European Journal of Human Genetics (2002) 10, 100–106

www.nature.com/ejhg

ARTICLE

Determination of the relative contribution of three genes – the cystic fibrosis transmembrane conductance regulator gene, the cationic trypsinogen gene, and the pancreatic secretory trypsin inhibitor gene – to the etiology of idiopathic chronic pancreatitis

Marie-Pierre Audrézet[1,6], Jian-Min Chen[2,6], Cedric Le Maréchal[2], Philippe Ruszniewski[4], Michel Robaszkiewicz[5], Odile Raguénès[1], Isabelle Quéré[1], Virginie Scotet[3] and Claude Férec*[,1,2,3]

[1]*INSERM-EMI 01 15, Génétique Moléculaire et Génétique Epidémiologique, Centre Hospitalier Universitaire, Brest, France;* [2]*INSERM-EMI 01 15, Génétique Moléculaire et Génétique Epidémiologique, Etablissement Français du Sang-Bretagne, Brest, France;* [3]*INSERM-EMI 01 15, Génétique Moléculaire et Génétique Epidémiologique, Université de Bretagne Occidentale, Brest, France;* [4]*Service de Gastroenterologie, Hopital Beaujon, Clichy, France;* [5]*Service d'Hepato-Gastro-Enterologie, Hopital de la Cavale Blanche, Brest, France*

In the last 5 years, mutations in three genes, the cystic fibrosis transmembrane conductance regulator (CFTR) gene, the cationic trypsinogen (PRSS1) gene, and the pancreatic secretory trypsin inhibitor (PSTI) gene, have been found to be associated with chronic pancreatitis (CP). In this study, using established mutation screening methods, we systematically analysed the entire coding sequences and all exon/intron junctions of the three genes in 39 patients with idiopathic CP (ICP), with a view to evaluating the relative contribution of each gene to the aetiology of the disease. Our results demonstrate that, firstly, 'gain-of-function' mutations in the PRSS1 gene may occasionally be found in an obvious ICP subject. Secondly, presumably 'loss-of-function' mutations in the PSTI gene appear to be frequent, with a detection rate of at least 10% in ICP and, finally, abnormal CFTR alleles are common: at least 20% of patients carried one of the most common CFTR mutations, and about 10% of patients were compound heterozygotes, having at least one 'mild' allele. Thus, in total, about 30% of ICP patients carried at least one abnormal allele in one of the three genes, and this is the most conservative estimate. Moreover, a trans-heterozygous state with sequence variations in the PSTI/CFTR genes was found in three patients. However, an association between the 5T allele in intron 8 of the CFTR gene and ICP remains unproven.
European Journal of Human Genetics (2002) **10,** 100–106. DOI: 10.1038/sj/ejhg/5200786

Keywords: cystic fibrosis transmembrane conductance regulator (CFTR); cationic trypsinogen gene (PRSS1); idiopathic chronic pancreatitis (ICP); pancreatic secretory trypsin inhibitor (PSTI/SPINK1); mutation analysis; trans-heterozygote

***Correspondence: C Férec, EFS-Bretagne, UBO, CHU, 46 rue Félix Le Dantec, 29275 Brest, France. Tel: +33 2 98 44 50 64; Fax: +33 2 98 43 05 55; E-mail: Claude.Ferec@univ-brest.fr**
[6]These authors contributed equally to this work.
Received 4 December 2001; revised 8 January 2002; accepted 16 January 2002

Introduction

Chronic pancreatitis (CP) is a potentially life-threatening disease. Although in most cases a causative factor can be determined, for example, long-term alcohol abuse, which accounts for about 70% of CP, or rarer hereditary, obstructive, or autoimmune causes, in about 10–30% of

patients the aetiology remains undetermined.[1] This latter category of the disease has traditionally been labelled as idiopathic CP (ICP).

