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Common *CFTR* Haplotypes and Susceptibility to Chronic Pancreatitis and Congenital Bilateral Absence of the Vas Deferens

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ABSTRACT: *CFTR* mutations enhance susceptibility for idiopathic chronic pancreatitis (ICP) and congenital bilateral absence of the vas deferens (CBAVD); however, it is unknown why *CFTR* heterozygotes are at increased disease risk. We recently showed that common *CFTR* variants are associated with aberrantly spliced transcripts. Here, we genotyped for common *CFTR* variants and tested for associations in two ICP (ICP-A: 126 patients, 319 controls; ICP-B: 666 patients, 1,181 controls) and a CBAVD population (305 patients, 319 controls). Haplotype H10 (TG11-T7-470V) conferred protection (ICP-A: OR 0.19, $P < 0.0001$; ICP-B: OR 0.78, $P = 0.06$; CBAVD OR 0.08, $P < 0.001$), whereas haplotype H3 (TG10-T7-470M) increased disease risk (ICP-A: OR 8.34, $P = 0.003$; ICP-B: OR 1.88, $P = 0.007$; CBAVD: OR 5.67, $P = 0.01$). The risk of heterozygous *CFTR* mutations carriers for ICP (OR 2.44, $P < 0.001$) and CBAVD (OR 14.73, $P < 0.001$) was fully abrogated by the H10/H10 genotype. Similarly, ICP risk of heterozygous p.Asn34Ser *SPINK1* mutation carriers (OR 10.34, $P < 0.001$) was compensated by H10/H10. Thus, common *CFTR* haplotypes modulate ICP and CBAVD susceptibility alone and in heterozygous *CFTR* and p.Asn34Ser mutation carriers. Determination of these haplotypes helps to stratify carriers into high- and low-risk subjects, providing helpful information for genetic counseling.
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KEY WORDS: *CFTR*; CBAVD; idiopathic chronic pancreatitis

Introduction

Genetic defects in the cystic fibrosis transmembrane conductance regulator (*CFTR*; MIM# 602421) gene encompass a wide disease spectrum including classic cystic fibrosis (CF), nonclassic CF and *CFTR*-related disorders presenting with single organ involvement of the lung, vas deferens, or pancreas [Knowles and Durie, 2002; Moskowitz et al., 2008]. The phenotype depends on organ-specific protein requirements, the amount of functional protein, which is influenced by the genotype, as well as genetic modifiers and environmental factors. Although classic CF is caused by two severe mutations, patients with milder phenotypes carry at least one mild mutation, resulting in partially preserved

Bernhard Steiner and Jonas Rosendahl contributed equally to this study.

Additional Supporting Information may be found in the online version of this article.

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CFTR function. A substantial fraction of patients with nonclassic CF and *CFTR*-related disorders, however, displays only one mutated *CFTR* copy. It is a yet unresolved issue why heterozygous carriers are at increased disease risk.

Idiopathic chronic pancreatitis (ICP) and congenital bilateral absence of the vas deferens (CBAVD) represent two monosymptomatic *CFTR*-related disorders. A mutation is identified in up to 30% of ICP patients and in about 80% of patients with CBAVD [Audrezet et al., 2002; Chillon et al., 1995]. The *5T* allele is a polymorphic variant with variable penetrance, causing less efficient exon 9 splicing and lower *CFTR* transcript levels. The mechanism of the partial penetrance of *5T* is partly due to variation in the length of the adjacent TG repeat, which further affects exon 9 splicing efficiency. Recent evidence indicates that synonymous variants and so-called "neutral" polymorphisms may disrupt alternative splice sites, such as exonic splicing enhancers (ESEs), and contribute to the level of functional protein [Cartegni and Krainer, 2002; Gavrilov et al., 1998]. In our previous study, we observed a highly significant association between aberrantly spliced *CFTR* transcripts and the presence of common variants, which caused changes of regulatory sequence ESE motifs [Steiner et al., 2004]. These results suggested that complex haplotypes of common *CFTR* variants may contribute to the final amount of functional protein and determine the variable expressivity of mild *CFTR* alleles. This prompted us to analyze *CFTR* variants and mutations in ICP patients and healthy controls from five European countries. We conducted a large replication study in an independent German ICP population and analyzed the same *CFTR* variants also in a cohort with CBAVD.

Materials and Methods

ICP Patients

The primary ICP study group consisted of patients from five different European countries (Czech, $n = 18$; France, $n = 47$; Italy, $n = 17$; Spain, $n = 31$; Switzerland, $n = 13$; in total, 126) (Table 1). Patients were retrospectively recruited for the genetic evaluation of their clinical diagnosis of ICP, and the results of their *CFTR* mutation testing have partially been published previously [Audrezet et al., 2002; Casals et al., 2004; Gomez et al., 2001; Truninger et al., 2001]. Healthy individuals, unrelated to the patients, from these countries served as controls (Czech, $n = 110$; France, $n = 92$; Italy, $n = 50$; Switzerland, $n = 67$; in total, 319). Patients were considered to have ICP if they had experienced two or more episodes of acute pancreatitis documented with clinical symptoms, serum amylase, and/or lipase levels three times above the upper limit and pancreatic edema, hemorrhage, or necrosis on sonography or computed tomography. In addition, at least two of the following criteria were required: pancreatic calcification, exocrine insufficiency, diabetes, characteristic ductal changes on ERCP, abnormal pancreatic secretin function test results, or histological confirmation of chronic pancreatitis. Patients were included only after the exclusion of the most common etiological factors including alcohol abuse, bile duct obstruction, metabolic disorders, and trauma. In children, ICP was diagnosed as reported previously [Witt et al., 2000]. Patients were excluded if they had a family history of pancreatitis and/or when the *PRSS1* (MIM# 276000) gene mutations p.Arg122His or p.Asn29Ile were detected.

