Mutations in serine protease inhibitor Kazal type 1 are strongly associated with chronic pancreatitis

J P H Drenth, R te Morsche, J B M J Jansen

Background: Although chronic pancreatitis is associated with risk factors such as alcoholism, hyper-parathyroidism, and hypertriglyceridaemia, little is known of the actual aetiology of the disease. It is thought that inappropriate activation of trypsinogen causes pancreatitis, and indeed in cases of hereditary pancreatitis mutations of cationic trypsinogen (PRSS1) have been described. As serine protease inhibitor Kazal type 1 (SPINK1) is a potent natural inhibitor of pancreatic trypsin activity, we hypothesised that SPINK1 mutations would be more common than expected among an unselected cohort of adult chronic pancreatitis patients.

Aims: To detect the prevalence of SPINK1 mutations in a cohort of chronic pancreatitis patients.

Methods: DNA was isolated from a cohort of 115 adult patients with chronic pancreatitis of alcoholic (n=72), hereditary (n=10), idiopathic (n=24), and miscellaneous (n=9) origin. We performed mutational analysis for two PRSS1 mutations (R122H, N29I) and four specific SPINK1 gene mutations (M1T, L14P, N34S, P55S) and compared the results with a control group of 120 healthy Dutch subjects.

Results: In six of the 10 patients that fulfilled the criteria for hereditary pancreatitis, but in none of the control subjects, mutations in the PRSS1 gene were found. In 14 patients we detected a SPINK1 mutation. Eleven patients were heterozygous for the N34S mutation and sequencing confirmed the homozygous state of N34S in a brother and sister. Two patients carried the P55S mutation, one as a compound heterozygote with N34S. The M1T and L14P SPINK1 mutations were not found in our cohort. The N34S mutation was detected in only two of 120 controls, while the P55S, M1T, and L14P mutations were absent in the same group. Patients with the N34S allele had a later onset of disease than those with PRSS1 gene mutations but earlier onset compared with the mutation negative group.

Conclusion: Identification of SPINK1 mutations in 12.2% of patients with adult alcoholic and idiopathic chronic pancreatitis suggests an important role for SPINK1 as a predisposing factor in adult chronic pancreatitis.
METHODS
Selection of patients
The study was approved by the hospital’s ethics committee. We first designed a computerised database of patients with chronic pancreatitis. We reviewed the records of all patients who had been referred from 1980 to 2000 to the University Medical Center St Radboud because of symptoms of chronic pancreatitis. A diagnosis of chronic pancreatitis was based on the presence of a typical history (recurrent upper abdominal pain, radiating to the scapula tip, relieved by leaning forward or sitting upright and increased after eating), suggestive radiological findings, such as pancreatic calcifications or pseudocysts, and/or pathological findings (pancreatic ductal irregularities and dilatations) revealed by endoscopic retrograde pancreaticography or magnetic resonance imaging of the pancreas before and after stimulation with secretin. In total, 165 records were available for review. We collected data with special emphasis on the cause of chronic pancreatitis, onset of symptoms, early (splenic vein thrombosis, pancreatic pseudocysts) and long term complications (steatorrhoea, diabetes mellitus), surgical procedures, use of narcotics, and smoking behaviour. Because our department actively participates in a project on pain management by thoroscopic splanchnic denervation we were able to divide the characteristics of pain into two types. Type A pain represents recurrent pain attacks while type B pain indicates unrelenting continuous pain. Patients who had an estimated daily intake of alcohol of more than 60 g (females) or 80 g (males) for more than two years were classified as chronic pancreatitis of alcoholic origin. Idiopathic chronic pancreatitis was diagnosed when precipitating factors such as alcohol abuse, gall stones, trauma, medication, infection, metabolic disorders, and positive family history were absent. Hereditary pancreatitis was defined when chronic pancreatitis was present in two or more family members. A letter was sent to these patients and their general practitioner explaining the goal of the study and a request to submit a blood sample. In total, 115 DNA samples were available for this study and all patients gave informed consent before enrollment.

