**PRSS1** and **SPINK1** mutations in idiopathic chronic and recurrent acute pancreatitis

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**METHODS:** The cationic trypsinogen gene (**PRSS1**; exons 2 and 3) and the serine protease inhibitor Kazal 1 gene (**SPINK1**; exon 3) were selectively amplified and sequenced from blood samples of 19 patients admitted to the Pancreas Clinic at our institution with chronic pancreatitis and/or idiopathic recurrent acute pancreatitis that were diagnosed or with onset before age 35. Fifty healthy volunteers served as controls. Whole blood samples were collected and gene specific sequences were amplified by polymerase chain reaction (PCR). All PCR products were subsequently sequenced in order to identify the presence of any mutations.

**RESULTS:** Nineteen patients with pancreatitis (14 males; median age 24 years, range 15-48 years) were included in this study, of which five showed the presence of gene mutations. Direct sequencing results indicated the presence of two previously unidentified mutations in exon 2 of **PRSS1** (V39E and N42S) in two patients with recurrent acute pancreatitis. Two cases had the N34S **SPINK1** mutation. Analysis of the relatives of one patient homozygous for this mutation showed that five of the six family members carried the N34S **SPINK1** mutation. Of these members, three were healthy heterozygous carriers and two were homozygotes (one sibling had diabetes, the other was healthy). Another patient was heterozygous for a novel **SPINK1** mutation located on exon 3 (V46D). All members from this patient’s family had normal genotypes, indicating that it was a de novo mutation. No mutations in either gene were present in the control subjects.

**CONCLUSION:** Two novel **PRSS1** mutations and one novel **SPINK1** mutation were identified in Mexican patients with early onset idiopathic recurrent acute pancreatitis.

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**Key words:** Cationic trypsinogen; **SPINK1**; **PRSS1**; Chronic pancreatitis; Recurrent acute pancreatitis; Hereditary pancreatitis

**Core tip:** Chronic and recurrent idiopathic pancreatitis has been associated with mutations in genes responsible for the synthesis of pancreatic proteases (**PRSS1**) and protease inhibitors (**SPINK1**). The distribution of these mutations varies among countries, but has not been examined in detail in Latin American countries. This study examined **PRSS1** and **SPINK1** in 19 Mexican subjects with chronic pancreatitis and/or idiopathic recurrent acute pancreatitis and identified two novel **PRSS1** mutations and one novel **SPINK1** mutation.
INTRODUCTION

Chronic pancreatitis (CP) is a progressive inflammatory disease that leads to fibrosis and different degrees of exocrine and/or endocrine insufficiency[1]. There are many factors contributing to disease development, including alcohol use[2], though some cases do not present any known risk factors and are classified as idiopathic. Hereditary pancreatitis is diagnosed in the case of a positive family history[3]. As early as 1952, the observation that CP clustered in certain families suggested a genetic component. However, identification of such genetic factors did not occur until 1996, when mutations in the cationic trypsinogen gene (PRSS1) were discovered in families with hereditary CP and in some cases of idiopathic CP[4,5]. Later, mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) mutations[6,7], trypsin inhibitor (SPINK1), and chymotrypsinogen C (CTRC) genes were described in both idiopathic CP and alcoholic CP[8,9].

PRSS1 mutations have been linked with hereditary pancreatitis, whereas mutations in the SPINK1 gene have been associated with pancreatitis of different etiologies[10]. However, mutations in SPINK1 are not always sufficient to induce pancreatitis, and additional pancreatitis-associated factors must be present in order to express the disease. For example, the commonly observed N34S mutation in SPINK1 by itself has no apparent functional effect[11,12]. CTRC mutations have been identified in patients with idiopathic CP and hereditary pancreatitis[13,14], as well as in subjects with alcoholic CP. The effect of these genes on pancreatitis likely results from an imbalance between pancreatic fluid composition[15,16] and pancreatic exocrine function[17,18]. However, mutations in the PRSS1, SPINK1, CFTR, CTRC, trypsin inhibitor, and chymotrypsinogen C genes have been associated with pancreatitis of different etiologies[13,14].