The replication study group consisted of 1,181 controls and 666 patients from Germany with the clinical diagnosis of ICP (Table 1). Again, the control subjects were unrelated in any way to patients. The diagnosis of ICP was based on the aforementioned criteria.

The study protocol was approved by the local medical ethic review committee at each participating center.

Table 1. Characteristics of Patients Groups and Number of Subjects Tested for the Various Genes

	Primary ICP study group	German ICP[a] replication group	CBAVD
Subjects			
Patients	126	666	305
Controls	319	1,181	319
Age of onset of disease (year, mean ± SD)	33.0 ± 16.0	30.6 ± 18.7	–
Genotyping CFTR mutations			
Patients	126	407	305
Controls	319	549	319
Poly-T tract and TG repeat in intron 8 and c.1408A>G (p.Met470Val)			
Patients	84	666	294
Controls	228	1,181	228

[a]Not all subjects included in the various groups with chronic pancreatitis were tested for *CFTR* mutations.

CBAVD Patients

Congenital bilateral absence of the vas deferens (CBAVD) accounts for approximately 3% of cases of male infertility in Caucasian populations. In about 85% of cases, CBAVD (MIM# 277180) is recognized as an autosomal recessive disorder associated with *CFTR* mutations. Clinical diagnosis of CBAVD was based on impalpable vas deferens on clinical examination and/or a missing segment of the vas on transrectal ultrasonography, a total absence of spermatozoa associated with low volume (< 1.5 ml), low pH (average < 6.8) and low concentrations of fructose in the ejaculate. Patients with renal abnormalities were excluded. The group consisted of 305 CBAVD patients: 213 from Southern France and 92 from Switzerland (Table 1). When available, parents of patients in whom a mutation had been detected were studied. Informed consent was obtained from all patients and their parents at the time of referral to the laboratory.

Genotyping

ICP patients

In the primary ICP study group, comprehensive *CFTR* (NM_000492.3) mutation screening was performed by denaturing gradient gel electrophoresis (DGGE), denaturing high-pressure liquid chromatography (dHPLC), or single-strand conformational polymorphism heteroduplex (SSCP-HD) analysis in all patients. We numbered the variants according to the current mutation nomenclature recommendations (www.hgvs.org/mutnomen) numbering the A of the ATG translation initiation codon as +1. In the primary ICP study group, 84 subjects were genotyped for five *CFTR* sequence variants: c.1210−12T(5_9) in intron 8 (according to the former nomenclature *5T - 9T*), c.1210−34GT(9_13) in intron 8 (TG9–TG13), c.1408A>G in exon 10 (p.Met470Val), c.2562T>G in exon 14a, c.4389G>A in exon 24. Criteria for the selection of these variants were based on the results of our previous study [Steiner et al., 2004] and the well described pathogenic role of the 5T and TG12/13 alleles in CBAVD [Groman et al., 2004].

In the German ICP replication group, 407 ICP patients and 549 controls were screened for 40 *CFTR* mutations and the five variants mentioned above. *CFTR* analysis was performed by melting curve analysis using fluorescence resonance energy transfer (FRET) probes.

ICP patients and control subjects were tested for the p.Arg122His and p.Asn29Ile in *PRSS1* (NM_002769.4) and for p.Asn34Ser in serin protease-inhibitor Kazal-type1 (*SPINK1*; NM_003122.3).

CBAVD Patients

In the CBAVD group, exhaustive analysis of the *CFTR* gene including (1) complete scanning of the 27 coding/flanking regions either by DGGE or DHPLC or sequencing, (2) screening for two frequent intronic mutations (c.1679+1.634A>G and 3717+12191C>T) by specific PCR-restriction tests or sequencing; and (3) searching of large rearrangements using a semiquantitative fluorescent PCR was performed. Results for c.1210−12T(5_9) and c.1210−34GT(9_13) in intron 8 and c.1408A>G in exon 10 (p.Met470Val) were available for 294 patients (96.4%) (Table 1).

