

CFTR, PRSS1 and SPINK1 Mutations in the Development of Pancreatitis in Brazilian Patients

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ABSTRACT

Context Mutations in cystic fibrosis transmembrane conductance regulator (CFTR), in cationic trypsinogen (PRSS1) and in serine protease inhibitor Kazal type 1 (SPINK1) genes have been associated with chronic pancreatitis (alcohol related, idiopathic and hereditary). However, the inheritance pattern is still not clear.

Patients Eighty-two unrelated Brazilian patients with chronic pancreatitis (alcohol-related disease in 64, idiopathic disease in 16, and hereditary disease in 2). Two hundred unrelated individuals with an ethnic distribution comparable to the patients were studied as controls.

Main outcome measure Detection of mutations in CFTR, PRSS1, and SPINK1 genes.

Results Mutations in the CFTR gene were found in 8 patients (9.8%) with chronic pancreatitis, 5 of them with idiopathic disease. Interestingly, the only clinical symptom in a male patient in the alcoholic group, who was a compound heterozygote ($\Delta F508/R170C$) for two CFTR mutations, was pancreatitis without infertility or pulmonary involvement. In the PRSS1 gene, the E79K change in exon 3 was found in one

patient (1.2%) with alcohol-related chronic pancreatitis. Four different alterations were identified in the SPINK1 gene.

Conclusions Mutations in the CFTR gene represent the major cause of idiopathic chronic pancreatitis in Brazilian patients. No mutation was found in the PRSS1 gene among our patients suggesting further genetic heterogeneity for hereditary and idiopathic chronic pancreatitis. Interestingly, the most frequent SPINK1 N34S mutation was not present in patients or controls. Moreover, the -253C allele for the SPINK1 gene was significantly more frequent in patients than controls ($P=0.004$), suggesting that it might represent a risk factor for the development of pancreatitis in our population.

INTRODUCTION

More than 1,000 mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) gene have been reported by the Cystic Fibrosis Genetic Analysis Consortium. They are responsible for widely variable manifestations, ranging from mild pulmonary disease with pancreatic sufficiency to severe pulmonary disease with pancreatic insufficiency [1, 2]. The high frequency of mutations in the CFTR gene has led to the recognition of its association with a variety of

conditions, including bronchiectasis, polyps, male infertility and chronic pancreatitis [3, 4, 5, 6, 7].

In addition, a rare autosomal dominant form of chronic pancreatitis is caused by mutations in the cationic trypsinogen (PRSS1) gene, mapped at 7q35 [8, 9]. The disease was characterized in patients with recurrent pancreatitis with onset in childhood, equal sex distribution, a family history of at least two other affected members, frequent presence of calcified stones in the pancreatic duct and no identified precipitating factors [10]. This gene and 7 other trypsinogen genes, including the cationic and anionic trypsinogen genes and their pseudogenes, are part of the T cell receptor beta chain (TCR-beta) gene complex. These trypsinogen genes are highly homologous, each residing within a tandemly duplicated 10 kb segment and each being composed of five exons. The first pathogenic mutation in the PRSS1 gene, a missense mutation (R122H) in exon 3 that segregated with the disease was reported by Whitcomb *et al.* [9]. Other mutations in this gene, also associated with hereditary pancreatitis, were observed by other groups [11, 12, 13].

Subsequently, the results of negative linkage and the absence of mutations in the PRSS1 gene in affected families indicated that other gene(s) might be involved in the pathogenesis of hereditary pancreatitis [11]. It was suggested that the serine protease inhibitor, Kazal type 1 (SPINK1) gene, also known as pancreatic secretory trypsin inhibitor (PSTI), located on chromosome 5 [14], is a potent protease inhibitor and a negative regulator of trypsin activity and may be a good candidate gene contributing to the development of pancreatitis [11]. Indeed, mutations in the SPINK1 gene have been associated with chronic pancreatitis [15, 16, 17, 18, 19]. However, the significance and inheritance pattern of SPINK1 mutations has been interpreted differently by different groups.

In order to determine the spectrum and the frequency of alterations in the CFTR, PRSS1 and SPINK1 genes and to establish genotype-phenotype correlation, we have analyzed a

group of 82 Brazilian patients affected with chronic pancreatitis.

PATIENTS AND METHODS

Patients

The patients were chosen from the Department of Gastroenterology of the University of São Paulo and DNA analysis was performed at the Human Genome Research Center, at the University of São Paulo. A total of 82 unrelated patients with chronic pancreatitis (64 males, 78.0%; 18 females, 22.0%. Mean±SD age: 49.7±10.2 years, range: 7-82) were included in the present investigation: 64 with alcohol-related disease, 16 with idiopathic disease, and 2 with the hereditary form. Sixty-two patients (75.6%) were Caucasian (from European descent), 19 (23.2%) were of mixed ethnicity (Caucasians, African-Brazilian, Indian-Brazilian) and 1 (1.2%) was Japanese.