Only in the last 3 years have molecular genetic studies uncovered a large piece of the puzzle. On the one hand, the observation that the pancreatic lesions of cystic fibrosis (CF) develop *in utero* and closely resemble those of CP stimulated two research groups to explore a possible relationship between mutations of the CF-causing gene – the cystic fibrosis transmembrane conductance regulator (CFTR; OMIM 602421) gene[2] – and CP.[3,4] This led to the important 1998 finding that about 20% of patients with ICP carry at least one CFTR mutation. On the other hand, the identification in 1996[5] of a 'gain-of-function' mutation in the human cationic trypsinogen (protease, serine, 1 (PRSS1); OMIM 276000) gene as a cause of the rare hereditary form of pancreatitis prompted the adoption of a candidate gene approach.[6,7] As a result, sequence variations in the human pancreatic secretory trypsin inhibitor (PSTI) gene – also known as serine protease inhibitor, Kazal-type, 1 (SPINK1; OMIM 167790) gene – were identified in 2000 and shown to be strongly associated with idiopathic and familial CP. Moreover, even 'gain-of-function' mutations in the PRSS1 gene have been detected in obvious ICP subjects.[8–10] Taken together, these findings highlight a significant contribution of genetic predisposing factor(s) to the pathogenesis of so-called ICP.

To date, most of the relevant studies performed mutational analysis of only a single gene, that is, the CFTR gene,[3,4,11–14] the PRSS1 gene (see a recent review[15]), or the PSTI gene.[6,7,16–20] Moreover, due to differences in the selection of participants, the number of participants analysed, and the mutation screening method used, the reported frequency of mutations in a given gene varied significantly among different studies. Clearly, a simultaneous analysis of all three genes in patients with a diagnosis of ICP would provide an estimation of the relative contribution of each gene to the etiology of this disease. It would also provide a unique opportunity to evaluate the frequency of the trans-heterozygous state with mutations in different genes in ICP patients. This study represents such an attempt, by systematically screening the entire coding sequence and all the exon/intron junctions of the three genes in 39 French patients with ICP.

Materials and methods

Patients

A total of 39 white French patients (26 male; 13 female) with ICP participated in the study. They were consecutively recruited in 1999 from multiple clinics in France. The disease was diagnosed following at least two separate episodes of abdominal pain typical of pancreatitis, an increase in serum amylase or lipase, and pathological sonographic findings. Exclusion criteria included the presence of precipitating factors, such as alcohol, gallstones, trauma, medication, infection or metabolic disorders, an age of greater than 45 years and a report of positive family history. The study was approved by the hospital's review board and all participants provided informed consent.

Mutation screening of the human PRSS1 gene

All of the five exons and exon/intron junctions of the PRSS1 gene underwent denaturing gradient gel electrophoresis (DGGE) analysis, as described elsewhere.[21]

Mutation screening of the human PSTI gene

The four exons and exon/intron junctions of the human PSTI gene were analysed using a previously established DGGE technique.[6]

Mutation screening of the human CFTR gene

The 27 exons and all of the intron/exon junctions of the CFTR gene were analysed according to a recently established denaturing high-performance liquid chromatography (DHPLC) technique.[22] In particular, a sweat test was done retrospectively and unrecognized CF-related lung disease was re-evaluated in patients who were found to be compound heterozygotes.

The intron 8 poly(T) variants were analysed according to the method of Friedman *et al*.[23]

Direct sequencing

Samples showing abnormal DGGE or DHPLC profiles were re-amplified from genomic DNA. Direct DNA sequencing was performed using the ABI PRISM™ BigDye™ Terminator Cycle Sequencing Kit (PE Applied Biosystems, Foster City, CA, USA) on an ABI PRISM 310 sequencer.

Statistical analysis

Comparison of mutation frequency between cases and controls was made using Fisher's exact test when theoretical values were lower than five (for PSTI mutations). Difference between expected and observed mutation frequencies was compared using a binomial distribution (for CFTR mutations and the intron 8 5T allele). All P values were two-tailed. A P value of lower than 0.05 was considered as significant.

The expected frequency of the most common CFTR mutations in the French population was calculated based on two estimates. The incidence of CF in the general population is estimated to be 1 out of 3500. Testing of 31 most common CFTR mutations as offered by a commercialised kit (OLA-PE Biosystem) detects about 85% of all CFTR alleles in France. Consequently, the expected frequency of the most common CFTR mutations is about 1 in 69 alleles ($\sqrt{}$(1 out of 3500) × 0.85=1.4%). The frequency of the 5T allele in the general population is estimated to be 5%.