DNA studies
We extracted genomic DNA from whole blood according to established protocols using the Puregene DNA isolation kit (Gentra Systems, Minneapolis, Minnesota, USA). In order to identify disease associated mutations we applied the following approach. We first screened the most prevalent mutations (N29I and R122H) in the PRSS1 gene. The N29I mutation was detected using an allele specific polymerase chain reaction (PCR). Patients were screened for the R122H mutation using a PCR-restriction fragment length polymorphism. Briefly, the 50 μl reaction mixture contained 200 ng of genomic DNA, 10 mM Tris HCl (pH 9.0), 50 mM KCl, 0.1% Triton, 2 mM MgCl2, 0.25 mM dNTPs, 100 ng of sense primer (5′-GATTGGGCAATAGCGGCAAG-3′) and 3.0 U Taq-DNA polymerase to generate a 555 bp fragment. Cycling conditions were an initial step at 94°C for five minutes, then 35 cycles at 94°C for 30 seconds, 60°C for 30 seconds, 72°C for 30 seconds, and a final elongation step at 72°C for five minutes. PCR products were incubated with 10 U BbrPl (Roche Diagnostics, Mannheim, Germany) for two hours at 37°C. The digested PCR samples were electrophoresed on a 3% RESp pense PCR agarose (Biodym, Landgraaf, the Netherlands) generating two fragments of 327 bp and 228 bp for the wild-type and two additional fragments of 208 bp and 20 bp when the R122H mutation was present.

In mutation negative patients with a positive family history for the disease, we sequenced all five exons of the PRSS1 using the primers shown in table 1. Cycling conditions were an initial step at 94°C for five minutes, then 35 cycles at 94°C for 30 seconds, 62.5°C/57.5°C (PCR 1/PCR 2) for 30 seconds, 72°C for one minute, and a final elongation step at 72°C for five minutes. The PCR products were purified after electrophoresis on an agarose gel with the Qaex II gel extraction kit (Qiagen, Hilden, Germany) With the purified products we performed sequencing with the Thermo Sequenase CyTMS5 Dye Terminator Kit (Amersham Pharmacia Biotech, Buckinghamshire, UK) according to the manufacturer’s manual and loaded them onto the ALFexpress DNA sequencer (Amersham Pharmacia Biotech).

Next we analysed four specific mutations in the SPINK1 gene: MIT, L14P, N34S, and P55S. We looked for these mutations in all chronic pancreatitis patients using an allele specific PCR. Briefly, a 50 μl reaction was prepared containing 200 ng genomic DNA, 10 mM Tris HCl (pH 9.0), 50 mM KCl, 0.1% Triton, 2 mM MgCl2, 0.25 mM dNTPs, 3.0 U Taq-DNA polymerase, and 100 ng of sense, antisense, and mutation primers (see table 2). Cycling conditions were an initial step at 94°C for five minutes and then 35 cycles at 94°C for 30 seconds, 59°C for 30 seconds, 72°C for 30 seconds, and a final elongation step at 72°C for five minutes. This allele specific PCR generated two fragments when the N34S mutation was present and one fragment when the wild-type was present. Hetero- or homozygosity of the N34S and P55S mutations were confirmed by complete sequencing of exon 3 of the SPINK1 gene using the sense primer as sequencing primer.

Population screening
To exclude the possibility that the observed mutations might be present in the population at large as a natural polymorphism, a population based study was performed. To this end 240 chromosomes of 120 unrelated healthy individuals of Dutch extraction were sequenced to detect the SPINK1 variants and the PRSS1 mutations R122H and N29I. Mean age of these healthy subjects was 35±11 years and 65 were female.

Statistical analysis
We used Fisher’s exact test to estimate differences in prevalence among the three groups. The Wilcoxon signed
ranks test was used to compare differences in age at onset. All p values were based on two sided comparisons. A p value of 0.05 was considered the lowest level of significance.

**RESULTS**

**Characteristics of the patients**

The clinical characteristics of the patients are shown in table 3. The cohort of 115 patients included 72 with alcohol related pancreatitis, 24 with idiopathic pancreatitis, 10 with a familial form of pancreatitis, and nine with various causes. There were 71 male and 44 female patients. Recurrent abdominal pain as a diagnostic problem or a therapeutic dilemma was the usual reason for referral.