MATERIALS AND METHODS

Subjects with CP and/or IRAP that were diagnosed or with onset before age 35 were prospectively and retrospectively enrolled in the study. For retrospective enrollment, the outpatient and inpatient database from the Pancreas Clinic at our institution was searched, and all eligible subjects were contacted by telephone. For prospective enrollment, all consecutive patients seen at our institution either as inpatients or at the outpatient Pancreas Clinic for CP or IRAP were included. Informed consent was obtained from the patients and 50 healthy volunteers who agreed to participate, and 20 cc of whole blood samples were then collected by peripheral vein puncture. Blood was stored at -70 °C for subsequent DNA extraction. This study was approved by the Institutional Review Board of the Instituto Nacional de Ciencias Médicas y Nutrición Salvador Zubirán.

Definitions

Acute pancreatitis was defined by the presence of two of the following: typical abdominal pain, three-fold elevation of serum pancreatic enzymes (amylase and/or lipase), and imaging evidence of acute pancreatitis. CP was considered if patients had any of the following predetermined criteria: histologic evidence (when available), imaging evidence from endoscopic retrograde cholangiopancreatography and/or magnetic resonance cholangiopancreatography with definitive evidence of CP according to the Cambridge classification, presence of pancreatic calcifications on computed tomography scan, plain abdominal X-rays, five or more CP-related findings on endoscopic ultrasound, and definitive pancreatic exocrine insufficiency according to a pancreolauryl test. IRAP was defined as the presence of two or more attacks of documented acute pancreatitis with no evident etiology after a thorough work-up and without imaging evidence of CP.

DNA extraction and gene- and exon-specific amplification

Whole blood (20 cc) was collected in K2EDTA BD Vacutainer tubes (Beckton Dickinson and Company, Franklin Lakes, NJ, United States). All blood specimens were processed at the Genomic Medicine Unit at the Instituto Nacional de Ciencias Médicas y Nutrición Salvador Zubirán. Genomic DNA was extracted using a standard salt precipitation protocol and assessed for quality and quantity by agarose gel electrophoresis. Exons 2 and 3 of the PRSS1 gene were amplified using primers and PCR amplification protocols previously reported by Nishimori et al[19] and Pho-Iam et al[20], respectively. Exon 3 of the SPINK1 gene was amplified using primers and polymerase chain reaction (PCR) amplification protocols as previously reported by Witt et al[21].

DNA sequencing

The PCR products from all samples were purified using a QiAquick PCR Purification Kit (Qiagen, Venlo, Limburg, The Netherlands) according to the manufacturer’s proto-
RESULTS

Nineteen subjects with either early onset CP or IRAP were identified and agreed to participate (14 males; median age 24 years, range 15-48 years) with a total of five instances of PRSS1/SPINK1 gene mutations (Table 1). Two mutations in PRSS1 were found: V39E in case 1 and N42S in case 6, both of which were in exon 2. The N34S SPINK1 mutation was identified in cases 8 and 19. The family of one of these patients, who was homozygous for the N34S mutation and had early onset chronic calcifying pancreatitis, was also examined, and six of the 14 additional members agreed to participate and provide blood samples for sequencing. Although no PRSS1 mutations were found in these family members, five of the six carried the same N34S SPINK1 mutation. Of these, both parents and one sibling were healthy and heterozygous for this mutation, whereas two siblings were homozygous (one was healthy and the other had developed diabetes at an early age). The other studied sibling had a normal genotype and was otherwise healthy. The fifth mutation was found in case 15, who was a heterozygote for a new SPINK1 mutation located on exon 3 (V46D). The family members of this patient were also studied, though no PRSS1 mutations were found, and the V46D or other SPINK1 mutations were not present. Moreover, no PRSS1 or SPINK1 mutations were identified in samples from the 50 healthy controls.

