Original Article

Spectrum of mutations and variants/haplotypes of CFTR and genotype–phenotype correlation in idiopathic chronic pancreatitis and controls in Chinese by complete analysis

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Mutations in cystic fibrosis transmembrane conductance regulator (*CFTR*) gene have been reported in patients with chronic pancreatitis. The authors examine whether the mutations and haplotypes of *CFTR* will increase the risk of developing idiopathic chronic pancreatitis (ICP) in Chinese and their genotype and phenotype correlations. Seventy-eight patients with ICP and 200 geographically and ethnically matched controls in Taiwan were analyzed. The entire 27 coding and intronic regions of the *CFTR* gene were identified using heteroduplex analytical techniques and confirmed by sequencing analysis. The presence of 125G/C, 1001+10C>T, IVSTn(TG)m, 1540A>G, c2694T>G, and c4521G>A were determined by directing sequencing. Abnormal *CFTR* allele was found to be thrice as frequent in ICP patients as in controls (22/156 vs 19/400, $p < 0.0001$). T5 allele was associated with early onset of ICP. In six-loci haplotype analysis, 13 common haplotypes were assembled in the 278 individuals tested. The 125G/1001+11C/TG12/470M/2694T/4521G haplotype was associated with risk of ICP (odds ratio 11.3; 95% confidence interval 2.3–54.6, $p = 0.008$) in Chinese. The mutation spectrum is different from other ethnic groups. A population-specific panel of *CFTR* changes should be recommended for targeted populations including ICP in Chinese. It is important to design suitable screening programs for different populations.

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Key words: *CFTR* – early onset – genotype – haplotype – idiopathic chronic pancreatitis – phenotype – 5T

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Genetic risk factors are attributed to a much more important role in the pathogenesis of chronic pancreatitis (CP) (1, 2). The cationic trypsinogen gene (*PRSSI*) (3) and serine protease inhibitor Kazal type 1 (*SPINK1*) (4) genes have been most extensively studied in CP. However, the frequency of hereditary pancreatitis that might be related to trypsinogen gene and serine protease inhibitor Kazal type 1 in Orientals is regarded relatively low and it is hardly to explain the genetic susceptibility of CP in Chinese. The cystic fibrosis transmem-

brane conductance regulator (*CFTR*) is a cAMP-regulated Cl^- channel that is expressed in many epithelial tissues (5). Loss of its function because of mutations in the *CFTR* (MIM 602421) gene may cause cystic fibrosis (CF). In addition, there are several other phenotypes associated with *CFTR* gene mutations, such as congenital bilateral absence of the vas deferens (CBAVD) and idiopathic chronic pancreatitis (ICP). More than 1000 different mutations and 200 polymorphisms have already been reported in patients with CF (updates

under <http://www.genet.sickkids.on.ca/cftr/app>). Classic CF is caused by two loss-of-function mutations in the *CFTR* gene, whereas patients with non-classic CF have at least one copy of a mutant gene that retains partial function of the *CFTR* protein. Individuals with CBAVD are reported to frequently carry mutations in the *CFTR* gene but show no classical CF clinical phenotype. The so-called 'mild' mutations that retain higher *CFTR* function have been reported to be associated with idiopathic pancreatitis (6, 7). These reports suggest that different types of mutations in the *CFTR* gene, causing different degrees of *CFTR* impairment, can be associated with widely different disease phenotypes. It is now well recognized that the spectrum of *CFTR*-related disease is much broader than previously thought (8).

In Asia, there are limited data about changes of *CFTR* gene in CP, including reports from Korean (9) and Japanese (10). One obstacle to comprehensive *CFTR* studies is the large size of its 27 exon spanning gene. So, most studies that have addressed the role of *CFTR* mutations in CP used commercially available screening panels of the 20–30 most common CF causing (rather than pancreatitis causing) *CFTR* mutations. Furthermore, the geographic distribution of *CFTR* mutations also varies worldwide.