**DNA studies**

Table 3 indicates the results from mutational screening of the PRSS1 and SPINK1 genes. We detected six patients who carried a mutant PRSS1 gene. Two patients who had the N29I allele were siblings while a third patient was their niece. The R122H mutation was detected in a mother and her daughter and in a third unrelated patient from a family with hereditary pancreatitis. We then sequenced the whole coding region of the PRSS1 gene in four other familial cases of chronic pancreatitis but were unable to demonstrate mutations in any of the five coding exons of PRSS1 in these patients. We then tested all patients for four SPINK1 mutations using an allele specific PCR. In total, 14 chronic pancreatitis patients (12.2%) had at least one SPINK1 mutation, and none of the PRSS1 mutation positive patients carried a SPINK1 mutation. We detected the N34S allele in 13 patients (fig 1). In 11 of these patients the N34S mutation was heterozygous (fig 2A) and in the remaining two, brother and sister, it was present in the homozygous state (fig 2B). Sequencing of the 240 control alleles from 120 patients identified two N34S heterozygous mutations (0.83% of alleles, 1.67% of the healthy cohort) (p<0.0001) A heterozygous P55S allele was found in two chronic pancreatitis patients. One patient was a compound heterozygote and carried the P55S and N34S on different alleles. The P55S variant was present in none of the control chromosomes tested (p<0.0001). The M1T and L14P mutations were not detected in any of the patient or control samples.

**Assessment of genotype-phenotype correlation**

Table 4 shows the cohort of chronic pancreatitis patients grouped according to the results of the DNA studies. Three groups were distinguished: those with a SPINK1 mutation (14 patients), those with a PRSS1 mutation (six patients), and those without any of the listed mutations (95 patients). The

![Figure 1](http://gut.bmj.com/)

**Figure 1** Allele specific polymerase chain reaction analysis of the N34S mutation on exon 3 of the SPINK1 gene. The wild-type SPINK band is located at 285 bp. If the N34S mutation is present as in patient Nos 2, 6, and 8, a second band at 190 bp appears. The left lane shows size markers.

![Figure 2](http://gut.bmj.com/)

**Figure 2** (A) DNA sequence electropherograms demonstrating a disease associated N34S SPINK1 mutation. Note the double peak at base 94 which results in a heterozygote A to G transition resulting in a substitution of asparagine by serine (N34S). (B) DNA sequence electropherograms demonstrating a disease associated homozygote N34S SPINK1 mutation. Note that at base 93 there is a single G indicating that A is replaced by G on both alleles. This results in a homozygous substitution of asparagine by serine (N34S).
causes of pancreatitis did not differ among the mutation negative group and those with SPINK1 mutations. In five patients with a mutated SPINK1 allele, chronic pancreatitis was associated with alcohol abuse although one patient reported an alcohol intake at the time of diagnosis of only 25 g/day, lower than that mostly associated with alcoholic pancreatitis. The other nine patients with a mutated SPINK1 allele were diagnosed as idiopathic (five patients), familial (two patients), hyperparathyroidism (one patient), and annular pancreas (one patient). The group that was heterozygous or homozygous for SPINK1 mutations had an earlier onset of the disease (11.5 and 18 years) but the course and severity of the disease were similar to heterozygous patients. Their parents, obligate N34S heterozygotes, did not suffer from symptoms suggestive of chronic pancreatitis. The mutation negative patients were similar to patients with a SPINK1 mutation with respect to abnormal findings on radiographic studies. Pancreatic pseudocysts were detected in 42 of 95 patients in the mutation negative group and in seven of 14 patients with a SPINK1 but in only one patient with PRSS1 mutations. Approximately 75% of patients from both groups underwent pancreatic surgery during the course of their disease, most commonly for pain management, and one third of patients from both groups were treated with thoracoscopic splanchnic nerve denervation. Compared with the mutation negative patients, diabetes mellitus as a sign of pancreatic endocrine failure was more common in patients with a SPINK1 mutation, although this did not reach statistical significance (table 4). Compared with the two other groups, patients with a mutant PRSS1 allele had a much earlier onset of symptoms (6–5 years), required more hospitalisations (16 per patient), and consumed more narcotics (p<0.05). All patients with PRSS1 gene mutations had a positive family history for the disease compared with two patients in the mutation negative group and two in the SPINK1 group. One patient who was compound heterozygote for the N34S and P55S SPINK1 mutations had a very severe disease. This 54 year old male suffered from alcoholic pancreatitis for eight years. Pancreatitis was complicated by exocrine and endocrine insufficiency, diffuse pancreatic calcifications, and severe type B pain. Because of exacerbation of his chronic pain computer tomography of the pancreas was performed which disclosed a 3 cm tumour compatible with a pancreatic adenocarcinoma. He died eight months later.

