggests variable phenotypes of the homozygous IVS3+2T>C mutation-associated CP. This IVS3+2T>C mutation has been observed with an average allele frequency of 6.3% in Japanese patients with idiopathic, familial, and alcoholic CP (Kume et al. 2005; Kereszterti et al. 2009), but is not specific to Japanese. It was also found in two patients with tropical pancreatitis in Bangladesh (Rossi et al. 2001) and Thailand (Snabboon et al. 2006), four patients with CP in Europe (Witt et al. 2000; Kalinin et al. 2006), three patients with familial pancreatitis in the United States (Pfützer et al. 2000), and one patient with pancreatitis in the United States (Keiles and kammesheidt 2006). Of these ten patients, eight patients carried the IVS3+2T>C mutation in a heterozygous form and the allele status was not stated in the remaining two patients. The clinical courses of these patients have been poorly described. A 16-year-old Thai female with tropical pancreatitis suffered from chronic upper abdominal pain and diabetes mellitus since she was 14 years old (Snabboon et al. 2006). She had diffuse calcifications throughout the pancreas. Three patients with familial pancreatitis in the United States had onset of pancreatitis at ages 10, 20, and 20, respectively (Pfützer et al. 2000). Because the clinical information was limited, it was difficult to compare the severity of phenotypes between the homozygous patients presented herein and the heterozygous ones reported in previous studies.

A human expressed sequence tag-based study showed that 0.69% of introns were flanked by GC-AG dinucleotides (Burset et al. 2000), indicating that the intron starting with GC could be properly spliced out in many genes. Therefore, SPINK1 transcript might be normally processed even after the IVS3+2T>C mutation was introduced. We have previously shown that a correctly spliced SPINK1 transcript could be detected, with predominant expression of a shorter SPINK1 transcript lacking exon 3, in gastric biopsy specimen of the patient 2 (Kume et al. 2006). The presence of correctly spliced transcripts is expected to be associated with a milder phenotype (Nissim-Rafinia and Kerem 2002). Variable levels of correctly spliced transcripts, which are translated to normal proteins in affected tissues, might be one explanation for the phenotypic variability in the IVS3+2T>C mutation-associated CP.

The IVS3+2T>C and other loss-of-function mutations including the c.27delC (Le Maréchal et al. 2004) have been considered collectively as the most severe alleles of SPINK1 (Chen and Ferec 2009). It has been suggested that loss-of-function SPINK1 alleles show varying penetrance, best exemplified by the case of the c.27delC mutation (Le Maréchal et al. 2004). In one family with hereditary pancreatitis, c.27delC segregated with the disease in two generations; the penetrance was as high as 75% (three of four carriers). However, in another family with familial CP, c.27delC was identified in two patients; the penetrance was 29% (two out of seven carriers). Thus, the most severe SPINK1 alleles appear to have a considerably lower penetrance than the most severe PRSS1 alleles, and SPINK1 has been defined as an intermediate penetrance gene (Chen and Ferec 2009). Along this line, it should be noted that there was no family history of pancreatitis in all patients. Alternatively, additional factors such as anatomical abnormalities, alcohol, and other pancreatitis-associated gene abnormalities are required for the development of pancreatitis in subjects carrying the heterozygous IVS3+2T>C mutation.

If one uses the age of symptom onset as a measure of the mutation severity, the IVS3+2T>C mutation has been suggested to be milder than the p.N34S mutation. The onset of the pancreatitis symptoms, the frequency and the onset of diabetes, the frequency of calcification and its onset, as well as the size of main pancreatic duct, were not different between the IVS3+2T>C mutation-positive and -negative patients (Masamune et al. 2007). In contrast, CP patients carrying the p.N34S mutation presented earlier symptom-onset and had more dilatation of the main pancreatic duct than those without the SPINK1 mutations. Patients carrying the p.N34S mutation required surgical and/or endoscopic intervention more often than those without the mutations. Indeed, 80% of the patients carrying the p.N34S mutation required extracorporeal shockwave lithotripsy, endoscopic pancreatic duct lithotomy, and/or surgical operation (Masamune et al. 2007). Patient 1 reported herein suffered from repeated attacks of pancreatitis and required surgery at the age of 16. Patient 3 required extracorporeal shockwave lithotripsy for the treatment of pancreatic stones. Thus, the phenotypes of patients 1 and 3 appear to be different from
previously reported patients, but rather similar to that of the p.N34S mutation-associated CP. Obviously, further studies are required to clarify the phenotypes of CP patients carrying the IVS3+2T>C mutation, especially focusing on the difference between the heterozygous and homozygous mutations.

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References