In addition to mutations, functional polymorphisms have also been detected within the *CFTR* locus in non-CF individuals that could also potentially result in alterations in *CFTR* gene expression (11). Some *CFTR* variants/polymorphisms and haplotypes (12) are regarded to be associated to CP (13, 14). Previous studies have demonstrated that TG repeat at the 3' end of intron 8 of *CFTR* gene is the binding site of the splicing factors (15), which determine the level of functional *CFTR* protein. The longer TG repeats in intron 8 increase the probability of alternative splicing of exon 9, which results in a production of non-functional *CFTR* protein (16). M470V is a common polymorphism in exon 10 in *CFTR* that affects intrinsic chloride channel activity (17). The cSNPs 2694T>G and 4521G>A may have affected pre-mRNA splicing by changing regulatory sequence motifs of exonic splice enhancers, leading to increased skipping of exon 9 and 12 and lower amounts of normal transcripts (18). Haplotype-based analysis has gained increasing attention in evaluating candidate gene in various clinical situations. Merits of haplotype study are more evident in the fine mapping of complex diseases and in identifying genetic variations that influence individual's susceptibility to diseases and responses to therapy. Therefore, it is advisable to use the haplotype-based

approach to address the role of *CFTR* variants in the risk of ICP.

In order to determine whether *CFTR* mutations, functional polymorphisms and haplotypes affect the risk of developing ICP in Chinese, we recruited 78 patients with ICP and 200 geographically and ethnically matched controls. All patients and control subjects were Chinese and from the same, narrowly confined area in Taiwan (19). We analyzed the entire coding region and intronic regions of the *CFTR* gene by denaturing high-performance liquid chromatography (DHPLC) (20–22), a simple, rapid, non-gel-based, non-fluorescence-based method that uses ion-pair reversed-phase liquid chromatography for detection of DNA variations sensitively and specifically. We also genotyped the common functional single-nucleotide polymorphisms of *CFTR* by direct sequencing in all subjects and controls. By this means, we could firmly determine that compound heterozygous mutation carriers, as well as heterozygotes with mild and uncommon mutations (previously regarded as unaffected CF carriers) have an increased risk of developing CP. Furthermore, we analyzed the *CFTR* haplotype patterns and genotype–phenotype correlations in ICP.

Participants and methods

Cases and controls

Patients and controls were recruited at a tertiary referral center (National Taiwan University Hospital) from July 2000 through December 2005. The patients referred to this hospital were residents of metropolitan Taipei, Taiwan, although some of them might have come from other cities around Taiwan. They were the so-called 'Taiwanese' or 'Taiwan Chinese.' Most of their ancestors moved to Taiwan from southeastern China about 500 years ago. They were not Taiwanese aborigines. The control subjects were from the same areas as the cases were recruited during the same time period from the hospital and had no history of acute pancreatitis, CP, diabetes mellitus (DM), pancreatic adenocarcinoma, and/or apparent biliary or pancreatic diseases. They were also no family history of pancreatitis and pancreatic adenocarcinoma. CP was defined as the presence of pancreatic calcifications or histological evidence of CP. The etiology of CP was classified with TIGAR-O system (2). All non-insulin-dependent diabetes mellitus was defined as fasting blood glucose >126 mg/dl and/or being administered at least one oral hypoglycemic agent. Patients who reported any known risk factor for

pancreatitis, such as a history of nutritive (including alcohol consumption of more than two drinks a day (20 g), biliary, metabolic (such as elevated serum triglycerides, calcium), autoimmune, medication with known pancreatic toxicity, pancreas or periampullary tumors, pancreatic duct anomaly or endocrine disorders, as well as subjects with any evidence of pulmonary diseases or CF, were excluded from the study. Patients identified as mutation carriers in the cationic trypsinogen (*PRSSI*) gene and serine protease inhibitor Kazal type (*SPINK1*) gene evaluated by complete sequencing of the coding region were also excluded. There were 78 cases with ICP (48 men and 30 women) and 200 control subjects (125 men and 75 women) recruited for analysis. The study was approved by the local institution committee, and the subjects gave their informed consent. The demographic and laboratory data were collected from the medical chart records.

DNA studies

Genomic DNA was extracted from leucocytes using the Viogene genomic DNA extraction kit (Viogene, Taipei, Taiwan), according to the manufacturer's instruction.

Polymerase chain reaction amplification and DHPLC heteroduplex analysis

The mutations of *CFTR* genes were examined using polymerase chain reaction (PCR) followed by DHPLC modified from our previous study (20, 21). PCR amplification of all 27 *CFTR* exons, including flanking intronic regions, was performed using gene-specific primers as previous described (7). PCR of target exons was performed in a 50- μ l volume containing 10 mmol/l of Tris-HCl, pH 8.3, 50 mmol/l of KCl, 1.5–4.5 mmol/l of $MgCl_2$, 50 mmol/l of dNTPs, 0.25 mmol/l of each primer, 100 ng of genomic DNA, and 1 U of BioThermStar DNA polymerase (Gene Craft GmbH, Munster, Germany). The PCR cycling reaction was initiated with a 10-min denaturation step at 95°C. Subsequent steps included a denaturation step at 94°C for 20 s and an extension step at 72°C for 45 s.