The criteria for a diagnosis of chronic pancreatitis was based on at least one of the following parameters: alterations of pancreatic parenchyma at ultrasound, alterations of pancreatic function determined by the secretin-erulein test, or histological alterations compatible with chronic pancreatitis.

An attack of pancreatitis was the usual presenting symptom and increasing pain was the usual reason for referral. Forty-four patients (53.7%) had diabetes, 16 (19.5%) had cysts and 12 (14.6%) had steatorrhea. No complementary exams were available for the remaining 10 patients. None of the patients had a diagnosis of typical cystic fibrosis and therefore sweat chloride tests were not routinely performed. Pulmonary problems were assessed through computerized tomography.

For the purpose of this study, smokers were defined as those who smoked 10 or more cigarettes per day. Alcoholic etiology was defined as a daily intake of at least 100 mL of ethanol by males and at least 80 mL of ethanol by females for 5 or more years, before

the first symptoms of pancreatitis. Idiopathic chronic pancreatitis was diagnosed when precipitant factors such as alcohol abuse, medication, infection, metabolic disorder, and positive family history were absent. A diagnosis of hereditary pancreatitis was made when the disease was present in at least three family members.

Controls

For the control group, 200 unrelated individuals (88 males, 44.0%; 112 females, 56.0%; $P < 0.001$ vs. patients. Mean \pm SD age: 35.0 ± 17.9 years, range: 21-77; $P < 0.001$ vs. patients) with an ethnic distribution comparable to the patients (155 Caucasian, 77.5%; 37 mixed ethnicity, 18.5%; 8 Japanese, 4.0%; $P = 0.354$ vs. patients) were selected at the Human Genome Research Center at the University of São Paulo. The controls included healthy relatives of patients with other genetic neuromuscular disorders, mainly muscular dystrophy, and professionals working at the University of São Paulo. None of the controls had any symptoms of pancreatitis.

DNA Analysis

EDTA blood samples were collected and the DNA was analyzed according to standard procedures. Mutation detection studies were carried out in all 27 exons of the CFTR gene, in the 5 exons of the PRSS1 gene and in the 4 exons of the SPINK1 gene through single-strand conformation polymorphism (SSCP) [20]. Because of the extremely high homology of the intronic sequence of the PRSS1 gene with that of the pseudogenes, a nested PCR strategy was employed.

A sample of 100 ng of genomic DNA was amplified under the following conditions: 20 nM Tris-HCl (pH 8.4), 50 nM KCl, 1.5 mM MgCl₂, 0.2 mM of each primer, and 2 units of Taq polymerase (Gibco BRL, Life Technologies, Grand Island, NY, USA) for 35 cycle. All PCR reactions were carried out in a total volume of 10 μ L.

Abnormally migrating fragments present in SSCP analysis were subsequently sequenced to confirm the presence of mutations. The PRSS1 gene in patients with hereditary pancreatitis was directly sequenced. Screening of the N34S mutation in the SPINK1 gene was also confirmed by restriction enzyme analysis as it creates a new site for TspRI [17]. Sequencing was performed according to the ABI standard protocol in a ABI 377 automated DNA sequencer (Applied Biosystems, Foster City, CA, USA).

The -253T>C and -164G>C variants of the PRSS1 gene were examined in the overall series of control subjects, while the other mutations of the SPINK1 gene were available in 150 control subjects only.

For the CFTR gene, only patients were analyzed. The frequency of CFTR variants of the T Tract length of intron 8 (IVS8Tn) was investigated. The analysis of the microsatellite IVS8Tn was made by PCR and the amplified fragments were subjected to electrophoresis at 1,500 V for 2 h in a 6.5% denaturing polyacrylamide gel.

ETHICS

All studies were done following patients' informed consent. The protocol was approved by the Ethics Committee of the University of São Paulo School of Medicine.

STATISTICAL ANALYSIS

Age was compared by means of the Student-t test. Genotype and gene frequencies, as well as gender, were compared between patients and controls by using contingency tables and were analyzed by means of the Yates' corrected chi-squared test or the Fisher's exact test (when at least one expected cell value in a 2x2 table was less than 5). Ethnic distribution was analyzed by the Pearson chi-squared test. Statistical analyses were performed by running the SPSS/PC+ version 5.0 package on a personal computer. Two-tailed P values than 0.05 were considered statistically significant.

