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ARTICLE

Mutation analysis of the cystic fibrosis transmembrane conductance regulator (*CFTR*) gene, the cationic trypsinogen (*PRSS1*) gene, and the serine protease inhibitor, Kazal type 1 (*SPINK1*) gene in patients with alcoholic chronic pancreatitis

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Susceptibility to alcoholic chronic pancreatitis (ACP) could be genetically determined. Mutations in cationic trypsinogen (*PRSS1*), cystic fibrosis transmembrane conductance regulator (*CFTR*), and serine protease inhibitor, Kazal type 1 (*SPINK1*) genes have been variably associated with both the hereditary and the idiopathic form of chronic pancreatitis (CP). Our aim was to analyze the three genes in ACP patients. Mutational screening was performed in 45 unrelated ACP patients and 34 patients with alcoholic liver disease (ALD). No mutation of *PRSS1* was found in ACP and ALD patients. Three mutations of *CFTR* were detected in four ACP patients with a prevalence (8.9%) not significantly different from that observed (3.0%) in ALD patients and from that expected (3.2%) in our geographical area. Neither compound heterozygotes for *CFTR* nor trans-heterozygotes for *CFTR/SPINK1* were found. One ACP patient (2.2%) was found to carry the most common mutation (*N34S*) of *SPINK1* compared to none of the ALD patients (P = NS). In five other patients (two with ACP and three with ALD) other rare variants, including *P55S*, were found. In contrast with the hereditary and the idiopathic forms of CP, in which mutations of *PRSS1*, *CFTR*, and *SPINK1* genes may occur, ACP is still a 'gene(s)-orphan' disease. The supposed genetic susceptibility to ACP relies on other yet unknown gene(s) which could affect the alcohol metabolism or modulate the pancreatic inflammatory response to alcohol abuse.
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Introduction

Chronic pancreatitis (CP) is a potentially life-threatening disease characterized by a progressive inflammatory disorder ultimately leading to irreversible morphological changes and permanent impairment of exocrine and endocrine functions. Most patients with CP suffer from relapsing attacks of abdominal pain and are at a markedly increased risk of developing maldigestion, diabetes mellitus, and pancreatic cancer.[1] Alcoholism is the most common etiologic factor in up to 70% of patients with CP.[2] Other causes include drugs, duct-obstructing lesions, and metabolic or autoimmune disorders. In the rare form of hereditary pancreatitis (HP), at least two major

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mutations (*R122H* and *N29I*) in the cationic trypsinogen gene (protease, serine 1, *PRSS1*; *OMIM*: 276000) have been identified.[3,4] *In vitro* biochemical studies suggest that these two mutations and additional pancreatitis-associated *PRSS1* mutations[5] may inhibit autolysis of trypsin and/or enhance autoactivation of trypsinogen, resulting in a gain of trypsin.

In up to 30% of patients, association with any of the aforementioned factors is lacking and the disease is classified as idiopathic (ICP). Some evidence exists that at least in a small proportion of patients with ICP, a mutation of one or both alleles of either the cystic fibrosis transmembrane conductance regulator (*CFTR*; OMIM: 602421) gene[6,7] or the serine protease inhibitor, Kazal type 1 (*SPINK1*; OMIM: 167790) gene[8,9] can be identified. Severe mutations in both alleles of the *CFTR* results in the commonly recognized cystic fibrosis (CF) clinical features of abnormal sweat chloride concentrations, pancreatic insufficiency, and progressive pulmonary disease. Among CF patients, two-thirds have a deletion of three-base pair between the nucleotides 1652 and 1655 with subsequent deletion of the phenylalanine amino acid at codon 508 (F508del), although approximately 1000 other mutations have been reported. Most *CFTR* mutations can be classified according to a 1–5 severity category system based on the presumed or demonstrated molecular consequences.[10] Typical CF patients with severe pancreatic impairment tend to have two severe mutations (ie, class I, II, or III), whereas CF patients with pancreatic sufficiency from birth tend to have at least one CF 'mild allele' (ie, class IV or V).[11]