Statistical Analysis

Testing for disease association of the genotypes of each single variant or haplotype was completed according to Sasieni [1997] using the online tool at http://ihg2.helmholtz-muenchen.de/cgi-bin/hw/hwa1.pl. The odds ratio (OR) for disease association was calculated in a heterozygous, homozygous or allele positivity (i.e., either homo- or heterozygous) model for each variant in a 1df test: for the heterozygous model: (Cases [WT−VT]∗Controls [WT−WT])/(Cases [WT−WT]∗Controls [WT−VT]); for the homozygous model: (Cases [VT−VT]∗Controls [WT−WT])/(Cases [WT−WT]∗Controls [VT−VT]) and for the allele positivity model: ((Cases [WT−VT]+Cases [VT−VT])∗Controls [WT−WT])/(Cases [WT−WT]∗(Controls [WT−VT]+Controls [VT−VT])). Additionally, allele frequency for each variant and conformity of the genotype distribution to the Hardy–Weinberg equilibrium was examined in each ICP, the CBAVD and the control groups. Test of linkage disequilibrium for all pairs of loci was done with the *Arlequin* Software (http://cmpg.unibe.ch/software/arlequin3/) using a test with 100,000 permutations and the EM algorithm [Excoffier et al., 2005]. Haplotypes consisting of three loci (the poly-T and -TG repeats and p.Met470Val) or five loci (plus the variants c.2562T>G and c.4389G>A) were estimated by the use of the PHASE algorithm (*PHASE*, version 2.1.1) based on Bayesian inference with the default settings [Stephens and Donnelly, 2003; Stephens et al., 2001] performing several independent runs from the whole population studied. Also, the estimated haplotype frequencies and the probabilities of the most likely pairs of haplotypes for each individual were obtained from the PHASE program. A standard stepwise logistic-regression procedure [Cordell and Clayton, 2002] was applied for evaluation of the relative importance of the different variants. The tests were performed in the statistical analysis package Stata 9.1 (http://www.stata.com/) with add-in routines for genetic analysis (dgc.genetics package: http://www-gene.cimr.cam.ac.uk/clayton/software/stata/). Two analyses of the data were undertaken: one in which the loci were modeled using alleles and the other in which they were modeled using genotypes. The most associated locus (i.e., the one with the smallest p value) was put in the logistic model. The other four loci were added one at a time, and a likelihood-ratio test was used to test whether either of them improved the model. A $P<0.01$ was considered suggestive of an improvement in the model. The main effect of each locus was calculated in a linear model with two parameters to allow an additive effect on a logit scale. Parameters for haplotype effects are also added in the model. With this framework and restricting the parameter(s) corresponding to the locus with association to main effects, we performed a 1-df or 2-df test for the additional effect of any single nucleotide polymorphism (SNP) or haplotype even after the effects of other SNPs have already been accounted for. To allow for multiple testing and to overcome the problem of correlated tests, we make use of the false discovery rate (FDR), by fixing the expected number of false positives among significant associations [Benjamini and Hochberg, 1995]. Specifically, if we select an uncorrected testwise significance level of α, the FDR is given by $N\alpha/k$, where N is the number of tests and k is the number of tests with a p value of less than α, so we fix a testwise significance level to obtain an overall FDR of 5%.

Results

We genotyped five *CFTR* sequence variants in the primary and replication study group: c.1210-12T(5_9) in intron 8 (according to the former nomenclature *5T–9T*), c.1210-34GT(9_13) in intron 8 (TG9–TG13), c.1408A>G in exon 10 (p.Met470Val), c.2562T>G in exon 14a, c.4389G>A in exon 24. The selection of the three SNPs and the two polymorphic variants are based on the results of our previous study, showing a highly significant association between the presence of these variants and aberrantly spliced *CFTR* transcripts [Steiner et al., 2004]. The amount of aberrantly spliced transcripts in individuals carrying these variants was similar to reported values for the 5T and TG12/13 alleles [Hefferon et al., 2004].

The statistical analyses first included association testing of the investigated *CFTR* sequence variants (Supp. Table S1). Next, the five variants were used to infer *CFTR* haplotypes and we explored their associations with ICP and CBAVD (Table 2, Supp. Tables S2 and S3). Finally, we performed a stepwise logistic regression procedure to evaluate the relative contribution to disease of each variant because haplotype analysis cannot distinguish between variants of primary functional importance and neighboring polymorphisms that are in linkage disequilibrium (LD).

Study in the Two Populations with ICP Patients

We investigated a primary study group including 126 ICP patients and 319 healthy individuals from five countries and conducted a large independent replication study consisting of 666 ICP patients and 1,181 controls (Table 1). To limit population stratification, patients and control subjects in the replication study were recruited only from Germany. In both ICP study populations, control groups were unrelated in any way to the patients.

CFTR Mutations

CFTR genotypes in patients and controls in the two ICP study groups are shown in Supp. Table S4. Due to lack of sufficient available DNA, not all subjects in the German replication group were genotyped for *CFTR* mutations. These subjects were excluded from the analysis of *CFTR* haplotypes in single mutant CFTR carriers. All mutations have previously been described (http://www.genet.sickkids.on.ca/cftr). According to a recent publication, we classified the *T5* allele as a mutation only when in *cis* with TG12 or TG13 [Groman et al., 2004].

CFTR mutations were identified in 8.4% of controls and 26.1% of patients in the primary ICP study group ($P<0.001$) compared to 6.9% of controls and 15.7% of patients ($P=0.004$) in the German ICP replication group (Table 3). No patient in both study groups carried two severe *CFTR* mutations. The majority of patients with any *CFTR* mutation had a mutation in only one copy of the gene in

Table 2. Number of Individuals and Odds Ratio of Common *CFTR* TGTM- Haplotypes for the Primary ICP Study Group, the German ICP Replication Group, Combined Sample Set and Patients with CBAVD