RESULTS

Age at the onset of symptoms varied widely, from 3 to 62 years (mean±SD: 37.8±8.5). Forty-nine patients were smokers (59.8%). Among the 16 patients with idiopathic chronic pancreatitis, two (12.5%) had congenital absence of the vas deferens and another two (12.5%) had lung disease.

A total of 13 changes were found: 7 in the CFTR gene (Δ F508/R851L, Δ F508/R170C, Δ F508/L206W, 2 N/ Δ F508, N/P205S, N/R31C and N/V920M), 2 in the PRSS1 gene (E79K and N246N) and 4 in the SPINK1 gene (-253T>C, -164G>C, -7T>G, c75C>T) (Table 1).

The CFTR Gene

Molecular analysis showed that 8 patients (9.8%) had mutations in the CFTR gene: 3 were compound heterozygotes (Δ F508/R851L, Δ F508/R170C and Δ F508/L206W) and 5 had mutations on just one allele (2 N/ Δ F508, N/P205S, N/R31C and N/V920M).

Among the 16 patients with idiopathic chronic pancreatitis, 5 (31.3%) had mutations in the CFTR gene (Δ F508/R851L,

Δ F508/L206W, N/ Δ F508, N/P205S and N/V920M). Two of these patients (Δ F508/L206W and N/P205S), were found to have congenital absence of the vas deferens in addition to chronic pancreatitis. One (Δ F508/R851L) referred only bronchitis in childhood and the last two (N/ Δ F508 and N/V920M) had no other additional signs.

Three of the 64 patients (4.7%) with alcohol-related chronic pancreatitis but with no pulmonary problems also had CFTR mutations: N/ Δ F508, N/R31C and compound heterozygote Δ F508/R170C. None of these 3 patients reported azoospermia.

The 5T variant was found in 8 patients (9.8%) and was present only in the 64 patients with alcohol-related chronic pancreatitis but not among idiopathic chronic cases. No CFTR mutations were found in these 8 heterozygotes for the 5T variant.

The PRSS1 Gene

No mutations in the PRSS1 gene were detected in the two patients with a family history of pancreatitis. The only change observed, among the 82 tested patients, was a single G->A transition at codon 235 in exon 3 (E79K) which was identified in a patient with

Table 1. Mutations and polymorphisms in the CFTR, PRSS1 and SPINK1 genes.

Gene	Localization	Mutation	Polymorphism	Frequency in patients' chromosomes	Frequency in controls' chromosomes	P value
CFTR	Exon 2	R31C		1/164 (0.6%)	-	-
	Exon 5	R170C		1/164 (0.6%)	-	-
	Exon 6	P205S		1/164 (0.6%)	-	-
		L206W		1/164 (0.6%)	-	-
	Exon 10	Δ F508		5/164 (3.0%)	-	-
	Exon 14a	R851L		1/164 (0.6%)	-	-
	Exon 15	V920M		1/164 (0.6%)	-	-
PRSS1	Exon 3		E79K	1/164 (0.6%)	1/300 (0.3%)	1.000 ^a
	Exon 5		N246N	47/164 (28.7%)	85/300 (28.3%)	1.000 ^b
SPINK1	Promoter		-253T>C	20/164 (12.2%)	20/400 (5.0%)	0.004 ^b
			-164G>C	4/164 (2.4%)	13/400 (3.3%)	0.788 ^a
	Exon 1		-7T>G	5/164 (3.0%)	8/300 (2.7%)	0.777 ^a
	Exon 2		c75C>T	1/164 (0.6%)	3/300 (1.0%)	1.000 ^a

^a Fisher's exact test

^b Yates' corrected chi-squared test

alcohol-related chronic pancreatitis, but with no family history. The same E79K mutation was found in one of the 150 control subjects (0.7%). A polymorphism, N246N, was also found in exon 5 of the PRSS1 gene with a similar ($P=1.000$) high frequency in patients (47.6%, 39 patients: 31 heterozygotes and 8 homozygotes) and controls (45.3%, 68 out of 150 subjects: 51 heterozygotes and 17 homozygotes) (Table 1).

The SPINK1 Gene

Four variants in the SPINK1 gene were found in our patients: -253T>C and -164G>C (in the promoter region), -7T>G (in the 5'UTR) and c75C>T (in exon 2). Three of these changes were found with similar frequencies among patients and normal controls (Table 1). All of them were heterozygote: the -164G>C variant was detected in 4 patients (4.9%) and in 13 of the 200 controls (6.5%); the -7T>G variant was detected in 5 patients with chronic pancreatitis (6.1%) and in 8 out of 150 controls (5.3%); the c75C>T variant, a C to T transition at position 75 of the cDNA, is a silent mutation which was observed in one patient (1.2%) with alcohol-related chronic pancreatitis and also in 3 out of 150 controls (2.0%).