SPINK1 is a peptide that is synthesized by pancreatic acinar cells and colocalizes with trypsinogen in the zymogen granules. *SPINK1* acts as the first line of defence against prematurely activated trypsinogen in the acinar cells by physically blocking the active site of trypsin.[8] The most known mutation (*N34S*) of the *SPINK1* gene is relatively common (up to 4% of general population)[12] and markedly increased (up to 25%) in ICP patients.[13] Apart from *N34S*, other variants (like P55S) have been detected in both the general population and patients with ICP.[8] The pathogenic role of *SPINK1* is still a matter of debate. Since the prevalence of ICP is low (1/16 000)[14] and the *SPINK1* gene mutations are relatively common, it has been speculated by some authors that the mutated *SPINK1* gene behaves as a disease modifier gene that either lowers the threshold for initiating pancreatitis or worsens the severity of pancreatitis caused by other genetic or environmental factors.[13] Other authors, however, assume that the *SPINK1* gene behaves as a disease inducer gene since the N34S mutation would render *SPINK1* incapable of inhibiting trypsinogen.[8] Functional investigations are currently underway in different laboratories to elucidate the exact pathophysiologic mechanism of *SPINK1* mutations.

The discovery of gene mutations that induce or predispose to CP led some researchers to investigate about a possible causal role of genetic factors in the occurrence and development of alcoholic chronic pancreatitis (ACP)[6,15–18]. Evidence for a genetic basis for ACP comes from epidemiological, laboratory, and clinical studies: only about only 5–10% alcoholics suffer from clinically recognized CP.[19] Although a linear correlation exists between the risk of developing CP and the quantity of alcohol consumption, there is no apparent threshold of toxicity.[20] Long-term, high-dose alcohol feeding of laboratory animals fails to cause CP.[21] Finally, black patients are two to three times more likely to be hospitalized for CP than alcoholic cirrhosis when compared to white patients.[22] To date, most of the relevant studies in ACP performed mutation analysis of one single gene, that is *PRSS1*, or *CFTR*, or *SPINK1*. Moreover, due to differences in the selection and number of participants and the mutation screening method used, the reported mutation rates observed in one single gene varied greatly among different studies. A simultaneous analysis of all three genes in patients with ACP would provide insights into the relative contribution of each gene to the etiology of this disease.

The aim of this study was to perform this kind of analysis by screening the most relevant mutations of the *PRSS1*, the *CFTR*, and the *SPINK1* genes in Italian patients with ACP. As controls, we screened patients from the same geographical area who were affected by alcoholic liver disease (ALD) without a clinically recognized pancreatic disease.

Materials and methods

Patients

Patients with ACP and ALD, who were consecutively admitted to our Institution between 2000 and 2002, were enrolled for genetic analysis.

ACP was defined as a condition characterized by: (1) at least two clearly separated episodes of pancreatitis (recurring abdominal pain with a more than five-fold increase of serum lipase and amylase); (2) a reported alcohol consumption higher than 80 g per day in men and more than 60 g per day in women for more than 2 years; (3) a negative family history of pancreatitis; (4) no other obvious causes of CP, such as trauma, drugs, and obstructive biliary diseases, and (5) evidence of pancreatic calcifications on abdominal X-ray film or CT.

ALD was defined as a condition characterized by: (1) a chronic (more than 1 year) impairment of liver function tests or presence of overt clinical signs of liver cirrhosis; (2) a reported alcohol consumption higher than 80 g per day in men and more than 60 g per day in women for more than 2 years; (3) no other recognizable causes of liver diseases such as viral hepatitis, drug-induced or autoimmune hepatitis, and metabolic diseases; (4) no clinical, biochemical, or radiological signs of CP; and (5) histological signs of ALD (whenever a liver biopsy was performed to confirm the clinical suspicion).

The study protocol was approved by the local Hospital's Review Board and all patients provided informed consent.

DNA extraction

Genomic DNA was extracted from whole blood according to the established protocols using the DNA Isolation Kit for Mammalian blood (Roche Diagnostic Corporation, Indianapolis, IN, USA).

Mutation screening of the PRSS1 gene

All the five exons and exon/intron junctions of the *PRSS1* gene were analyzed by means of denaturing gradient gel electrophoresis (DGGE) analysis, as described by Chen *et al*.[9] Samples showing abnormal DGGE profiles were sequenced using the ABI Prism 377 DNA Sequencer (Applied Biosystems, Foster City, CA, USA).