DISECUSSION

The results of this study identified two previously undocumented PRSS1 mutations. The first mutation, N42S, represents the substitution of one polar hydrophilic amino acid with another similar one. However, the second novel mutation identified, V39E, represents the substitution of a non-polar hydrophobic amino acid to a negatively charged polar one, which could induce a conformational change in the final synthesized molecule. Although functional studies are needed to elucidate the effect of these mutations on protein structure, expression and/or secretion as well as their contribution to the pathogenic mechanisms of pancreatic injury, the results from the current study suggest that mutations in the PRSS1 gene are sufficient to induce pancreatic disease. Indeed, PRSS1 gene mutations have been directly implicated in the pathophysiology of hereditary and idiopathic CP by producing an autolysis-resistant trypsin and/or facilitating auto-activation.[17,18] However, the presence and contribution of mutations in other exons, genes or environmental factors remains unclear and should not be ruled out.

Recent reports from India[22] and Japan[23] indicated that SPINK1 mutations confer strong genetic susceptibility to developing CP, but alone do not cause the disease. Some SPINK1 mutations alter peptide expression or binding affinity, though the disease-causing biochemical defect of the N34S mutation remains unknown.[21,22] In the current study, one case with the SPINK1 N34S mutation had apparently unaffected family members, two healthy siblings who were homozygous for the same mutation, and another sibling and both parents who were heterozygous. Thus, SPINK1 mutations may require other associated genetic and/or environmental risk factors in order to promote pathogenicity. It is possible that these mutations impact the phenotypic presentation of the disease, with patients developing CP at earlier ages[8,9,12], as seen in this early onset CP population.

The sequencing results of this study identified a novel SPINK1 mutation (V46D) in a patient with established calcific CP and no other evident predisposing factors. Computational simulations could indicate if this mutation likely aborts SPINK1 protein synthesis, in contrast to other previously described mutations that reduce the enzymatic activity[25]. It appeared to be a de novo mutation, as none of the family members had it nor did they present any clinical manifestations of pancreatitis, though no paternity tests were run. In addition, neither the family members nor the affected individual had prior history of exposure to pancreatic disease-related risk factors. As the presence of mutations in other exons or genes remains unknown, a direct causal effect of this new mutation needs to be validated.

In agreement with previous studies worldwide[21,23], the current study provides further support that the frequency, nature and type of mutations vary among populations. This is the first Mexican study to explore the genetics of

| Patient case No. | Sex | Age, in years | Age at symptom onset or diagnosis, in years | Clinical presentation | Mutation |
|-----------------|-----|--------------|---------------------------------------------|----------------------|----------|
| 1               | Female | 45          | 8                                           | IRAP, pancreatic calcifications at age 15, Puestow procedure at age 22 | V39E PRSS1 |
| 6               | Male  | 26          | 20                                          | IRAP, pancreatic calcifications at age 22 | N42S PRSS1 |
| 8               | Female | 26          | 26                                          | IRAP, pancreatic calcifications and dilated main pancreatic duct | N34S SPINK1 |
| 15              | Male  | 20          | 16                                          | IRAP, pancreatic calcifications | V46D SPINK1 |
| 19              | Male  | 15          | 12                                          | Abdominal pain, jaundice, pancreatic calcifications | N34S SPINK1 |

IRAP: Idiopathic recurrent acute pancreatitis.
early onset idiopathic CP in Hispanics. Although still a minority in the United States\textsuperscript{13} and European countries, the Hispanic population has shown a steady and continuous growth rate, and thus the results provide valuable information to health care workers responsible for the medical attention of such minorities. The main limitations of this study include the small sample population, incomplete sequencing of the entire \textit{PRSS1} and \textit{SPINK1} genes, and absence of testing for \textit{CFTR} and \textit{CTRC} mutations. However, the findings of this study are consistent with previous reports and identify new pancreatitis-related mutations.

**CONCLUSION**

Mexican subjects with idiopathic CP and IRAP present similar mutations in the \textit{PRSS1} and \textit{SPINK1} genes as reported in other populations. This study identified three novel mutations, two in \textit{PRSS1} and one in \textit{SPINK1}, which may be unique to the Mexican population. The novel V46D \textit{SPINK1} mutation may play a direct causal role of pancreatitis, though this finding needs to be validated by future functional studies.

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