**DISCUSSION**

Our investigation had two main objectives. Firstly, to assess whether SPINK1 mutations are associated with chronic pancreatitis and secondly, to determine whether patients who carry a SPINK1 mutation follow a different disease course compared with SPINK1 negative patients. We found a strong association between chronic pancreatitis and SPINK1 alleles in a cohort of 115 patients who were referred to us because of symptoms of chronic pancreatitis. In these patients the frequency of a SPINK1 mutation was seven times higher than that of a group of healthy controls. Most patients with a SPINK1 mutation had the N34S variation (13/14) but there were two patients with the P55S allele. Possession of these

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**Table 4** Distribution of various clinical parameters among patients with chronic pancreatitis grouped according to the results of mutational screening for the SPINK1 and PRSS1 genes

<table>
<thead>
<tr>
<th></th>
<th>SPINK1</th>
<th>PRSS1</th>
<th>Mutation negative</th>
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</thead>
<tbody>
<tr>
<td><strong>Demographics</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>n</td>
<td>14</td>
<td>6</td>
<td>95</td>
</tr>
<tr>
<td>Sex [M/F]</td>
<td>10/4</td>
<td>0/6</td>
<td>61/34</td>
</tr>
<tr>
<td>Age (y)</td>
<td>41 (12)</td>
<td>26 (7)</td>
<td>49 (11)</td>
</tr>
<tr>
<td>Age of onset (y)</td>
<td>26 (18)</td>
<td>6 (5)</td>
<td>38 (11)</td>
</tr>
<tr>
<td>Duration of symptoms (y)</td>
<td>14 (8)</td>
<td>20 (9)</td>
<td>11 (9)</td>
</tr>
<tr>
<td><strong>Cause</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Alcoholic</td>
<td>5 (36%)</td>
<td>67 (71%)</td>
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</tr>
<tr>
<td>Idiopathic</td>
<td>5 (36%)</td>
<td>19 (20%)</td>
<td></td>
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<tr>
<td>Familial</td>
<td>2 (14%)</td>
<td>6 (100%)</td>
<td></td>
</tr>
<tr>
<td>Annular pancreas</td>
<td>1 (7%)</td>
<td>4 (4%)</td>
<td></td>
</tr>
<tr>
<td>Pancreatic divisum</td>
<td>7 (50%)</td>
<td>34 (35%)</td>
<td></td>
</tr>
<tr>
<td>Trauma</td>
<td>3 (3%)</td>
<td></td>
<td></td>
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<tr>
<td>Hyperparathyroidism</td>
<td>1 (7%)</td>
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<tr>
<td><strong>Hospital admissions</strong></td>
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<tr>
<td>Average number of hospitalisations per patient</td>
<td>5</td>
<td>16</td>
<td>4</td>
</tr>
<tr>
<td>Total number of hospitalisations</td>
<td>70</td>
<td>95</td>
<td>271</td>
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<tr>
<td><strong>Clinical characteristics</strong></td>
<td></td>
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<tr>
<td>Smoking</td>
<td>8 (67%)</td>
<td>3 (50%)</td>
<td>69 (73%)</td>
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<tr>
<td>Pancreatic pseudocysts</td>
<td>7 (50%)</td>
<td>1 (17%)</td>
<td>42 (44%)</td>
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<tr>
<td>Pancreatic duct abnormalities</td>
<td>7 (50%)</td>
<td>1 (17%)</td>
<td>46 (48%)</td>
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<tr>
<td>Diabetes mellitus</td>
<td>7 (50%)</td>
<td>0</td>
<td>30 (31%)</td>
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<tr>
<td>Steatorrhoea</td>
<td>7 (50%)</td>
<td>1 (17%)</td>
<td>34 (35%)</td>
</tr>
<tr>
<td><strong>Pain</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>5 (36%)</td>
<td>2 (40%)</td>
<td>25 (26%)</td>
</tr>
<tr>
<td>B</td>
<td>8 (57%)</td>
<td>2 (40%)</td>
<td>52 (54%)</td>
</tr>
<tr>
<td><strong>Therapy</strong></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Narcotics</td>
<td>11 (79%)</td>
<td>6 (100%)</td>
<td>69 (72%)</td>
</tr>
<tr>
<td>Pancreatic surgery</td>
<td>11 (79%)</td>
<td>3 (50%)</td>
<td>70 (73%)</td>
</tr>
<tr>
<td>Splanchnic nerve denervation</td>
<td>4 (29%)</td>
<td>2 (33%)</td>
<td>31 (33%)</td>
</tr>
</tbody>
</table>