DHPLC heteroduplex analysis

DHPLC was performed on a WAVE DNA fragment analysis system equipped with a DNA-sep column employing a UV-C scanner to detect eluted DNA (Transgenomic, Crewe, UK) (23). Prior to loading, PCR product was denatured by heating to 94°C for 5 min and allowed to

reanneal slowly by reducing the temperature 1°C each minute until the temperature reached 25°C for a total period of 70 min (23). The resolution temperature for each sequencing was determined using DHPLCMELT software (<http://insertion.stanford.edu/melt.html>). Product displaying abnormal elution profiles was subjected to sequence analysis.

Sequence analysis

Purified PCR product was sequenced using BigDye Terminator sequencing chemistry (Applied Biosystems) employing primers used in the initial amplification step. An ABI3100 automatic DNA sequencer (PE Applied Biosystems Inc., Foster City, CA) was used to analyze the product of the sequencing reaction. The sequence obtained was compared with the published sequence database (updates under <http://www.genet.sickkids.on.ca/cftr/app>).

Variants/polymorphisms/genotyping of *CFTR*

The presence of 125G/C, 1001+10C>T, IVSTn(TG)m, 1540A>G, c2694T>G, c4521G>A in *CFTR* whatever the DHPLC displayed as abnormal elution profiles were determined by directing sequencing.

Haplotype analysis

Haplotype-based approach was used to provide a better understanding of the disease-associated *CFTR* variations. Seven *CFTR* diallelic polymorphic loci were studied to determine the haplotypes for risk of ICP. The expectation-maximization(EM)-based haplotype frequency estimations and permutation-based hypothesis testing procedures were performed based on previous work in our institution (19, 24). The significance level was $p < 0.05$ for the omnibus test and 1×10^{-4} (0.05/64) for individual haplotype analyses (64 haplotypes for six loci).

Statistical analysis

The between-group demographic data were compared by the Student's unpaired *t*-test for continuous data and by the χ^2 test for categorical data. Statistical analysis of genotype distribution and allele frequencies was performed by χ^2 square test or the Fisher's exact test (SPSS for WINDOWS 11.5; SPSS, Chicago, IL). Hardy-Weinberg equilibrium was tested among the

controls and among the group with CP to confirm the control as suitable. The multiple step-wise logistic regressions were applied to determine the independent risk factor related with the presence of ICP with enrolling age and sex as adjustment to prevent confounding bias. All tests were two tailed with statistical significance setting at the level of $p < 0.05$.

Results

We conducted a comprehensive study involving a cohort of 78 patients with ICP as well as a geographically and ethnically matched 200 control subjects. All 27 exons, including flanking intronic regions, were analyzed by DHPLC analysis. The median age of ICP was 45.00 ± 11.528 years and control group 42.00 ± 9.679 years, $p = 0.523$). The distribution of gender between patients with ICP and controls were not significant. The median age of onset of disease in patients with ICP was 36.0 ± 17.1 years.

CFTR mutations

We identified a total number of 22 abnormal *CFTR* alleles, which amounts to thrice the allele frequency in healthy controls (19 abnormal *CFTR* alleles in 200 controls; 14.1% vs 4.8%, $p < 0.0001$, Table 1). Among 78 patients with ICP, there were 19 patients (19/78, 24.4%) with single abnormal allele and three patients (3/78, 3.8%) with two abnormal alleles. All our patients were heterozygous for a single *CFTR* mild/uncommon mutation. All the identified mutations were mild mutation of *CFTR*, which might produce a small amount of functional *CFTR* and was regarded as to be associated with pancreatic phenotype that was concordant with previous report (6, 7). A total of 20 IVS8-5T (one homozygote in control group and the others all heterozygotes) and nine other

mutations were identified (Table 1). The IVS8-5T mutation in ICP accounts for 27.2% (six out of 22) of all identified *CFTR* mutant alleles. In patients with ICP carrying 5T, the mutant allele was all associated with 12 or 13 TG repeats. The other mutations were identified in heterozygous patients who were all homozygous for 7T in intron 8. These mutations include I556V, G to A 3849+45, N287Y, I125T, E217G, S895N, G1069R, and Q1352H that have been found in patients with CP or CBAVD (<http://www.genet.sickkids.on.ca>) (Table 1).

