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Germline Mutations in *CFTR* and *PSTI* Genes in Chronic Pancreatitis Patients

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Mutations in the cationic trypsinogen, cystic fibrosis transmembrane conductance regulator (*CFTR*) and pancreatic secretory trypsinogen inhibitor (*PSTI*) genes have recently been associated with chronic pancreatitis. This paper investigates the frequency of *CFTR* and *PSTI* gene mutation in patients with idiopathic and alcoholic chronic pancreatitis, the clinical course of patients with these two kinds of disease, and examines the clinical differences between carriers and noncarriers of mutation. In idiopathic pancreatitis a significant increase was found in mutation frequency both in the *CFTR* gene (13%) and N34S mutation in the *PSTI* gene (3.9%), as well as an increase in familial disposition to pancreatic disorders. In alcohol-induced pancreatitis an increase in calcification, exocrine insufficiency, and diabetes mellitus was observed. In conclusions, mutations in the genes investigated are involved in causing idiopathic pancreatitis. Such mutations have no connection either with the age at onset or the clinical course of the disease.

KEY WORDS: alcoholic chronic pancreatitis; idiopathic chronic pancreatitis; *CFTR* mutations; *PSTI* mutations.

Chronic pancreatitis is an inflammatory disease that causes structural and progressive damage to the organ, resulting in a permanent deficit both to the exocrine and endocrine components (1). Alcohol is the main risk factor and principal cause, although the mechanism responsible for disease onset and progressive damage to the pancreas remains unclear.

In recent years three genes have been identified as being involved in disease onset: the cationic trypsinogen gene, responsible for some cases of hereditary pancreatitis (2–4), and the CFTR (5–8) and PSTI (9, 10) genes, involved in sporadic idiopathic chronic pancreatitis. Mutations in the cationic trypsinogen gene reduce physiological inactivation of trypsin that forms within the pancreas; cationic trypsin then remains active and resistant to autolysis (3). The succession of events from trypsinogen activation leading to digestion of the pancreas depends, therefore, on the defense mechanisms of the acinar cell, ie, the ability to control oxidative stress, prevent intracellular hypercalcemia, inhibit trypsin, and repair damage.

Hereditary pancreatitis, however, is the cause of less than 1% of chronic pancreatitis. About 30% of the cases do not have a known cause and are defined as idiopathic. Cystic fibrosis (CF), is the most frequent recessive autosomal genetic disease found in Caucasians. Its characteristic features are chronic pulmonary disorder; pancreatic fibrosis, which usually leads to gland insufficiency; obstructive azoospermia in males due to atresia of the vas deferens; and an increase in chloride concentrations in the sweat. Cystic fibrosis attacks different organs in different ways, but foreseeable in function of the CFTR genotype

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(11). Genotypes that reduce CFTR protein function to 1% of its normal value cause typical cystic fibrosis, characterized by pulmonary disorders, pancreatic insufficiency, congenital bilateral absence of the vas deferens (CBAVD), and sweat test alteration. Genotypes that reduce CFTR protein function to 5% of normal values cause cystic fibrosis without pancreas insufficiency, while those that reduce it to 10% cause CBAVD.

Variant T5 of intron 8 is an example of a *CFTR* allele causing CBAVD or chronic pancreatitis without CF pulmonary disorder when it is associated with two polymorphisms: TG12 in the (TG)m tract situated immediately upstream from the poly-T tract in intron 8 and the V codon at position 470 in exon 10. PSTI is a protein that prevents trypsinogen and proteolytic cascade activation within the pancreatic cell (12). Mutations that alter the protein or that reduce its effect have been found in sporadic and chronic hereditary pancreatitis patients (9, 10).

Our paper investigates mutation frequency in *CFTR* and *PSTI* genes in patients with alcoholic and idiopathic chronic pancreatitis and compares them with the general population. The clinical course of these two kinds of pancreatitis were evaluated and clinical differences were sought between *CFTR* and *PSTI* gene mutation carriers and noncarriers.