Results

Mutation analysis of the PRSS1 gene

All 39 patients with ICP were analysed for PRSS1 mutations by DGGE. Only the known c.365G>A (CGC>CAC; R122H, originally termed R117H in the chymotrypsin numbering system[5,24]) mutation was found in a 42-year-old male subject (Table 1). He had suffered the disease from the age of 6 years and, to date, no family members have been reported to have pancreatitis.

Mutation analysis of the PSTI gene

These patients were analysed for PSTI mutations by DGGE. Four patients (10.3%; 4 out of 39) were found to carry at least one allele of the known c.101A>G variant, which is presumed to result in a N34S missense mutation,[6,7] as compared with 3 out of 200 unrelated blood donors[6] (1.5%; P=0.0149). In addition, the known −41G>A[6] variant and a novel variant, namely −2C>A, in the 5′-untranslated region of the PSTI gene were each identified once in different patients (Table 1).

Table 1 Sequence variations identified in the PRSS1, PSTI, and CFTR genes in 39 patients with ICP

Patient	*PRSS1*	*PSTI*	*CFTR Mutant*	*PolyT*
1	–[a]	–	–	7T/7T
2	–	–	F508del/R352Q	9T/7T
3	–	–	F508del/P5L	9T/7T
4	–	–	c.4575+2G>A	9T/7T
5	–	–	–	7T/7T
6	–	N34S[b]	–	7T/7T
7	–	–	–	7T/5T
8	–	–	F508del/Q1476X	9T/7T
9	–	–	–	7T/7T
10	–	–	–	7T/7T
11	–	–	–	7T/7T
12	–	–	–	7T/7T
13	–	–	V562I	7T/5T
14	–	−2C>A	W1282X	7T/5T
15	–	–	IVS3-6T>C	7T/7T
16	R122H	–	–	7T/7T
17	–	–	–	9T/7T
18	–	–	–	7T/5T
19	–	–	–	7T/7T
20	–	N34S/N34S	–	7T/7T
21	–	–	–	9T/5T
22	–	–	–	7T/7T
23	–	–	E217G/A1136T	9T/7T
24	–	–	–	7T/7T
25	–	–	–	ND[c]
26	–	–	–	ND
27	–	N34S	IVS18−20T>C	9T/7T
28	–	–	F508del	9T/7T
29	–	–	–	7T/7T
30	–	–	N1303K	ND
31	–	–	G542X	9T/7T
32	–	–	–	7T/5T
33	–	–	F508del	9T/7T
34	–	−41G>A[d]	–	7T/7T
35	–	–	–	9T/7T
36	–	–	–	9T/7T
37	–	–	–	7T/7T
38	–	N34S	L967S	7T/7T
39	–	–	–	7T/5T

[a]Indicates two wild alleles. [b]N34S is in linkage disequilibrium with the IVS1-37T>C and the IVS3-66_-65insTTTTvariants.[7,17] [c]ND, not done due to insufficient DNA samples. [d]−41G>A is in linkage disequilibrium with the c.174C>T (C58C) variant.[6]

Mutation analysis of the CFTR gene

All of the 39 patients were screened for CFTR mutations by DHPLC. A total of 18 'abnormal' CFTR alleles were identified in 14 subjects, among whom four (patients 2, 3, 8, and 23) were compound heterozygotes (Table 1). In particular, eight patients (20.5%; 8 out of 39) carried one of the most common CFTR mutations. This is 7.4 times the expected carrier rate of 1 out of 35 (2.8%; $P<0.001$).

None of the four compound heterozygotes were found to have unrecognised CF-related pulmonary symptoms following re-evaluation. However, a sweat test done retrospectively was positive in two of them, at 90 mmol/L for the patient with a F508del/P5L genotype and at 80 mmol/L for the patient with a F508del/Q1476X genotype.

The 5T allele was present in seven of the 36 patients tested (Table 1). The frequency (9.7%; 7 out of 72) of this allele in ICP was therefore nearly two times greater than the rate of 5% expected in the general population (P=0.093).