Genotype and phenotype correlations

The age of onset was younger in patients with ICP who carrying T5 allele (27.5 ± 12.9 , $p = 0.009$, Table 2). Furthermore, these patients carrying T5 allele were all with TG12 repeat in intron 8. A trend of earlier onset was noted with the increase of number of *CFTR* mutations but did not reach statistical significance (Table 2). In subgroup analyses for patients with ICP, the age, gender, diabetes mellitus, existence of calcification or pancreatic stones and pseudocyst formation were not significantly associated with the existence or numbers of *CFTR* mutations. The presence of DM, calcification and pseudocyst were also not associated with the existence of *CFTR* mutations.

Polymorphisms

Poly T

The 7T was the most common (94.9% in ICP and 96% in controls) and hence the 7T/7T was a dominant genotype in Chinese (Table 3), similar to Japanese (10) and Vietnamese (12). The T5 (3.6%, 20/556) and T9 alleles (0.7%, 4/556) were much less frequent than the T7 allele (Table 3). The T6 allele was found with a frequency about 0.1% (1/556). One alleles of 6T was

Table 1. *CFTR* gene mutation in 78 patients with ICP and 200 control subjects

Mutation	Location	Nucleotide alteration	Predicted effect	No. (%) in ICP	Controls (%)
I556V	Exon 11	A to G 1798	Amino acid substitution	7 (8.9)	2 (1)
IVS8-5T	Intron 8	deletaion of 2T between 1342-12 and 1342-6	Aberrant splicing	6 (7.7)	14 (7)
G to A 3849+45	Intron 19	G to A at 3849+45	mRNA splicing defect	3 (3.8)	2 (1)
N287Y	Exon 6b	A to T 991	Amino acid substitution	2 (2.6)	0 (0)
I125T	Exon 4	T to C 506	Amino acid substitution	1 (1.3)	0 (0)
E217G	Exon 6a	A to G 782	Amino acid substitution	1 (1.3)	0 (0)
S895N	Exon 15	G to A 2816	Missense mutation	1 (1.3)	0 (0)
G1069R	Exon 17b	G to A 3337	Amino acid substitution	1 (1.3)	0 (0)
Q1352H	Exon 22	G to C at 4188	Amino acid substitution	0 (0.0)	1 (0.5)

ICP, idiopathic chronic pancreatitis.

Table 2. Distribution of various clinical parameters among patients with ICP based on *CFTR* mutation

	Total (<i>n</i> = 78)	No mutation (<i>n</i> = 59)	With mutation (<i>n</i> = 19)	One mutation (<i>n</i> = 16)	Two mutation (<i>n</i> = 3)	T5 mutation (<i>n</i> = 6)
Age of onset (median, range 95% CI)	36.0 ± 17.1	38 ± 15.6	32 ± 21.7	34 ± 22.8	29.0 ± 3.8	27.5 ± 11.6*
Clinical parameters						
DM	39	32	7	6	1	3
Chronic calcification pancreatitis	52	41	11	10	1	4
Pseudocyst formation	15	13	2	2	0	0
Acute pancreatitis	56	41	15	13	2	5
Episodes of acute attack	1 ± 2.5	1 ± 2.8	2 ± 1.5	2 ± 1.2	2 ± 3.1	2 ± 1.3

CFTR, cystic fibrosis transmembrane conductance regulator; CI, confidence interval; DM, diabetes mellitus; ICP, idiopathic chronic pancreatitis.

**p* = 0.009, compared with patients with ICP without T5 mutation.

found in normal subjects. The sequence analysis indicates that three alleles of 6T probably resulted from a deletion of thymidine from the 7T and the other one by a point mutation from T to A. The frequency distribution of poly T in alcoholic or ICP was not different from that in normal subjects.

TG repeats

The (TG)11 and (TG)12 were dominant TG repeats in Chinese and the ratio was roughly 1:1 (Table 3). Their frequency in CP was similar to that in normal subjects. The frequency distributions of genotypes of (TG)11/11, (TG)11/12, and (TG)12/12 were not significantly (*p* = 0.181) different among normal subjects and patients with idiopathic pancreatitis (Table 3).