MATERIALS AND METHODS

In our study we enrolled 77 consecutive adult patients with chronic pancreatitis under care in the Pancreatological Ward at the A.S.O. San Luigi Gonzaga Hospital at Orbassano and in the Gastroenterological Ward at the A.S.O. Mauriziano Umberto I in Turin from January 1996 to July 2001. Most of them were symptomatic; only a minority was identified incidentally by imaging findings suggestive of chronic pancreatitis. The patients were divided into two groups according to whether their clinical history was positive for drinking (>80 g/day for at least three years). Twenty-one patients (17 males and 4 females, average age 56 years) were suffering from alcoholic chronic pancreatitis (ACP) and 56 (31 males and 25 females, average age 48 years) from idiopathic chronic pancreatitis (ICP). Chronic pancreatitis was diagnosed according to the morphologic characteristics of the pancreas, determined using either radiological (US, CT, MNR, ERCP) or histologic examination. These were then associated to clinical criteria and hyperamylasemia during abdominal pain, dyspepsia, exocrine insufficiency (with or without steatorrhea), and diabetes mellitus.

The case history of each patient was evaluated for family history with pancreatic disorders (chronic or acute pancreatitis, pancreatic carcinoma, and CF); biliary lithiasis (gallstones, microlithiasis, "biliary sand," or previous cholecystectomy due to cholelithiasis); complications (pseudocysts, pseudoaneurysms, inflammatory masses), and surgery. Finally, the age at onset was investigated; for eight patients it was not possible to set a precise date for disease onset.

Genomic DNA for analysis of *CFTR* and *PSTI* was initially isolated from peripheral blood samples by conventional methods for molecular analysis of DNA. A sample of whole blood was drawn from each patient and stored at −20°C until DNA extraction by a standard method. Screening for *CFTR* gene mutations was carried out in all patients using denaturant gradient gel electrophoresis (DGGE) of all 27 exons as described by Fanen et al (13), and samples presenting band shifts were directly sequenced (ABI Prism 377, Perkin Elmer). The intron 8 polypyrimidine tract length (poly-T) was analyzed in all 77 patients as described in Chillon et al (14).

The (TG)m length was determined in those patients carrying the poly-T T5 allele by sequencing the intron 8/exon 9 junction from a PCR product amplified using primers 9i-5 and 9i-3 (15).

All patients were examined to establish the presence of mutation N34S in the *PSTI* gene by amplifying genomic DNA with oligonucleotides PSTIex3F (5′-CCC AAT CAC AGT TAT TCC CC-3′) and N34Hind (5′-CCT GGT GCA TCC ATT AAG TTG A-3′). Since the amplifying product has a site for the restriction enzyme HindII, if mutation N34S is present it is revealed by digestion. The exon 3 sequence of the *PSTI* gene confirmed the presence of the mutation.

Fecal elastase 1 (ELA-1) was determined for all patients using ELISA (ScheBo-Tech GmbH, Wettemberg, Germany). The antibody is specific for human ELA-1 and does not cross-react with the elastase of animal origin found in exogenous pancreas extracts (16). Samples of feces collected during acute pancreatitis or during recurrent attacks were not examined. Values of fecal ELA-1 < 200 μg/g in at least two different determinations were considered as indicating exocrine insufficiency.

The mutation frequency found in our patients was compared with that of the general population, and data were analyzed using the χ^2. All values of $P < 0.05$ were considered statistically significant. The frequency of the various clinical characteristics of ACP was compared with the frequency of ICP. The same comparison was made between mutation-carrying and noncarrying patients. Data found by χ^2 test were considered statistically significant at $P < 0.05$.

The statistical significance of the comparison between mutation carriers and patients without mutation, as compared to age at disease onset and fecal elastase values, was determined using the Students t test (significant values for $P < 0.05$).