Results from the PRSS1 and SPINK1 gene analysis were used to divide the cohort of chronic pancreatitis: SPINK1, patients who had at least one mutated SPINK1 allele (N34S and/or P55S); PRSS1, patients who had the N291 or R122H PRSS1 mutation; Mutation negative, patients who did not have one of the mentioned mutations (wild-type). Percentages refer to the proportion of patients within each given group. Type A pain represents recurrent pain attacks while type B pain indicates unrelenting continuous pain.
alleles has clinical consequences as carriers develop their disease earlier in life than patients without this mutation but later than patients with PRSS1 gene mutations. Although we detected minor differences in the clinical presentation between the groups that were mutation negative and SPINK1 positive, the phenotype of chronic pancreatitis was very similar when judged on the basis of the individual patient. Our study extends previous work because we included adult patients with chronic pancreatitis due to various causes. Indeed, our chronic pancreatitis cohort reflects the panel of patients seen in daily clinical practice as it has been estimated that in developed countries 60–70% of patients with chronic pancreatitis have a history of excessive alcohol intake while in 30% the disease is regarded as idiopathic. 

Despite these criteria, there is considerable confusion about the amount of alcohol intake that usually increases the risk of developing pancreatitis. Data vary from 25 g/day to more than 80 g/day for more than five years. It might be reasonable to postulate that SPINK1 mutations render patients more susceptible to risk factors such as alcohol, hypercalcaemia, and anatomical variations for the development of pancreatitis.

Trypsinogen is regarded as the initiating molecule in the current model of the pathogenesis of pancreatitis. Mutations in PRSS1 are thought to result in either reduced degradation of the active trypsin or facilitated activation of trypsinogen. In this pathogenetic model SPINK1 acts as an inhibitor of activated trypsin and our findings confirm the important role of premature protease activity in the pathogenesis of pancreatitis. Indeed, incubation of equimolar quantities of trypsin and SPINK1 produces an inactive complex and it has been estimated that SPINK1 can inactivate 10–20% of active trypsin. Mutations in the SPINK1 gene may have functional consequences. Probably the mutations alter the protein structure so that it affects the efficient construction of an inactive complex.

It is not clear whether SPINK1 gene mutations lead to a distinct form of pancreatitis with a separate mendelian inheritance pattern. The M1T mutation which destroys the translational initiation codon of SPINK1 has been observed in an anatomical variations for the development of pancreatitis. Another mutation, 17G77G, located in exon 4, was also present in a single case. One intron mutation (IVS3–2>T>C) was described in three patients from one study. Given the very low frequency of these mutations, it is unlikely that testing these mutations in our cohort would have significantly altered the results presented here.

In conclusion, our study identified mutations of the SPINK1 gene as a risk factor for chronic pancreatitis. Furthermore, it has increased our understanding of the pathogenesis of pancreatitis, and it may increase our understanding of the clinical behaviour of the disease.

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