The M470V polymorphism

Several reports have suggested that the most frequent *CFTR* polymorphism, M470V, played a role in modulating CFTR protein level at both transcriptional and translational levels independent of intron 8 polythymidine genotype (17). Our results did not show a significant difference (*p* = 0.316) in genotype and allele frequency distributions of M470V in Taiwanese patients with ICP and matched controls (Table 3). The results indicate that the wild-type M470 allele is not the more common allele in Taiwanese population (46.2% vs 53.8% for M to V allele frequency), similar to those in Taiwanese patients with CP (40.8% vs 59.2%). The frequency distribution of M/M, M/V, and V/V genotypes in chronic pancreatitis was not statistically significant (Table 3).

5T and its adjacent polymorphic TG repeats

Because the disease penetrance of 5T is affected by its adjacent polymorphic TG repeats, the number of TG dinucleotide repeats in intron 8 of

each patient was determined by direct sequencing whatever the DHPLC displayed as abnormal profiles. The distribution of 5T/6T/7T/9T, 10/11/12/13TG and M470V alleles were shown in Table 4. The 5T alleles were not significantly increased in patients with ICP (6/156 vs 14/400; *p* = 0.581). The distribution of TG repeat in each poly T was shown in Table 4. It was found that in all patients carrying 5T, the mutant allele was mostly associated with 12 TG repeats (Table 4). Allelic variations of 5T–12TG were similarly frequent in the patient group.

Haplotype analysis and association with ICP

Haplotypes were assembled using the genotype data obtained from the 278 tested samples and the haplotype program based on the permutation test as our previous studies (19, 24, 25). Six loci consisting of five diallelic variants and the poly TG repeats in intron 8 were analyzed.

Table 5 displays the results of six-loci-estimated haplotype frequency analyses for the *CFTR* polymorphic sites in cases and controls for the entire population. With six loci, there should be $2^6 = 64$ haplotypes. However, because there were linkage disequilibriums in this small region, some haplotypes did not exist or had very low frequencies, and we only listed haplotypes with frequency >0.001. The omnibus haplotype profile test (25) was significant ($\chi^2 = 26.28184$, *p* = 0.008), which indicated the overall haplotype frequency profile difference between cases and controls was significant, and thus there might be some disease-predisposing haplotypes in patients with ICP.

Accordingly, in the individual haplotype analyses, we identified haplotypes with significantly higher haplotype frequency in the cases than in the controls at the significance level *p* < 0.05 by permutation tests [the results would not be

Table 3. Allele frequency of poly T, poly TG repeat and M470V

	No. of alleles (%)	Poly T					M470V					Poly TG						
		T5	T6	T7	T9		470M	470V	M/M	M/V	V/V	TG10	TG11	TG12	TG13	11/11	11/12	12/12
Control subjects	400	14 (3.5)	1 (0.2)	384 (96.0)	2 (0.5)		163 (40.8)	237 (59.2)	37 (18.5)	84 (42)	79 (39.5)	3 (0.8)	181 (45.3)	212 (53.0)	4 (1.0)	33 (16.5)	104 (52.0)	45 (22.5)
ICP	156	6 (3.8)	0 (0.0)	148 (94.9)	2 (1.3)		72 (46.2)	84 (53.8)	18 (23.1)	38 (48.7)	22 (28.2)	3 (1.9)	70 (44.9)	78 (50.0)	5 (3.2)	18 (23.0)	27 (34.6)	22 (28.2)

ICP, idiopathic chronic pancreatitis.

significant if the stringent Bonferroni correction for probability value were used ($p < 0.05/64$ for 64 individual haplotype analyses were performed)]. All these significant haplotypes had high haplotype frequencies (>0.01).

The 125C/1001+11C/TG11/470V/2694T/4521G haplotype was a dominant haplotype in the Chinese. The 125G/1001+11C/TG12/470M/2694T/4521G haplotype was associated with risk of chronic pancreatitis with a odds ratio (OR) 11.3 (95% confidence interval (CI) 2.3–54.6) that indicated a large association effect ($p = 0.008$). When we analyzed the loci that composed the haplotypes, we found that 125G>C was the potential significant loci associated with ICP in our population.