RESULTS

Our case history identified *CFTR* gene mutations in 10 ICP patients (13%), 8 males and 2 females (Table 1). All mutations (W1282X, N187K, R352Q, ΔF508, R75Q, R31C, 621+2T->G, I197V, K68N, R1162X) were found in heterozygotes, indicating that these patients are carriers of a single mutation. The frequency of mutation carriers of the *CFTR* gene in the

TABLE 1. PATIENTS CARRYING THE *CFTR* MUTATION*

Pt	*Sex*	*Age (yr)*	*Age at onset (yr)*	*Alcohol (g/day)†* (≤10)	(10–40)	(40–80)	*Familial*	CFTR *mutations*	*Exocrine insufficiency*	*Diabetes mellitus*
T.B.	M	59	23	(≤10)			No	W1282X	Yes	No
B.G.	M	40	29	(≤10)			Yes	N187K	No	No
E.P.	M	40	34	(≤10)			No	R352Q	No	Yes
D.N.	M	53	47		(10–40)		No	R75Q	Yes	No
R.L.	F	57	44	(≤10)			No	R31C	No	No
T.F.	M	56	*‡	(≤10)			No	621 + 2T → G	Yes	No
F.G.	M	54	46		(10–40)		No	I197V	Yes	No
V.M.	M	65	*†		(10–40)		No	K68N	Yes	No
B.L.	F	57	56		(10–40)		Yes	ΔF508	No	Yes
T.G.	M	25	24	(≤10)			No	R1162X	No	No

*This table shows the characteristics of chronic pancreatitis patients, carriers of CFTR mutations.
†No patient drank >40 g/day.
‡Not known.

general population is 4% (1/25) (17). Therefore, the mutation frequency was significantly higher as compared to the general population ($\chi^2 = 6.79$, $P = 0.009$).

The presence of polymorphism T5 was found in eight male ICP patients (Table 2). In six the polymorphism is associated to TG12 (haplotype TG12/T5); two are carriers of haplotype TG11/T5. The frequency of the T5 allele carriers in the general population is around 10% (18); we found a similar value (10.4%; 8/77) in our patients, but the relatively small number of patients studied (77) is insufficient to state that the frequencies are the same.

The N34S mutation was found in three cases (two females and one male) of ICP (Table 3). In a fourth patient (a woman with a form of idiopathic pancreatitis), HindII enzyme digestion showed weight bands different than expected for mutation N34S. The subsequent exon 3 sequencing in this patient revealed the insertion of an adenine after nucleotide 98 (98insA). This creates a restriction site for the HindII enzyme leading to a TAC (tyrosine) codon change in position 33 in TAA (stop) thus producing a truncated protein. The frequency of mutation N34S in the general population is 0.77% (10). Our investigation, however, produced a statistically significant mutation frequency of 3.9% (3/77) ($\chi^2 = 6.81$, $P = 0.009$).

None of the patients examined was homozygous for one mutation or heterozygote for two mutations. No mutation was found in ACP patients.

As regards the clinical characteristics of ACP and ICP patients, an increased frequency of pancreatic disease was found in the idiopathic patients' families ($\chi^2 = 3.74$, $P = 0.04$) (Figure 1). In particular, 19 of 56 ICP patients had a history of familial pancreatic disease. Among these, two are carriers of a mutation in the *CFTR* gene, one in the *PSTI* gene, two showed T5 polymorphism (one with genotype TG11/T5 and one with genotype TG12/T5). Among ACP patients, on the other hand, increased frequencies were found in calcification ($\chi^2 = 14.75$, $P = 0.0001$), exocrine insufficiency ($\chi^2 = 3.73$, $P = 0.05$) and diabetes