Discussion

The main objective of this study was to determine the relative contribution of the three known pancreatitis-associated genes to the etiology of so-called ICP. In order to achieve this goal, a prerequisite was to choose suitable techniques for accurately and completely screening the three genes. In this study, the simple PRSS1 and PSTI genes – the former is composed of only five exons encoding 247 amino acids[25] and the latter consists of only four exons encoding 79 amino acids[26] – were subjected to our previously established DGGE analyses, which have been demonstrated to be both sensitive and efficient, as well as capable of detecting known mutations and identifying novel variants.[6,10,21] In contrast, it is not easy to perform a complete analysis of the CFTR gene, which consists of 27 exons encoding 1480 amino acids.[27] Indeed, to date, most of the relevant studies have investigated only a selected subset of the most common CFTR mutations in CP. Taking advantage of a recently established DHPLC technique,[22] we performed a systematical analysis of all 27 exons and all the exon/intron junctions of the CFTR gene in a total of 39 ICP patients. Based upon our results, several main estimates can be established.

'Gain-of-function' PRSS1 mutations are rare in ICP

While PRSS1 mutations are often found in patients with hereditary pancreatitis, they can also be identified in subjects with ICP, albeit with an exceptionally low

detection rate.[8–10] The present study confirmed this: we identified only the c.365G>A (CGC>CAC; R122H) mutation once out of the 39 ICP patients. In this regard, it is important to note that the same R122H mutation was also detected in one out of 39 well-characterised ICP patients in a just published study.[28] Moreover, this mutation was found in a patient diagnosed for 20 years as having alcoholic pancreatitis and with no family history of pancreatic disease.[29] Furthermore, a gene conversion event, a c.365~366GC>AT two base pair nucleotide change, which also results in a R122H mutation in PRSS1, was also detected in one Belgian with ICP.[30]

Presumably 'loss-of-function' PSTI mutations are frequent in ICP

In the present study, four patients (three heterozygotes and one homozygote) were found to carry the c.101A>G (N34S) mutation, representing a carrier frequency of 10.3%, which is significantly higher than that (1.5%) in the control population (P=0.0149). This frequency is higher than the 4.3%[20] and 5.0%,[31] similar to the 9.4%,[19] but much lower than the 18.8%,[7] 23.1%,[28] 25.9%,[16] and 28.6%,[18] reported in other studies. This discrepancy may be mainly due to the difference in the composition of patients chosen in these studies.

In addition, the known −41G>A[6] variant and a novel variant, namely −2C>A, both in the 5′-untranslated region of the PSTI gene, were each identified once (Table 1). Neither variant was present in 400 control chromosomes, evaluated by DGGE analysis. Importantly, the −41G>A variant has been identified several times in our newly recruited patients with CP (unpublished data). The location of the −41G>A variant is also interesting: it is 19 nucleotides downstream from the major transcription start point and immediately upstream of one of the three minor transcription start points of the PSTI gene.[26] Thus, the −41G>A variant may predispose to pancreatitis by lowering the transcription efficiency of the gene. As for the −2C>A variant, if it had any functional consequence, it would most probably affect the translation efficiency of the protein. These hypotheses could be tested by in vitro transcription and translation analyses.

Therefore, between 10.3% (4×N34S only) and 15.4% (plus 1×−41G>A and 1×−2C>A) of the 39 ICP patients carried at least one abnormal PSTI allele.

CFTR mutations are common in ICP

In order to avoid certain potential confounding factors in interpreting the data, we will discuss our results only in comparison with those observed in patients with ICP and, firstly, consider the detection frequency of the well-defined, most common CFTR mutations.

The well-defined, most common CFTR mutations are significantly increased in ICP A strong association between CFTR mutations and ICP was reported in most of the previous studies (Table 2). With respect to the one negative report,[13] the limited number of samples analysed should be taken into account. Additionally, none of the genotyped F508del and R117H mutations were found in 14 Japanese patients with ICP,[14] a not surprising result given that these alleles are extremely rare in the Japanese population.