Discussion

The present study is the first comprehensive report on Chinese with phenotype–genotype correlations. The mutations in the *CFTR* gene account for 14.1% (22/156) of the total alleles and 24.4% (19/78) of patients with ICP compared with 4.8% (19/400) of the alleles and 9.5% (19/200) in controls. The *CFTR* mutation spectrum in the Chinese population was found to be quite different from those observed in the Caucasian populations. Weiss et al. (7) have conducted a complete *CFTR* gene study by sequencing which enrolled 67 patients with ICP and 60 controls in Germany. The mutation rate of allele (including combination of 5T allele with 12TG repeats) was 18.7% vs 9.2% in ICP and controls (25/134 vs 11/120, $p < 0.05$), compared with our 14.1% vs 4.8% in ICP and controls. Cohn et al. (26) also reported an increased risk of ICP in *CFTR* carriers by analyzing 52 patients with ICP and identified 18 pathogenic *CFTR* alleles in 15 subjects. These similarities implied that complete study of the large *CFTR* genes with comparison of ethnic geographic control subjects might detect the association of *CFTR* with ICP (27) and other *CFTR*-related disease phenotypes (28, 29).

In the literature, a number of studies have shown an increased incidence of *CFTR* mutations in patients with chronic pancreatitis (30, 31). However, most of them were not comprehensive investigations of the role of *CFTR* mutations because of the limited number of CF-causing mutations analyzed using screening panels for the diagnosis of CF, existence of geographical and ethnic variations in *CFTR* mutation carrier frequencies, and the lack of reliable data on the frequency of uncommon

Table 4. Distribution of TG repeat in poly T

Poly T	5T		6T		7T		9T	
	ICP (n = 6)	Control (n = 14)	ICP (n = 0)	Control (n = 1)	ICP (n = 156)	Control (n = 384)	ICP (n = 0)	Control (n = 2)
TG repeat								
10	0	0	0	0	1	2	0	1
11	0	0	0	0	71	181	0	0
12	6	10	0	1	82	200	0	1
13	0	4	0	0	2	1	0	0

ICP, idiopathic chronic pancreatitis.

CFTR mutations in healthy control populations. Bombieri et al. (32) conducted a study analyzing *CFTR* mutations in healthy control by denaturing gradient gel electrophoresis approach that identified 30 *CFTR* mutations and 17 5T alleles. Differently, the population samples were gathered from four different regions in southern Europe and the observed mutation frequencies and their distribution were different from those in our control population.

Our data showed the very different distribution of *CFTR* mutations compared with previous report in both ICP and control groups from western countries. The 20 most common European mutations and the nine CF-causing mutations (severe mutation such as F508) were not detected in our cases and controls, similar to previous reports from Japanese (10). It suggested that if we used commercially available screening panels to identify *CFTR* alterations, we would have missed most of the mutations in our population. According to our study, the risk of developing ICP for a heterogenous abnormal *CFTR* carrier (regardless of its severity) is

increased approximately ninefolds over that of the normal population (95% CI 3.36–23.9, $p < 0.001$). All our mutations are belonging to ‘mild’ mutations compatible with previous studies (6), including I556V, G to A 3849+45, I125T, E217G, N287Y, S895N, G1069R, and Q1352H. Among them, we found a significantly higher accumulation of the I556V allele in our patients with ICP. I556V that was originally identified in patients with moderate pulmonary disease and sufficient pancreatic function (33) was found in seven patients; the frequency of I556V in chronic pancreatitis (7/78, 8.9%) was significantly ($p < 0.0001$) higher than that of controls (2/200, 1%). The G to A 3849+45 which were originally identified in Irish patients with moderate to severe pulmonary disease and sufficient pancreatic function (34) was found in three patients with ICP. The frequency of G to A 3849+45 in chronic pancreatitis (3/78, 3.8%) was also higher ($p = 0.029$) than that of controls (2/200, 1%). E217G was previously identified in a Polish CF patient with pancreatic sufficiency (data from CFGAC). The E217G mutation partially decreased membrane

Table 5. Six-loci haplotype frequency estimates of *CFTR* in patients with ICP and controls and significance levels of comparison from permutation tests