	ICP								
	Primary study group			Replication group			Combined sample set		
	No. of individuals and ODDS			No. of individuals and ODDS			No. of individuals and ODDS		
Haplotypes	Controls ($N = 228$)	Patients ($N = 84$)	P value	Controls ($N = 1{,}181$)	Patients ($N = 666$)	P value	Controls ($N = 1{,}409$)	Patients ($N = 750$)	P value
Homozygous model									
H3 (TG10-T7-M)	2 (0.9%)	6 (7.1%)	0.003*	38 (3.2%)	38 (5.7%)	0.007[a]	40 (2.8%)	44 (5.9%)	0.0004*
	8.34 (1.64–42.47)			1.88 (1.18–2.99)			2.19 (1.41–3.41)		
H10 (TG11-T7-V)	74 (32.5%)	10 (11.9%)	<0.0001[a]	364 (30.8%)	179 (26.9%)	0.06	438 (31.1%)	189 (25.4%)	0.002*
	0.19 (0.09–0.41)			0.78 (0.59–1.02)			0.66 (0.52–0.85)		
Heterozygous model									
H3 (TG10-T7-M)	62 (27.2%)	19 (22.6%)	0.60	366 (31.0%)	214 (32.1%)	0.38	428 (30.4%)	233 (31.1%)	0.42
	0.85 (0.47–1.54)			1.10 (0.89–1.35)			1.24 (1.06–1.45)		
H10 (TG11-T7-V)	97 (42.5%)	33 (39.3%)	0.009[a]	576 (48.8%)	334 (50.2%)	0.47	673 (47.8%)	367 (48.9%)	0.12
	0.47 (0.27–0.83)			0.91 (0.72–1.17)			0.84 (0.67–1.05)		

	CBAVD		
	No. of individuals and ODDS		
Haplotypes	Controls ($N = 228$)	Patients ($N = 294$)	P value
Homozygous model			
H3 (TG10-T7-M)	2 (0.9%)	13 (4.4%)	0.01[a]
	5.67 (1.26–25.50)		
H10 (TG11-T7-V)	74 (32.5%)	18 (6.1%)	<0.0001[a]
	0.08 (0.04–0.15)		
Heterozygous model			
H3 (TG10-T7-M)	62 (27.2%)	93 (31.6%)	0.17
	1.31 (0.89–1.92)		
H10 (TG11-T7-V)	97 (42.5%)	102 (34.7%)	<0.0001[a]
	0.34 (0.23–0.52)		

[a]Significant after correction for multiple testing using an overall false discovery rate (FDR) of 5%.

Table 3. Number of Individuals and Odds Ratio of *CFTR* mutations for the Primary ICP Study Group, the German ICP Replication Group, Combined Sample Set and Patients with CBAVD

	ICP								
	Primary study group			Replication group			Combined sample set		
	No. of individuals and ODDS			No. of individuals and ODDS			No. of individuals and ODDS		
Mutations	Controls ($N = 319$)	Patients ($N = 126$)	P value	Controls ($N = 549$)	Patients ($N = 407$)	P value	Controls ($N = 868$)	Patients ($N = 533$)	P value
Homozygous model									
CFTR	2 (0.6%)	8 (6.3%)	<0.0001	1 (0.2%)	7 (1.7%)	0.007[a]	3 (0.3%)	15 (2.8%)	<0.0001[a]
	12.56 (2.62–60.18)			10.43 (1.28–85.14)			9.21 (2.65–31.98)		
Heterozygous model									
CFTR	25 (7.8%)	25 (19.8%)	0.0001[a]	37 (6.7%)	57 (14.0%)	0.0001[a]	62 (7.1%)	82 (15.4%)	<0.0001[a]
	3.14 (1.72–5.73)			2.30 (1.48–3.55)			2.44 (1.72–3.46)		

	CBAVD		
	No. of individuals and ODDS		
Mutations	Controls ($N = 319$)	Patients ($N = 305$)	P value
Homozygous model			
CFTR	2 (0.6%)	201 (65.9%)	<0.0001[a]
	637.96 (153.12–2,658.01)		
Heterozygous model			
CFTR	25 (7.8%)	58 (19.0%)	<0.0001[a]
	14.73 (8.39–25.85)		

[a]Significant after correction for multiple testing using an overall false discovery rate (FDR) of 5%.

both ICP cohorts (75.8 and 89.1%, respectively). ICP risk associated with *CFTR* mutations was lower in the German replication group (heterozygous model OR 2.30, 95% CI (confidence interval) 1.48–3.55; homozygous model OR 10.43, 95% CI 1.28–85.14) compared to the primary ICP study group (OR 3.14, 95% CI 1.72–5.73; OR 12.56, 95% CI 2.62–60.18) (Table 3 and Fig. 1A).

CFTR Variants and Their Haplotypes

Single-variant association testing (Supp. Table S1) showed evidence of an association for the p.Met470Val and the c.1210–34GT[10] variant. The wild-type M allele and the c.1210−34GT[10] variant are occurring more frequently in patients than controls in the primary ICP group. Genotype distribution of all variants was in Hardy–Weinberg equilibrium in both study groups.

We identified 43 different haplotypes and their distribution differed significantly between cases and controls ($P<0.05$). The majority of haplotypes was rare (<2% frequency) (Supp. Tables S2 and S3).