In the present study, we identified four well-defined CFTR mutant alleles in eight patients (5×F508del, 1×W1282X, 1×N1303K, and 1×G542X; Table 1). These mutations would have also been identified by the widely used genotyping method (Table 2). Thus, 20.5% (8 out of 39) of our study cohort carried at least one of the most common CFTR mutations. This is 7.4 times the expected frequency (P<0.001) and agrees well with most of the previous studies.

Complete analysis of the entire coding sequence of the CFTR gene identified more variants, of significant importance being the revelation of a second 'mild' CFTR allele in a significant fraction of the patients having a 'severe' CFTR mutation As indicated in Table 2, most of the previous studies used the genotyping method to analyse a subset of the most common CFTR mutations in ICP. Therefore, it is possible that more comprehensive DNA testing might detect additional mutations. It is also possible that some of the patients having one abnormal CFTR allele may actually be compound heterozygotes with a second mutation that was not detected. Having analysed the 27 exons of the CFTR gene, we are able to address these issues.

Table 2 CFTR mutations and 5T allele in ICP

Study	*CFTR mutations*[a]	*5T allele*	*Comment*
Sharer *et al*[3]	15~20% (9~12 out of 60)[b]	Unable to calculate	Tested for 22 CFTR mutations
Cohn *et al*[4]	25.9% (7 out of 27)	9.3% (5 out of 54)[c]	Tested for 17 CFTR mutations
Castellani *et al*[11]	20% (6 out of 30)	3.3% (2 out of 60)	Tested for 18 CFTR mutations
Ockenga *et al*[32]	27.3% (3 out of 11)	4.5% (1 out of 22)	Tested for more than 30 CFTR mutations
Arduino *et al*[12]	15% (3 out of 20)	12.5% (5 out of 40)	Tested for 12~31 CFTR mutations
Pallares-Ruiz *et al*[13]	0% (0 out of 10)	10% (2 out of 20)	Scanning of 27 exons by DGGE
Present study	20.5% (8 out of 39)	9.7% (7 out of 72)	Scanning of 27 exons by DHPLC

Certain studies included patients with CP resulting from different causes, where only the data concerning ICP were extracted. [a]Only the well-defined, most common CFTR mutations were considered. [b]Expressed as carrier frequency (number of patients with at least one abnormal allele/total number of patients tested). [c]Expressed as allele frequency.

Firstly, we found a total of 10 additional alleles (R352Q, P5L, c.4575+2G>A, V562I, IVS3-6T>C, E217G/A1136T, IVS18-20T>C, and L967S; Table 1) that would have been missed by the conventional genotyping method. Despite a lack of conclusive evidence, we believe at least some of these additional variants may have functional consequences. For example, R352Q, P5L, and A1136T occur in strictly conserved residues (refer to Figure 2 in Chen *et al*[33]). In particular, R352Q is within the stringently conserved motif T351-R352-Q353, which is immediately downstream of TM6 and has been suggested to loop back into the chloride channel, narrowing the lumen and thereby forming both the major resistance to current flow and the anion selectivity filter;[34] A1136T is within TM12; c.4575+2G>A, occurring just two nucleotides downstream of the translation stop codon, may affect the interplay between splicing and polyadenylation or alter the mRNA stability; Q1476X is a nonsense mutation. Moreover, we would like to add that any functional consequence of these additional variants should be considered in the context of ICP rather than in the context of the typical CF disease, since a well-characterised polymorphism in typical CF may act as a predisposing factor to atypical CF disease.

Secondly, we found four patients to be compound heterozygotes, among whom three (patients 2, 3 and 8) would have been labelled as F508del carriers by the genotyping method, while no mutation would have been detected in the fourth (patient 23). Although a sweat test was positive in the patients (both were males) with F508del/P5L (90 mmol/l) and F508del/Q1476X (80 mmol/l), neither of them showed evidence of CF-related lung disease. Moreover, no atypical CF presentations such as sinusitis and congenital absence of the vas deferens were reported. Thus, as in the case of the two previously reported compound heterozygotes, F508del/R117H[4] and I336K/R75Q,[32] none of our four compound heterozygotes could be described as carrying two 'severe' CFTR mutations. Still, one may argue that the F508del/Q1476X genotype represents two 'severe' alleles, since Q1476X is an obvious nonsense mutation resulting in a truncation of the last four amino acids of the CFTR protein. This argument would be negated by a recent combined analysis of the CFTR (refer to Functional Delineation of the C-tail in Chen *et al*[33]).