Mutation screening of the CFTR gene

The 31 most frequent mutations (*F508del, I507del, G551D, G542X, N1303K, 1717-1G>A, W1282X, R553X, R347P, R347H, R334W, 3849+10kb C>T, R117H, 621+1G>T, A455E, S549N, R560T, S549R, V520F, Q493X, 3849+4A>G, 1078delT, R1162X, 3659delC, 3905insT, Y122X, 2183delAA>G, 2789+5G>A, 1898+1G>A, 711+1G>T, and G85E*) were examined with the polymerase chain reaction (PCR) followed by an oligonucleotide ligation assay (OLA, Applied Biosystems, Foster City, CA, USA) and finally a sequence-coded separation. Other seven mutations, frequently observed in Italian CF patients (*Q552X; 711+5G>A; 2790-2A>G; S589N; T338I; 1898+3A>G, and 1717-8G>A*), were examined by sequencing. Using both methods in the general population from the same geographical area (San Giovanni Rotondo) in which our study was performed, Gasparini *et al*.[23] found a carrier frequency of *CFTR* mutations of 3.22%. The *CFTR* polymorphic intron 8 polyT region was analyzed according to the method described by Chillon *et al*.[24] This sequence contains five, seven, or nine thymidines (*5T, 7T, 9T*, respectively). The *5T* allele is associated with decreased splicing efficiency of intron 8, which results in the reduced levels of normal *CFTR* mRNA.

Mutation screening of the SPINK1 gene

Molecular study of the *SPINK1* gene was performed by PCR amplification and sequencing of the entire coding region and the intron/exon junctions, as described by Witt *et al*.[8] All mutations were confirmed by means of RFLP (restriction endonuclease: *TaaI*, MBI Fermentas, Germany) or PCR with confronting two pair primer (PCR-CTPP)[25] assays.

Statistical analysis

Univariate analysis was preliminarily performed for sex, age, age at symptoms' onset, symptoms' duration, smoking habits, and alcohol consumption by sorting patients according to their disease (ACP or ALD). Within each group, patients were then sorted into three subgroups according to the mutation analysis results: one subgroup without *CFTR* or *SPINK1* mutations and the other two subgroups with either *CFTR* or *SPINK1* mutations. Univariate analysis was carried out among the three subgroups for sex, age, age at symptoms' onset, symptoms' duration, smoking habits, and alcohol consumption.

Student' s *t*-test was used for age, age at symptoms' onset, symptoms' duration, and alcohol consumption. Pearson's χ^2-test was used for sex and smoking habits. Comparison of mutation frequency between ACP and ALD patients was performed by means of Fisher's exact test (expected values lower than 5 per cell). A *P*-value less than 0.05 was considered to indicate statistical significance.

Results

Patients

A total of 45 unrelated Caucasian patients (44 males; mean age: 38 years) with ACP and 34 patients (28 males; mean age: 44 years) with ALD were studied. We did not find any significant difference between the two groups of patients as far as alcohol consumption (160 ± 40 *vs* 140 ± 40 g/day in men with ACP and ALD, respectively) and smoking habits (26 (57.8%) ACP and 19 (55.9%) ALD patients had a current or past smoking history) was considered. Furthermore, no specific association was observed between any demographic or clinical subjects' characteristics and gene mutations (Table 1).

Mutation analysis of the PRSS1 gene

Mutation analysis of *PRSS1* was performed in all patients with ACP and ALD. No mutation of the gene was found in both patient groups (Tables 2 and 3).

Mutation analysis of the CFTR gene

Mutation analysis of *CFTR* was performed in all ACP patients and in all ALD patients except one due to insufficient DNA sample. In four ACP patients, three mutations were detected in heterozygous state (Table 2) with a prevalence rate of 8.88%. This prevalence was not significantly different from the expected prevalence of 3.22% ($P = 0.64$) in our geographical area. No patient with ACP carrying *CFTR* mutations had the *5T* allele (Table 2). A single mutation in the heterozygous state was detected in one ALD patient with a prevalence rate (3.03%) similar to that expected in the general population. This patient also had a *5T* allele in the heterozygous state (Table 3). Neither compound heterozygotes for *CFTR* nor trans-heterozygotes for *CFTR/SPINK1* genes were found in our study population (Tables 2 and 3).