Stepwise logistic regression procedure showed that the strongest protective effect was associated with TG11-T7 (OR 0.67, 95% CI 0.52–0.86, $P=0.002$) and adding p.470Val (χ^2 1.64 [2 df]; $P=0.44$), c.2562T (χ^2 1.07 [2 df]; $P=0.59$) and c.4389A (χ^2 1.16 [2 df])] $P=0.56$) made no significant independent contribution. This was due to the fact that TG11-T7 was predominantly combined with p.470Val (p.470Val: 97%; p.470Met: 3%). The almost complete LD between TG11-T7 and p.470Val suggests a haploblock of these three variants (calculated pairwise linkage disequilibrium: Pair (c.1210−34GT[9_13], c.1210−12T[5_9]): χ^2 41.80 (4 df); Pair (c.1210−34GT[9_13], p.Met470Val): χ^2 63.57 (2 df); Pair [c.1210−12T[5_9], p.Met470Val): χ^2 203.93 [2 df]; all P values <0.001). This finding was confirmed using the LD information from the HapMap (http://hapmap.ncbi.nlm.nih.gov/). The strongest risk effect was associated with TG10-T7 (OR 2.06, 95% CI 1.33–3.20, $P=0.001$). The addition of p.470Met (χ^2 6.07 [2 df]; $P=0.04$) but not of c.2562G (χ^2 2.28 [2 df]; $P=0.32$) and c.4389A (χ^2 2.04 [2 df]; $P=0.36$) improved model fit. Based on these findings, we performed association testing with the TGn-Tn-M/V-haplotype, as shown in Table 2.

In the homozygous model, the haplotype H3 (TG10-T7-p.470Met) was associated with an eightfold increased ICP risk in the primary ICP study group (OR 8.34, 95% CI 1.64–42.47, $P=0.003$) (Table 2). The risk effect of H3 was confirmed in the ICP replication group, although the size effect was less pronounced (OR 1.88, 95% CI 1.18−2.99, $P=0.007$). Notably, the risk effect of the H3/H3 genotype (OR 2.2, $P<0.001$) was of similar size like the risk in *CFTR* mutation heterozygotes (OR 2.4, $P<0.001$) (Fig. 1A).

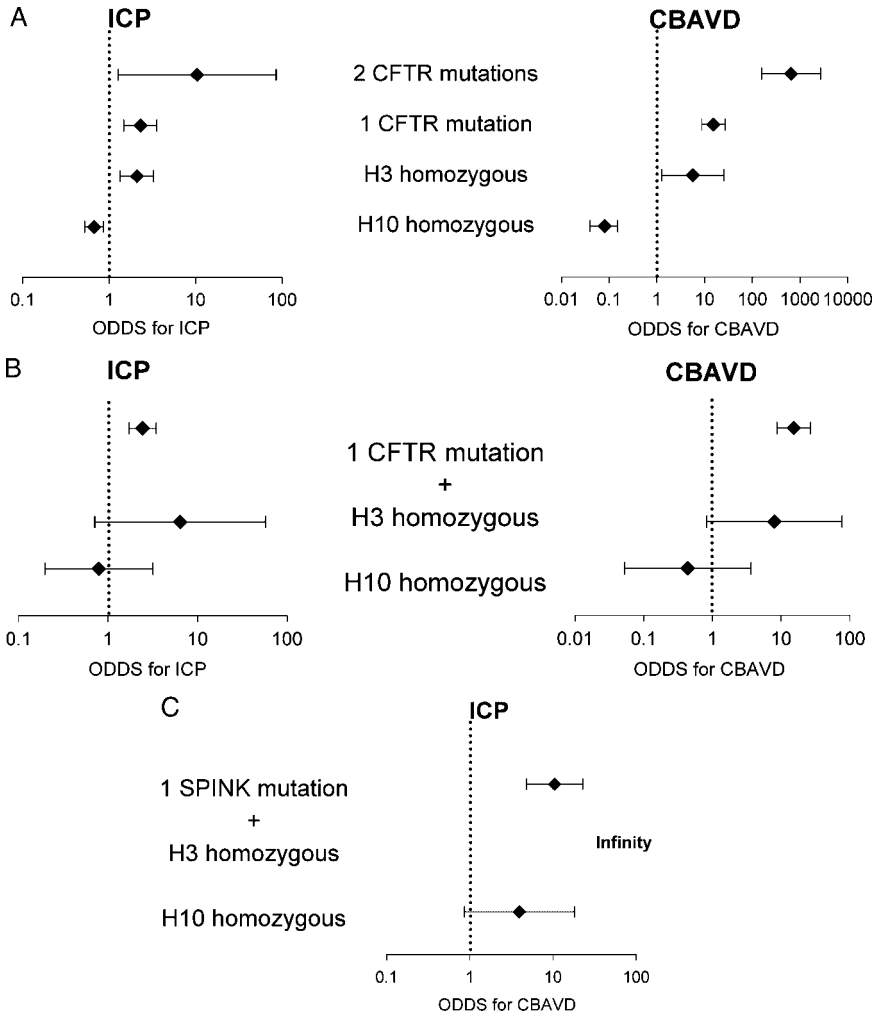

Figure 1. Associations of CFTR mutations and the TG-T-M-Haplotypes H3 and H10 with idiopathic chronic pancreatitis (ICP) and congenital bilateral absence of the vas deferens (CBAVD).

We assessed the additive risk effect of H3 in relation to the presence of none, one or two mutant *CFTR* alleles (Supp. Fig. S1). Notably, a significant adverse effect associated with the genotype H3/H3 was observed in patients without *CFTR* mutations, rendering it very unlikely that this effect was caused by linkage with mutations searched for. The protective genotype H10/H10 (TG11-T7-p.470Val), observed in almost a third of controls, was associated with a fivefold risk reduction in the primary ICP group (OR 0.19, 95% CI 0.09–0.41, $P<0.0001$) (Table 2). The protective effect associated with this genotype approached significance in the ICP replication group (OR 0.78, 95% CI 0.59–1.02, $P=0.06$) and was confirmed in the combined sample set (OR 0.66, 95% CI 0.52−0.85, $P=0.002$).