The -253C allele was significantly more frequent in patients (22.0%, 18 patients: 16 heterozygotes and 2 homozygotes) than in the control group (10.0%, 20 heterozygotes in 200 controls) ($P=0.004$).

DISCUSSION

A possible bias of our study may be due to the age and gender distribution of the two groups of subjects: the controls were on the average younger than the patients, as well as being prevalently male. However, for idiopathic pancreatitis (chronic as well as inherited), the onset of the symptoms is not correlated to age and sex. On the other hand, in the case of alcoholic pancreatitis, although older patients might have had a longer exposure to alcohol, the onset usually occurs following heavy ingestion of alcohol and may therefore be observed in young adults as well. In addition,

although age cannot influence the frequency of mutations and polymorphisms, it should be taken into account that the younger controls presented a lower risk and exposure of developing chronic pancreatitis.

On the other hand, the fact that the relative proportion of males among patients with alcoholic pancreatitis is greater than among females (64:18) is probably because men drink more than women. Although among the controls there are more women than men (112:88), the sample is large enough to circumvent this difference.

The CFTR Gene

Clinically chronic pancreatitis may occur in 1 to 2% of patients with cystic fibrosis [1]. However, previous studies have shown that 13.4 % of patients with chronic pancreatitis and 37% of patients with idiopathic chronic pancreatitis had mutations in the CFTR gene [6, 7]. This is the first reported study in Brazilian patients affected with chronic pancreatitis. Molecular analysis showed that 9.8% of the total group of patients had mutations in the CFTR gene: 3 were compound heterozygotes ($\Delta F508/R851L$, $\Delta F508/R170C$ and $\Delta F508/L206W$) and 5 had mutations on just one allele (2 N/ $\Delta F508$, N/P205S, N/R31C and N/V920M). The most common mutation was $\Delta F508$, in accordance with other population studies including our previous study on Brazilian patients [21]. Among the 16 patients with idiopathic chronic pancreatitis, 5 had mutations in the CFTR gene ($\Delta F508/R851L$, $\Delta F508/L206W$, N/ $\Delta F508$, N/P205S and N/V920M). Two of these patients ($\Delta F508/L206W$ and N/P205S), were found to have congenital absence of the vas deferens, a condition associated with cystic fibrosis mutations in addition to chronic pancreatitis. One ($\Delta F508/R851L$) referred only bronchitis in childhood and the last two (N/ $\Delta F508$ and N/V920M) had no other additional signs. Three (4.7%) of the 64 patients with alcohol-related chronic pancreatitis but with no pulmonary problems also had CFTR mutations: N/ $\Delta F508$, N/R31C and compound heterozygote $\Delta F508/R170C$.

None of these 3 patients reported azoospermia. This observation caught our attention, in particular, the last patient who was a compound heterozygote ($\Delta F508/R170C$) and in whom the only clinical symptom was pancreatitis. This patient is the father of two children (both heterozygote for the $\Delta F508$ mutation) indicating apparently normal fertility despite carrying two mutations in the CFTR gene.

The 5T variant which reduces the efficiency of exon 9 splicing and reduces the expression of functional CFTR [22] was found in 8 of 164 chromosomes (4.9%), which does not differ from the allelic frequency of this variant (5.0%) in the general population [23]. No CFTR mutations were found in these 8 heterozygotes for the 5T variant. Interestingly however, it was present in 8 (12.5%) of the 64 patients with alcohol-related chronic pancreatitis but not among the idiopathic chronic cases, contrary to previous studies which found a higher frequency of this variant (9.3%) in this group of patients [6].

Ductal obstruction is the primary event in idiopathic chronic pancreatitis and cystic fibrosis. The damage is amplified when bicarbonate decreases pH within the acinar space and lumen of the ductules. The CFTR protein participates in bicarbonate secretion by directly conducting bicarbonate ions across the apical membranes of pancreatic duct cells. A defect in bicarbonate secretion by the pancreatic duct could contribute to this aspect of the disease [24]. Previous studies have associated idiopathic chronic pancreatitis with CFTR mutations [6, 7, 25, 26] while others did not show any association between CFTR mutations and alcohol-related chronic pancreatitis [7, 27]. More recently, a high frequency of CFTR mutations in patients with alcohol-related chronic pancreatitis was reported suggesting that mutations in the CFTR gene could be a risk factor for alcohol-related chronic pancreatitis [28]. The present study confirms the strong association between mutations in the CFTR gene and idiopathic chronic pancreatitis in Brazilian patients.