TABLE 2. POLY-T POLYMORPHISM CARRIERS*

Pt	*Sex*	*Age (yr)*	*Age at onset (yr)*	*Alcohol (g/day)†* (≤10)	(10–40)	(40–80)	*Familial*	*Poly-T*	*Exocrine insufficiency*	*Diabetes mellitus*
N.G.	M	44	34	(≤10)			No	TG12T5	Yes	No
C.S.	M	43	42		(10–40)		No	TG11T5	Yes	No
G.M.	M	39	33		(10–40)		Yes	TG12T5	Yes	No
M.R.	M	60	56		(10–40)		No	TG12T5	Yes	No
P.N.	M	69	64	(≤10)			No	TG12T5	Yes	Yes
A.G.	M	59	42	(≤10)			No	TG12T5	Yes	Yes
P.V.	M	54	*‡	(≤10)			No	TG12T5	No	No
S.U.	M	52	50	(≤10)			Yes	TG11T5	No	No

*This table shows the genotypic and phenotypic characteristics of chronic pancreatitis patients, carriers of the T5 allele.
†No patient drank >40 g/day.
‡Not known.

TABLE 3. PATIENTS CARRYING N34S AND 98INSA *PSTI* MUTATIONS*

Pt	*Sex*	*Age (yr)*	*Age at onset (yr)*	*Alcohol (g/day)*	*Familial*	PSTI *mutation*	*Exocrine insufficiency*	*Diabetes mellitus*
T.L.	F	32	29	(≤10)	Yes	N34S	No	No
D.G	M	56	39	(≤10)	No	N34S	Yes	Yes
O.S.	F	34	33	(≤10)	No	98insA	No	No
T.M	F	70	63	(≤10)	No	N34S	No	No

*This table shows the genotypic and phenotypic characteristics of chronic pancreatitis patients, carriers of PSTI mutations.

mellitus ($\chi^2 = 9.64$, $P = 0.001$), as compared to the idiopathic forms (Figure 1).

No significant difference was found in the clinical characteristics of patients with or without the ICP mutation.

DISCUSSION

The increase in *CFTR* and *PSTI* gene mutations found in this investigation, as compared to the general population, suggests they are involved in disease onset. Mutations of these genes are present in about 40% of the ICP patients investigated (22/56), while none were found in those with ACP. Furthermore, mutations in the *CFTR* gene are more frequent in males than in females.

Of the 10 *CFTR* gene mutations found, three belong to class I (W1282X, 621+2T->G, R1162X) and are associated to absence of or reduced proteic synthesis. One mutation belongs to class II (ΔF508) and is characterized by an intracellular protein maturation and translocation defect. Four mutations were found in patients with mild forms of CF (R31C, K68N, R75Q, and R352Q). The remaining two (N187K and I197V) are missense mutations that have never been observed before.

The remarkable variety of mutations observed, although many are only mild, is in agreement with