5'UTR	Intron 6b	Intron 8	Exon 10	Exon 14a	Exon 24	Overall (%)	Control (%)	Cases (%)	p Value	OR
125GC	1001+11CT	Poly TG	M470V	2694T>G	4521G>A					
C	C	(TG)11	v	T	G	0.43243	0.43833	0.41564	0.452	
C	C	(TG)12	M	T	G	0.35326	0.34961	0.3731	0.488	
C	C	(TG)12	v	T	G	0.12277	0.14104	0.0672	0.008	0.43 (0.25–0.74)
C	C	(TG)11	M	T	G	0.02319	0.02102	0.02868	0.722	
C	C	(TG)12	v	T	G	0.02134	0.02063	0.02559	0.722	
G	C	(TG)12	M	T	G	0.0172	0.005	0.04689	0.001	11.3 (2.3–54.6)
C	C	(TG)12	M	T	G	0.0142	0.01373	0.01287	0.943	
C	T	(TG)12	M	T	G	0.00539	0	0.01282	0.066	
C	C	(TG)12	v	T	A	0.0036	0.0025	0.00641	0.574	
G	C	(TG)11	v	T	G	0.00259	0	0.01081	0.063	
C	C	(TG)11	M	T	G	0.00223	0.00314	0	0.499	
C	C	(TG)12	M	G	G	0.0018	0.0025	0	0.474	
C	T	(TG)12	v	T	G	0.00001	0.0025	0	0.247	

CFTR, cystic fibrosis transmembrane conductance regulator; ICP, idiopathic chronic pancreatitis; OR, odds ratio; UTR, untranslated region.

$p = 0.008$ in omnibus test.

protein expression and anion transporting activities by 60–70%. The mutation spectrum in Chinese in our study had shown difference from that observed in East Asia also. Q1352H and E217G and M470V are considered to be strongly associated with chronic pancreatitis in Korean (9). I556V, I125T and Q1352H are reported to be associated chronic pulmonary disease from Singapore (35). In our study, only one control subject with Q1352H, which implied that the Q1352H might not associated with ICP in Chinese. S895N was found to be associated with CBAVD in Taiwanese (36). The 2694T>G is reported to be related to Chinese origin in a patient with CF (37).

Interestingly, we found the T5 allele was associated with early onset of ICP in our population. All the T5 allele was associated with the TG12 or the TG13 alleles in our population, a combination that is known to increase aberrant exon 9 splicing. In contrast, T5 was mainly associated with the TG11 allele in Caucasians (38). In recent publications, the differential formation of secondary structures by dinucleotide repeats TG12 or TG13 has been reported to affect *CFTR* splicing efficiency (39) and therefore to be of relevance for the discrimination between benign and pathogenically relevant 5T alleles (38). Therefore, in addition to compound heterozygous patients, subjects who carry one mild *CFTR* mutation or a 5T–12TG combination must now be considered to be at an increased risk of developing ICP.

With the increase of mutation numbers, there seemed to be a trend of earlier onset of ICP in Chinese. Maire et al. (40) reported that the prevalence of *CFTR* mutations was higher in patients with ICP before age of 35 in French population (35.5% in patients ≤ 35 years against 15.1% in those > 35 years, $p = 0.06$) but they did not mentioned whether some distinct mutation/abnormal allele was associated with the early onset of age. In our study, *CFTR* mutations were not associated with the presence of calcification, pseudocyst and pancreatic duct structures that were different to those reported from United States (41).

CFTR variants/polymorphisms

Poly T, poly TG and M470V in intron 8

The 5T alleles were not significantly increased in patients with ICP and controls. The majority of the 5T allele was associated with the TG12 or the TG13 alleles in our population (Table 4), a combination that is known to increase aberrant exon 9 splicing. In contrast, T5 was mainly associated

with the TG11 allele in Caucasians (38). In recent publications, the differential formation of secondary structures by dinucleotide repeats TG12 or TG13 has been reported to affect *CFTR* splicing efficiency (39) and therefore to be of relevance for the discrimination between benign and pathogenically relevant 5T alleles (38). Therefore, in addition to compound heterozygous patients, subjects who carry one mild *CFTR* mutation or a 5T–12TG combination must now be considered to be at an increased risk of developing ICP. We found one alleles of 6T in normal subjects. The 6T repeat has been found in a Korean patient with bronchiectasis (9) and it was very rare in Caucasians. As the 6T was found only in both CP and normal subjects in our study, its role in chronic pancreatitis is worthy to be further investigated.

Our patients and controls had a higher frequency (50% and 53% allele in ICP and control) of the (TG)12 repeat (Table 3) compared with Europeans (10.5% in disseminated bronchiectasis and 1% in control) (42). However, the frequency of the (TG) allele is similar to that in Japanese (10). The proportion of the (TG)11/11, (TG)11/12, and (TG)12/12 genotype was similar in patients with idiopathic pancreatitis and normal subjects in our population. Comparisons of genotypes of M470V alone showed no significant difference between patients with ICP and normal subjects, similar in Japanese (Table 3).