The PRSS1 Gene

Recent reports indicate an association between several missense mutations in the PRSS1 gene and hereditary pancreatitis [9, 11, 12, 13]. These or other mutations in the PRSS1 gene were not detected in our two patients with a family history of hereditary pancreatitis. The only change observed among the 82 tested patients was a single G->A transition at codon 235 in exon 3 (E79K) which was identified in a patient with alcohol-related chronic pancreatitis, but with no family history. The same E79K mutation was also found in one of 300 normal chromosomes, suggesting that it may be a rare polymorphism. This change was described previously in patients with idiopathic chronic pancreatitis but was also found subsequently in normal controls [29] in accordance with the present results. In addition, a neutral polymorphism, N246N, was also found in exon 5 of the PRSS1 gene with a similar high frequency in patient (28.7%) and control (28.3%) chromosomes. According to some investigators, PRSS1 mutations might have a role in idiopathic chronic pancreatitis but not in alcohol-related chronic pancreatitis [29, 30]. No mutation in the coding region of the PRSS1 gene was found in our patients, suggesting the existence of another gene for hereditary and idiopathic chronic pancreatitis [30, 31] or the possibility of mutations in non-coding regions.

The SPINK1 Gene

Previous studies have identified variants in the SPINK1 gene, in particular, the missense mutation N34S, which has been found with a high frequency in chronic pancreatitis patients [15, 32, 33, 34, 35]. Very recently, it has been shown that this mutation increases the susceptibility for fibrocalculous pancreatic diabetes in subjects from the Indian sub-continent although it is not known if this mutation is directly causative of chronic pancreatitis [36]. Interestingly, this change

was not found in our patients or in 400 control chromosomes and, therefore, does not seem to increase the susceptibility for pancreatitis in our population. However, we found four variants in the SPINK1 gene among our patients: -253T>C and -164G>C (in the promoter region), -7T>G (in the 5'UTR) and c75C>T (in exon 2). Two of them, c75C>T and 164G>C in the promoter region, were apparently not reported previously.

Three of these changes were found with similar frequencies among patients and normal controls and probably represent neutral polymorphisms. The -164G>C variant, was detected in 4 of 164 patient chromosomes (2.4%) and in 13 of 400 control chromosomes (3.3%). The -7T>G variant, which probably does not affect the acceptor splice site was detected in 5 of 164 chromosomes of the patients with chronic pancreatitis (3.0%) and in 8 of 300 control chromosomes (2.7%). The c75C>T variant, a C to T transition at position 75 of the cDNA, is a silent mutation observed in one chromosome of patients with alcohol-related chronic pancreatitis (0.6%) and also in 3 of 300 control chromosomes (1.0%). Therefore, these three variants do not seem to increase the susceptibility for pancreatitis.

However, the -253T>C variant caught our attention. The -253C allele was significantly more frequent in patients than in controls (12.2% vs. 5.0%; P=0.004). Although the -253T>C variant is localized in the non-coding region, alterations in these regions have been associated with other diseases; for example, the IVS8Tn variant in the CFTR gene [22] was associated with cystic fibrosis, and a polymorphism in the transcriptional control region upstream of the serotonin transporter (5-HTT) gene [37, 38] was associated with different psychiatric disorders [39, 40].

The observation that the -253T>C genotype was 2.4 times more frequent among patients than controls led us to suggest that this polymorphism might result in a different transcriptional efficiency conferring an increased risk of developing pancreatitis in Brazilian patients. Other population studies

detected this alteration in patients and in controls with similar frequencies [11, 16, 19] which might be due to ethnic differences in different populations. Interestingly, the N34S polymorphism in the SPINK1 gene which is apparently common (4.4% of non-diabetic subjects) in Southern India [36] and other populations [32, 33, 34, 35] was not found in 300 Brazilian control chromosomes. This observation was confirmed by two different methods. However, other polymorphisms in the promoter region of the serotonin transporter (5-HTT) gene also showed significantly different distributions in the Japanese [39] as compared to Brazilians [40] indicating that these population differences are probably a common finding. In short, the present study adds to the notion that the contribution of genetic factors to the pathogenesis of complex conditions such as pancreatitis may differ in different populations. In addition, it would be of great interest to confirm by means of functional analysis whether or not the -253T>C genotype increases the susceptibility for developing pancreatitis in Brazilian patients.

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Abbreviations SSCP: single-strand conformation polymorphism; TCR-beta: T cell receptor beta chain gene

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