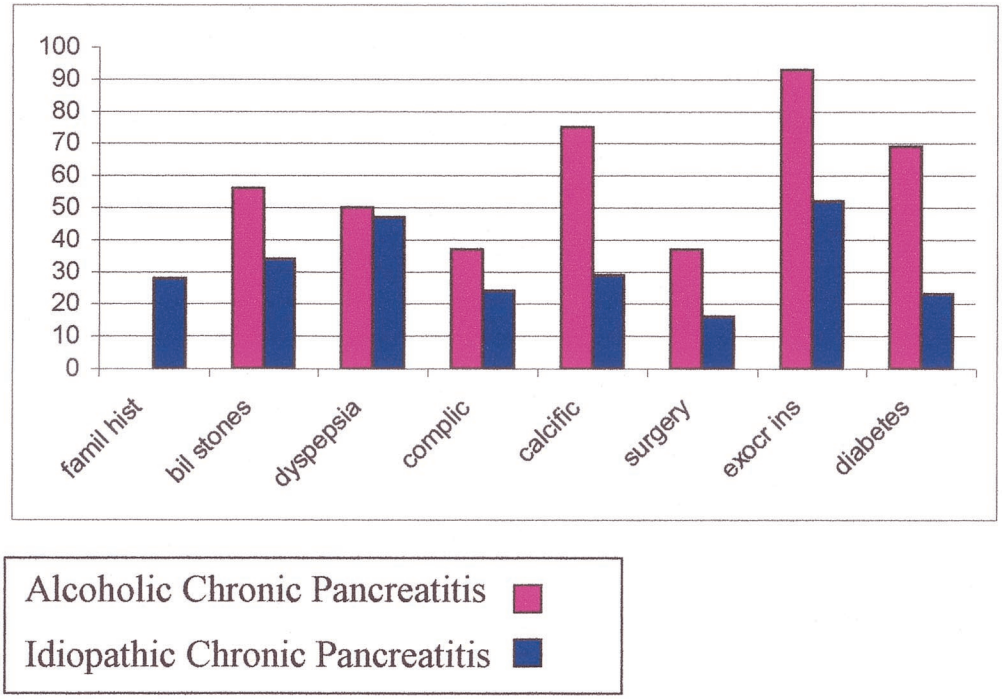

Fig 1. The diagram compares the clinical characteristics of alcoholic chronic pancreatitis (ACP) and idiopathic chronic pancreatitis (ICP) patients and gives the percentage in frequency of familial pancreatic disorder, pancreatic calcification, exocrine insufficiency, and diabetes mellitus. Comparison between the two groups gave a statistically significant result ($P < 0.05$).

recently published data on some case histories where no high frequency of severe mutations (such as ΔF508 and R117H) was found in patients with chronic pancreatitis (19, 20).

In six patients the T5 is associated in *cis* with TG12, a "polyvariant mutated gene," which is observed in CBAVD patients but rare in the general population. The T5 and TG11 association, however, found in two patients, is frequent in the healthy population and very likely does not correspond to a gene having any pathogenic role. No evident correlation was found between the presence of the T5 intronic polymorphism and chronic pancreatic damage.

Furthermore, our data seem to confirm the role the PSTI gene mutations have in causing chronic pancreatitis, in agreement with other papers (9, 10). As regards the meaning of the new mutation 98insA, mutations in the *PSTI* gene, and mutation N34S in particular, result in "loss of function" of the protein and determine the disease since the quantity of inhibitor of the trypsinogen is reduced. In light of this, 98insA must be the mutation responsible for the disease since it produces a truncated, therefore insufficient, protein.

No significant difference was found in age at onset among either ICP or ACP patients in our investigation. We observed higher frequencies of pancreatic calcification and exocrine and endocrine insufficiency in ACP patients. These, however, do not seem to be connected to the seriousness of the disease, but rather, may depend on the fact that the ACP patients were on average 10 years older than the ICP patients, even though the age at onset for both groups is comparatively the same. We also found more familial pancreatic disorders in the idiopathic patients. Further, the age at disease onset of mutation carriers and noncarriers did not vary significantly, and nor did their clinical picture. As opposed to patients with mutation in the cationic trypsinogen, the presence of mutations in the *CFTR* and *PSTI* genes in idiopathic patients does not seem to be connected either to age at onset or clinical course of the disease itself.

No significant difference was found in comparing the clinical characteristics of CP mutation carrier and noncarrier patients. This observation is in contrast with a recent study by Truninger et al (21), in which the frequency of pancreatic calcifications was higher in patients with alcoholic chronic pancreatitis without *CFTR* mutations.

Chronic pancreatitis is a multifactorial etiopathogenic disease so it is likely that one or more additional environmental factors are involved which, over long periods of exposure, contribute in damaging the pancreas. The low penetrance of the mutations in the genes investigated remains to be explained and, therefore, the risk the patients' families run in getting the disease. So far it is not clear how these people should be followed.

Another result of our work demonstrating the high frequency of mutation in the *CFTR* gene in males as compared to females: this fact suggests that there may be other factors connected to sex that have some influence in causing pancreatitis. Mutations in the *PSTI* and *CFTR* genes do not seem to be involved in the alcoholic forms of pancreatitis, as described in other works (5, 22, 23). In these cases the environmental factor probably has a specific role in bringing about the disease.

In conclusion, our investigation confirms the role genetic mutations play in creating idiopathic chronic pancreatitis.

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