Study in the Population with CBAVD Patients

To further explore the observed adverse and protective effects associated with CFTR mutations and the H3 and H10 haplotypes, we examined a large cohort of CBAVD patients (Table 1).

CFTR Mutations

CFTR genotypes are shown in Supp. Table S5. Susceptibility to develop CBAVD due to *CFTR* mutations was much higher compared to ICP (heterozygous model OR 14.73, 95% CI 8.39–25.85, $P<0.001$; homozygous model OR 638, 95% CI 153–2658, $P<0.001$) (Table 3 and Fig. 1A). Again, no patient carried two severe *CFTR* mutations and the most significant adverse effect associated with the genotype H3/H3 was found in patients without a mutant *CFTR* allele (Supp. Fig. S1).

CFTR Variants and Their Haplotypes

In the homozygous model, haplotype H3 was associated with an increased disease risk (OR 5.67, 95% CI 1.26–25.50, $P=0.01$), whereas the haplotype H10 conferred a highly significant and strong protective effect (OR 0.08, 95% CI 0.04–0.15, $P<0.0001$) (Table 2). Again, a significant effect of H3/H3 was found in patients without any *CFTR* mutation (Supp. Fig. S1).

CFTR Haplotypes in Single Mutant *CFTR* Carriers

In mono-symptomatic *CFTR*-related phenotypes, there is a substantial fraction of patients carrying only one mutant *CFTR* allele; however, it is a yet unresolved issue why *CFTR* heterozygotes, suggested to produce 50% of functional protein, are at increased disease risk. We therefore investigated the effect of H3 and H10 in heterozygous *CFTR* carriers of the combined ICP sample set and the CBAVD cohort. As shown in Figure 1B, the increased risk associated with a single mutant *CFTR* allele for ICP (OR 2.44, 95% CI 1.72–3.46, $P<0.001$) and CBAVD (OR 14.73, 95% CI 8.39–25.85, $P<0.001$) was fully abrogated by the H10/H10 genotype (ICP OR 0.79, 95% CI 0.20–3.17; CBAVD OR 0.44, 95% CI 0.05–3.66). The risk for ICP increased in the presence of H3/H3 genotype in the ICP study group (OR 6.37, 95% CI 0.71–57.19; CBAVD OR 0.44, 95% CI 0.05–3.66) but not in the CBAVD patients (OR 8.00, 95% CI 0.83–77.22). By logistic regression analysis, we confirmed the effects of H3 or H10 haplotypes in patients with *CFTR* mutations. The homozygous H3/H3 haplotype significantly improved the fit of the multiplicative model and elevated the risk for ICP (χ^2 11.13 [2 df]; $P=0.004$), whereas, the H10 haplotypes reduced the risk for ICP (χ^2 13.20 [2 df]; $P=0.001$). As both haplotypes H3 and H10 show independent influences, this finding point to a real haplotype effect. The presence of opposite functional intronic variants or unidentified *CFTR* mutations in close linkage to H3 or H10 seems unlikely.

Epistasis Between *CFTR* and *SPINK1* in ICP

Similar to single mutant *CFTR* carriers, it is poorly understood why only some p.Asn34Ser heterozygotes develop ICP. We therefore genotyped for this mutation and investigated the effect of H3 and H10 in p.Asn34Ser heterozygotes. The p.Asn34Ser *SPINK1* mutation was identified in 7/540 controls (1.3%) and in 97/708 ICP patients (13.7%, $P<0.001$). No controls, but 13/708 ICP patients (1.8%) were p.Asn34Ser homozygous. ICP risk was increased by about 24-fold in homozygous (OR 23.56, 95% CI 1.40–397.20, $P<0.001$) and by 10.5-fold in single p.Asn34Ser carriers (OR 10.47, 95% CI 4.80–22.83, $P<0.001$). As shown in Figure 1C for p.Asn34Ser heterozygotes, the H3/H3 genotype was found in 6/84 ICP patients, but in none of the controls increasing disease risk to infinity. The H10/H10 genotype conferred protection against the development of ICP (OR 3.95, 95% CI 0.86–18.09).

Distribution of the Mutated Regions in Patients with Two *CFTR* Mutations

CFTR mutations were found in ABC transporter domains 1 and 2 (NBD1 and NBD2), the ABC transmembrane domains (TMD1 and TMD2) and the regulatory domain (RD). The majority of mutations was located in NBD1, rendering the distribution of mutations in this domain suitable for statistical analysis. The percentage of patients with two mutations in the NBD1 is significantly lower in ICP patients (i.e., 1 out of 15 patients with 2 *CFTR* mutations; 6.7%) than in patients with CBAVD (86/195; 44.1%, $P=0.005$), but there is no difference in patients with one mutation in NBD1 and the second in NBD2 (ICP: 1/15 [6.7%]; CBAVD: 8/195 [4.1%], $P=0.49$) (Fig. 2A). The localization of the mutated amino acid or exon is presented graphically in Figure 2B. The size of the area corresponds to the number of patients.