One may also argue that the two patients with the F508del/P5L (90 mmol/l) and F508del/Q1476X (80 mmol/l) genotypes had, in fact, typical CF disease, according to the diagnostic criteria established by a consensus panel of the Cystic Fibrosis Foundation.[35] Regarding this issue, we agree with the view that 'we need to have both a CF 'disease' and a CF 'syndrome'.'[36] In fact, 'Expansion of the spectrum of disease associated with the CFTR mutant genes creates a need for revision of the diagnostic criteria for CF and a dilemma for setting nosologic boundaries between CF and other diseases with CFTR aetiology.'[37]

An association between 5T allele in intron 8 of the CFTR gene and ICP is not yet established

The 5T allele reduces the efficiency of exon 9 splicing and thereby reduces the expression of functional CFTR.[38,39] A relation between susceptibility to CP and the presence of the 5T allele has been controversial (Table 2). Here we detected a frequency of 9.7% (7 out of 72) of the 5T allele in ICP and this was about two times greater than the rate of 5% expected in the general population. But this difference remains unsignificant (P=0.093) because of the small size of analysed samples. Therefore, an association between the 5T allele and ICP remains unproven. Nevertheless, the 5T allele, if combined with a CFTR mutation, may significantly increase the risk. In this regard, a F508del/5T genotype was identified three times[3,4] and Q493X/5T[3] and R553X/5T[3] once each in patients with ICP. In this study, the 5T allele was found in a patient having the W1282X mutation (Table 1).

Trans-heterozygous state with sequence variations in different genes exists in certain subjects

To date, a combination of either a PRSS1 mutation plus a CFTR mutation,[28,40] a PRSS1 mutation plus a PSTI mutation,[41] or a CFTR mutation plus a PSTI mutation[28,31] has been occasionally reported. These rare cases strongly suggest an interaction between different susceptibility loci in the pathogenesis of the disease. Here, we identified three patients (numbers 14, 27, and 38; Table 1) who are trans-heterozygotes of PSTI/CFTR mutations or variations. Tracing the origin of these sequence variations in the parents and long-term follow up of these trios may offer a unique opportunity to evaluate the synergistic involvement of two distinct genes.

In summary, this study has systematically analysed three pancreatitis-associated genes in a cohort of 39 French patients with ICP. Our results demonstrates that about 30% of the patients labelled as ICP had, in fact, a genetic defect. Here, it should be remembered that this is the most conservative estimate since we did not include those uncommon variants identified in the PSTI and CFTR genes, some of which must surely have functional consequences. Moreover, the promotor region and the vast intronic sequences of the two genes remain largely unexplored, and certain mutations, such as large deletions, could not be identified by the DGGE or DHPLC techniques. Thus, taking into consideration these factors, it is not unreasonable to predict that more ICP patients may eventually be found to carry at least one abnormal allele in one of the three genes and that, at the same time, more compound heterozygotes and trans-heterozygotes may be found. Long-term follow up of these patients, including heterozygotes, homozygotes, compound heterozygotes, and trans-heterozygotes, would certainly improve our understanding of the complex nature of ICP. Finally, it is believed that the number of so-called ICP patients will be further reduced when new predisposing genetic factors are identified.

Electronic-database information

Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/omim; The Cystic Fibrosis Mutation Data Base, http://www.genet.sickkids.on .ca/cftr/; The Cationic Trypsinogen and Pancreatic Secretory Trypsin Inhibitor Genetic Variant Database, http://www.uni-leipzig.de/pancreasmutation/.

Acknowledgments

This study is dedicated to Claudine Verlingue. We thank all the patients who participated in this study and are grateful to JF Bretagne (Rennes), R Delcenserie (Amiens), C Faure (Paris), P Jonveaux (Nancy), and (Lyon) Lachaux A for sample contributions. This work was supported by the INSERM and the Projet Hospitalier de Recherche Clinique (PHRC).

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