Mutation analysis of the *SPINK1* gene

Mutation analysis identified the most known mutation (*N34S*) of *SPINK1* in one ACP patient (2.2%). compared to none of the ALD patients ($P =$ NS). *P55S* (a likely

Table 1 Demographic and clinical data

ACP	All	*CFTR Mut*	*SPINK1 Mut*	*Mut negative*
N	45	4	3	38
Sex (M/F)	44/1	4/0	3/0	37/1
Age (years)	38±5	36±3	37±4	38±5
Age at onset (years)	30±5	29±6	30±5	32±6
Symptoms' duration (years)	8±2	7±3	7±3	6±3
Alcohol (g/day)	160±43	130±20	133±23	166±44
Smokers	26	3	2	21
ALD				
N	34	1	3	30
Sex (M/F)	28/6	1/0	3/0	24/6
Age (years)	44±6	46	39±7	44±6
Age at onset (years)	36±7	35	33±11	37±6
Symptoms' duration (years)	8±3	11	7±4	6±3
Alcohol (g/day)	140±41	140	133±23	140±43
Smokers	19	1	1	17

Table 2 Sequence variations identified in the *PRSS1*, *CFTR*, and *SPINK1* genes in 45 ACP patients

			CFTR		
Patients	*n*	*PRSS1*	*Mutant*	*Poly T*	*SPINK1*
1	1	—[a]	*F508del*	ND[b]	—
2	1	—	*G542X*	7/9	—
3	1	—	*711+1G>T*	7/7	—
4	1	—	*711+1G>T*	7/7	—
5	1	—	—	7/7	*P55S*
6	1	—	—	5/7	*IVS2-23A>T*
7	1	—	—	7/9	*N34S*
8	1	—	—	7/7	ND
9–10	2	—	—	5/7	—
11–37	27	—	—	7/7	—
38–45	8	—	—	7/9	—

[a]indicates two wild alleles. [b]ND, not done due to insufficient DNA sample.

Table 3 Sequence variations identified in the *PRSS1*, *CFTR*, and *SPINK1* genes in 34 ALD patients

			CFTR		
Patients	*n*	*PRSS1*	*Mutant*	*Poly T*	*SPINK1*
1	1	—[a]	—	ND[b]	*P55S*
2	1	—	—	7/7	*P55S*
3	1	—	—	7/7	*MIT*
4	1	—	*G85E*	5/7	—
5	1	—	—	5/5	—
6	1	—	—	5/9	—
7–8	2	—	—	5/7	—
9–27	19	—	—	7/7	—
28	1	—	—	7/7	ND
29–34	6	—	—	7/9	—

See Table 2.

polymorphism) and two other rare variants (*IVS2-23A>T* and *M1T*) were found in five patients (two with ACP and three with ALD) (Tables 2 and 3).

Discussion

The pathogenesis of ACP is still an unresolved problem. Several different theories have been made such as duct obstruction by protein plugs, direct toxicity of ethanol, and oxidative stress.[26] Very recently, fatty acid ethyl esters, which are by-products of the nonoxidative metabolism of ethanol in the pancreas, have been proposed as possible etiologic factors.[27] However, none of these mechanisms has yet found a solid experimental support to gain wide acceptance.

As already mentioned, evidence for a genetic basis for ACP comes from epidemiological, laboratory, and clinical studies.[19–22] Although susceptibility to alcoholic pancreatic damage could be inherited, until now no clear association between any gene mutation(s) and occurrence of ACP in alcoholics has been found. In particular, during the last 6 years, three single genes (*PRSS1*, *CFTR*, and *SPINK1*) have been investigated in patients with ACP.