Discussion

In this study, we performed an extended *CFTR* haplotype association analysis at the TGm, Tn, and p.Met470Val locus in patients with ICP and CBAVD and healthy controls. Our data suggest that the common *CFTR* haplotypes TG10-T7-p.470Met (H3) and TG11-T7-p.470Val (H10) contribute significantly to CBAVD and ICP susceptibility, both alone and in heterozygous *CFTR* and p.Asn34Ser mutation carriers. Replication in independent cohorts and reducing population heterogeneity is crucial to confirm variant-disease associations [Ioannidis, 2007]. We therefore performed haplotype association testing in two independent ICP populations and explored the association of specific haplotypes in CBAVD, a more homogeneous *CFTR*-related disorder. Our genetic study demonstrates different size effects and significance levels, but the disease modulating effects associated with the haplotypes H3 and H10 are consistent across all three populations.

The TG11-T7 haplotype has been shown to give about 14% miss-splicing compared to 5% of the TG10-T7 haplotype [Cuppens et al., 1998]. The allele at the p.Met470Val locus is known to affect CFTR function with the methionin variant showing increased intrinsic chloride channel activity, but slower

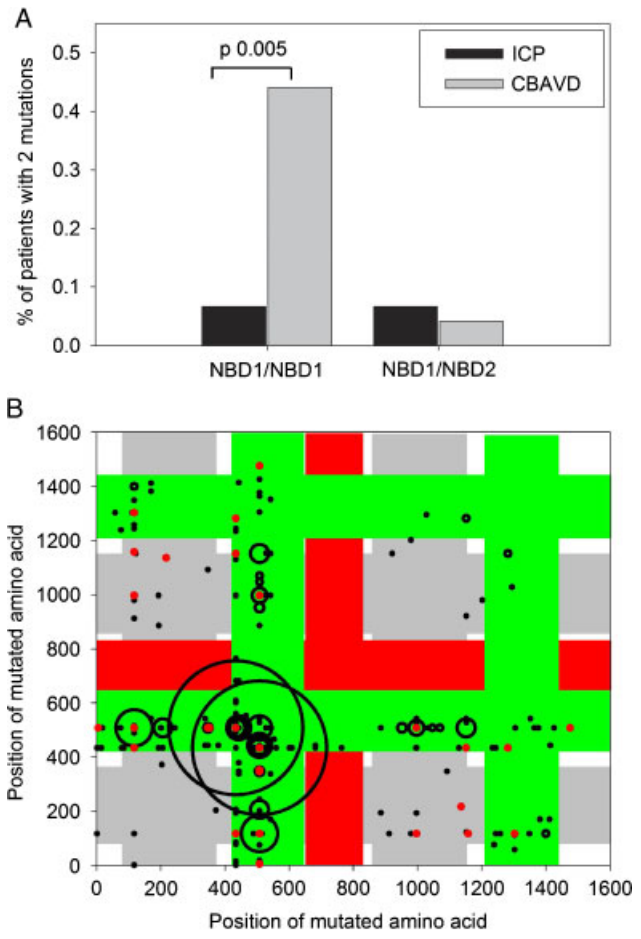

Figure 2. Distribution and localization of the mutated aminoacid or exon in the CFTR gene in patients with idiopathic chronic pancreatitis (ICP) and congenital bilateral absence of the vas deferens (CBAVD) with 2 mutations. **A:** Percentage of patients with either two mutations in the NBD1 or one in NBD1 and the other in NBD2. **B:** Localization of the two mutation according to position of the mutated amino acid or exon (ICP: red dots; CBAVD: black dots). The colored bands represent the following functional domains: in gray: ABC tramsmembrane domains (gray), ABC transporter domains (NBD1 and NBD2) (green), regulatory domain (red).

maturation compared to the valine variant [Cuppens et al., 1998]. In the present study, the haplotype TG11-T7-p.470Val was associated with a protective effect, whereas the TG10-T7-p.470Met allele increased disease risk for ICP and CBAVD. A recent study reported similar findings in patients with primary sclerosing cholangitis, that is, the TG11-T7 allele and p.470Val were less frequent in patients than controls [Henckaerts et al., 2009]. Three studies of ICP patients with an Asian ethnical background also underline the importance of the p.Met470Val variant and the importance of haplotype analysis [Chang et al., 2007, 2008; Lee et al., 2003]. Disease protection associated with the valine allele has also been shown in respiratory disorders [Stankovic et al., 2008; Tzetis et al., 2001]. These results from *CFTR*-related disorders demonstrate that the role of CFTR in epithelial cells extends well beyond chloride permeability [Guggino and Stanton, 2006]. Accumulating evidence suggests that CFTR influences diverse cellular processes by interaction with a number of proteins. These interactions may affect localization, processing, and trafficking of CFTR within cells. Our data suggest that the TG10-T7-p.470Met haplotype enhances ICP and CBAVD susceptibility. The slower maturation of the p.470Met allele is maybe not considerably affecting the intrinsic chloride channel activity of the pancreatic or testicular cells, but impairing the tissue- and development-specific maturing and turnover of a functional CFTR channel.

The frequency of *CFTR* mutations and their risk effect was higher in CBAVD compared to ICP, suggesting that the *CFTR*-related pathway is less common in ICP and its genetics to be more complex. Another difference between ICP and CBAVD concerned the distinct mutation distribution in the *CFTR* domains in patients with two mutant alleles. This result raises the possibility that beside organ-specific protein requirements also different distributions of *CFTR* mutations might explain why subjects develop ICP or CBAVD. Further studies are required to understand selectivity for functionally nonequivalent *CFTR* domains and the associated phenotype.