Evolution of (TG)n-M470V

When the haplotype frequencies of (TG)n-M470V were compared, there was a marked difference between Chinese and European populations. Our (TG)n-M470V is very similar to Japanese; about 2:3 of the M and V haplotype. There is about 70% of the M haplotype in European individuals, and haplotypes with (TG)11 and (TG)12 were about 30% and 3%, respectively (42). Furthermore, (TG)10-V470, which was not detected in this study, occupies 8% and 16% of the V haplotype in Greece and France. Thus, after separation from the white ancestors, the Chinese ancestors had received higher selective pressure for longer TG repeats or lower expression of CFTR proteins. One of the hypotheses for the selection pressure postulated by Japanese was the demand of less fluid loss related to Cl[−] channel in diarrheal diseases and less sweat fluid and electrolyte loss in the warm and humid climate in East Asia such as Japan. Long TG repeats may be a characteristic of Asian populations.

The genotypes that produce proteins with higher *CFTR* function, such as M/M470 with (TG)11/11 or 11/12 and M/V470 with (TG)11/11, were either absent or significantly smaller in Chinese, while a considerable number of these genotypes were present in Greek and French individuals (data not shown). Most M/M470 genotypes were associated with (TG)12/12, which reduces the amount of intact CFTR proteins. In the genotype (TG)11/11-V/V470, both genes express proteins with low intrinsic channel activity. Most genotypes in our population consisted of the (TG)11-V470 and (TG)12-M470 haplotype, which result in lower *CFTR* function owing to a decrease in the amount of intact protein from one gene and lower intrinsic channel activity of the protein coded by the other. Hence, the majority of Chinese have genotypes that cause lower *CFTR* function, similar to those of Japanese and Vietnamese (12). The genetic distance between Asians populations is as much shorter as that between Asians and Caucasians.

CFTR haplotypes

The overall *CFTR* function *in vivo* is determined by its genotype. Genotyping based on one single functional polymorphism is not sufficient to explain the association of chronic pancreatitis and *CFTR* gene. Additional mild mutation or/and polymorphism on these genetic backgrounds might further reduce *CFTR* function. In the present study, we found a distinct haplotype that confers host susceptibility of ICP in Chinese consisted of previously reported functional polymorphisms. Haplotypes assembled by the 125G/C, 1001+11C>T, TG repeats, M470V, 2694T>G, 4521G>A of *CFTR* could arbitrarily be classified into 13 common types in Chinese (Table 5). The six-loci haplotype analysis showed that the 125G/1001+11C/TG12/470M/2694T/4521G haplotype was associated with ICP in Chinese (OR 11.3; 95% CI 2.3–54.6, $p = 0.008$). When we analyzed the loci that composed the haplotypes, we found that 125G>C was the potential significant loci associated with ICP (Table 5). In this study, we observed that the G to C at 125 variation is strongly associated with ICP in Chinese (OR 13.9; 95% CI 3.1–62.2) which was reported to be associated with ICP in the first time. Functionally, the G to C change may destroy the unique *Ava*I site in exon 1. In Taiwanese patients with CBAVD, there was one patient carried G to C at 125 of *CFTR* gene and it was not known whether it was associated with CBAVD or not in Chinese. Functional analysis

of the significance of 125G/C of *CFTR* awaits further study.

In summary, this is the first *CFTR* study including patients with ICP and controls in Chinese by comprehensive analysis. We found that there were marked differences in the mutation spectrum between different ethnic populations in both ICP and controls. Our study has demonstrated that carriers of mild mutations of *CFTR* and a distinct haplotype carry an increased risk of developing ICP in Chinese. T5 allele is associated with early onset of ICP in our population. To our knowledge, the inheritance pattern of ICP is complex and partially understood. This indicates that the contribution of other factors (such as environmental factors) and interaction with other genetic determinants might affect the disease severity. It remains to be studied and elucidated what the cellular mechanism of mild mutation/functional polymorphisms of *CFTR* is and how it interact with to other genes to induce chronic pancreatitis. Because of a specific profile of *CFTR* mutation/haplotype, a population-specific panel should be recommended for targeted populations including ICP in Chinese. Such information is important to be able to design suitable screening programs for different populations.

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Competing interest

Nothing to declare.

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