Screening for mutations of *PRSS1* has been always negative[16,17,28] confirming that *PRSS1* mutations have no role in determining or predisposing to pancreatic damage by alcohol. Mutations of *CFTR* (including mutations in the noncoding sequence of thymidines in intron 8) have been detected in patients with ACP, but their prevalence is similar to that expected in the general population.[6,15,17,29] Finally, the most common mutation (*N34S*) in *SPINK1* has been found more frequently associated in patients with ACP than in healthy controls or in patients with alcoholism but without CP.[18,30] However, this finding has not been confirmed in any another study.[12]

In the present study, we performed a simultaneous analysis of all the three genes in patients with ACP. Our working hypothesis was that the pancreatic damage due to high alcohol intake could be due to either an abnormal allele in one of the three genes or a combination of multiple mutations occurring in the two alleles of the same gene (compound heterozygote) or different genes (trans-

heterozygote). This hypothesis has already been successfully tested in both ICP and tropical calcific pancreatitis (TCP). The latter is an idiopathic, juvenile, nonalcoholic form of CP widely prevalent in several tropical countries. By simultaneously analyzing 39 ICP subjects for common mutations of *CFTR*, *SPINK1*, and *PRSS1*, Noone *et al*.[31] found that about 60% of their patients had at least one mutation in either *CFTR* or *SPINK1*, or both. Interestingly, the risk of pancreatitis was increased approximately five-fold by having one *CFTR* mutation, 20-fold by having *SPINK1* N34S mutation, 40-fold by having two *CFTR* mutations (compound heterozygotes), and 900-fold by having N34S and two *CFTR* mutations. By using a similar approach, Audrézet *et al*.[32] found that at least 30% of 39 French patients with ICP carried at least one abnormal allele in one of the three genes, with a compound heterozygote state for CFTR in four patients and a trans-heterozygote state for *SPINK1/CFTR* genes in other three patients. Very recently, Chandak *et al*.[33] found that about half of the patients suffering from TCP carried a mutation (predominantly the *N34S*) in the *SPINK1* and no mutation in the *PRSS1* gene. These findings are consistent with the hypothesis that all these genes may be involved in the pathogenesis and phenotypic variability of both ICP and TCP. Unfortunately, our findings in ACP were disappointing since only seven patients were found to carry at least one mutation in the heterozygous state in either *CFTR* or *SPINK1*. Moreover, no compound heterozygote or trans-heterozygote was observed. The prevalence of these mutations was not significantly different between patients with ACP and those with ALD. Three patients also had the *5T* allele in intron 8, but none of them showed a concurrent mutation in *CFTR*.

Mutational screening of *PRSS1* and *SPINK1* genes was performed by techniques similar to those adopted by other research groups. Consequently, methodological problems are not likely to be involved in our negative findings. In contrast, we did not perform a complete analysis of the *CFTR* gene, which consists of 24 exons encoding 1480 amino acids, but we investigated only a selected subset of the most common *CFTR* mutations in CF. Therefore, minor ('mild') mutations in the *CFTR* gene cannot be completely excluded in our patients since the entire gene would have been sequenced. It would be tempting to speculate that atypical *CFTR* alleles are likely to be associated with a higher residual *CFTR* function, which by itself is unable to induce pancreatic damage but in combination with a heavy alcohol intake could be sufficient to determine pancreatitis. Nevertheless, the functional consequence of minor variants is still unknown especially when no concurrent major mutation in the *CFTR* gene is detected in the same patient. Moreover, one would speculate that an alcoholic drinker who simultaneously carries a major mutation of the *CFTR* gene in one allele and a minor mutation of the same gene in the other allele is likely to be more affected by an atypical form of CF with CP than ACP. In the present study, the prevalence of mutations of *CFTR* in ACP patients was similar to that observed in ALD and expected in normal population from the same geographical area.

Finally, two potential drawbacks of the present work should be pointed out. The lack of a significant difference in the prevalence of gene mutations between ACP and ALD patients could be affected by a beta error due to the small sample size. The use of ALD patients as pathological controls does not afford us to extrapolate the conclusions of this study to a healthy population. Future studies should be designed with large sample sizes including healthy controls in order to avoid these potential shortcomings.

In summary, this study shows that mutations in *PRSS1*, *CFTR*, and *SPINK1* genes are occasionally found in patients with ACP, but their prevalence is not significantly increased in comparison with alcoholics who develop ALD without clinical, biochemical, or radiological signs of CP. In striking contrast with the HP and the so-called ICP in which mutations of these genes occur at variable frequency, ACP is still a 'gene(s)-orphan' disease. The supposed genetic susceptibility to pancreatic alcohol damage should be searched in other yet unknown genes that could affect the alcohol metabolism or modulate the pancreatic inflammatory response to alcohol abuse.

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