In the study by Groman et al. [2002], no differences in clinical features and sweat chloride concentrations were observed between nonclassic CF patients with and without *CFTR* mutations. It was hypothesized that factors other than *CFTR* defects can cause such phenotypes. Our data indicate that individuals without mutant *CFTR* may have an increased risk to develop ICP (OR 2.70, 95% CI 1.44–5.03, $P = 0.001$) or CBAVD (OR 3.94, 95% CI 1.25–12.44, $P = 0.01$), depending on the TG-T-p.Met470Val haplotype. The fact that an independent positive or negative risk effect of the homozygous H3 and H10 genotypes was observed in subjects with and without *CFTR* mutations, renders it very unlikely that this effect was caused by linkage with unidentified mutations. Thus, ICP and CBAVD may be *CFTR* related despite negative comprehensive mutation testing. All our patients had isolated ICP and CBAVD at the time of diagnosis; however, pulmonary or gastrointestinal CF manifestations may emerge later in their life. ICP patients experienced at least two episodes of acute pancreatitis, a rare (1.4%), but potential first complication among patients with CF [De et al., 2005]. Sweat test was performed in 16 of our ICP patients and displayed normal values. Because not all patients were tested for laboratory evidence of *CFTR* dysfunction, we cannot totally exclude that few ICP patients may develop CF complications later in their life, although this would be difficult to define because nasal potential difference and sweat chloride levels often display intermediate levels in *CFTR*-related disorders. There is increasing evidence that the majority of patients with isolated CBAVD also displays features of mild respiratory disease (such as sinusitis, nasal polyposis, chronic cough) or digestive manifestations (pancreatitis) when carefully examined by specialized clinicians [Claustres, 2005]. In our series of infertile patients, complete clinical evaluation was done in almost 30% and associated symptoms were reported in 42 cases. Moreover, sweat test was performed in 29 CBAVD patients; values were positive in 11, intermediate in 9, and negative in 6 cases.

An increased proportion of single mutant *CFTR* carriers have been reported in patients with asthma, chronic rhinosinusitis, and bronchiectasis, challenging the concept that *CFTR* heterozygotes are asymptomatic [Dahl et al., 1998; Pignatti et al., 1995; Wang et al., 2005]. Limitations of these studies include lack of investigating for rare mutations and no testing on healthy controls. Similarly, it is poorly understood why only some subjects heterozygous for p.Asn34Ser develop ICP. Our data show an increased susceptibility for ICP and CBAVD in single mutant *CFTR* and p.Asn34Ser carriers; however, homozygosity for haplotype H10 conferred protection, whereas ICP risk was strongly enhanced by the presence of H3/H3 (Fig. 1B and C). Thus, we provide evidence for a genetic interaction between *CFTR*

and *SPINK1* and the variable penetrance of p.Asn34Ser and *CFTR* mutations heterozygotes might partly be explained by common *CFTR* haplotypes.

Our study provides data, which are helpful for genetic counseling. Given the high frequency of healthy *CFTR* (~5%) and p.Asn34Ser (~1%) heterozygotes in Caucasians, it is important to reliably detect carriers and identify those at risk to develop ICP and CBAVD. According to our results, we propose that the analysis of TG-T-p.Met470Val haplotypes may help to stratify single-mutant *CFTR* and p.Asn34Ser carriers into a high- and low-risk group. Due to novel techniques such as intracytoplasmatic sperm injection, CBAVD men are able to father children. Because commercial tests only analyze common CF-causing, but not mild CFTR mutations, the carrier status of a CBAVD partner may remain undetected and a couple may still have a risk of having a CF child. Our results show a risk reduction for subjects with no mutant *CFTR* and the H10/H10 genotype (ICP: OR 0.75, 95% CI 0.53–1.04, $P = 0.08$; CBAVD: OR 0.44, 95% CI 0.18–1.07, $P = 0.06$). In subjects with no mutant *CFTR* and no H3/H3 genotype, there was a risk reduction of 60% for ICP (OR 0.39, 95% CI 0.21–0.71, $P = 0.006$) and of 75% for CBAVD (OR 0.26, 95% CI 0.08–0.80, $P = 0.01$). Thus, lack of mutant *CFTR* combined with the H3/H3 genotype renders it very unlikely that a *CFTR* defect is the underlying disease causing mechanism. In a familial segregation study presenting two brothers with an identical *CFTR* haplotype, but different phenotypes, one being fertile and the other having CBAVD, it was suggested that another gene is implicated in the pathophysiology of CBAVD [Mercier et al., 1995]. Here we show evidence that the analysis of TG-T-p.Met470Val haplotypes provides important information in single mutant carriers on whether ICP and CBAVD are *CFTR* related or rather the result of genetic defects at other loci. Uncovering the underlying disease-specific genetic pathogenesis is not only important for genetic counseling, but also to develop new therapeutic strategies.

In summary, we provide evidence that common TG-T-p.Met470Val haplotypes contribute significantly to ICP and CBAVD susceptibility both alone and in epistasis with *SPINK1*. The analysis of few variants may help to identify clinically relevant haplotypes, which provide more informative genetic counseling, particularly for heterozygous *CFTR* and p.Asn34Ser mutation carriers. In addition, knowledge of these haplotypes provides information on the underlying disease-causing mechanism, that is, whether patients with ICP or infertile men have *CFTR*-related disease or not. The exact pathobiology of the TG-T-p.Met470Val haplotypes remains to be investigated.

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