Mutational Analysis of ATP8B1 in Patients with Chronic Pancreatitis

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Abstract

**Background:** Mutations in genes encoding cationic trypsinogen (PRSS1), pancreatic secretory trypsin inhibitor (SPINK1) and chymotrypsinogen C (CTRC) are associated with chronic pancreatitis. However, in many patients with a familial chronic pancreatitis pattern suggesting a genetic cause, no mutations in either of these genes can be found, indicating that other, still unknown, associated genes exist. In this respect ATP8B1 is an interesting candidate due to its strong expression in the pancreas, its supposed general function in membrane organization and the higher incidence of pancreatitis in patients with ATP8B1 deficiency.

**Methods:** We analyzed all 27 ATP8B1 coding exons and adjacent non-coding sequences of 507 chronic pancreatitis patients by direct sequencing. Exons that harbored possible relevant variations were subsequently sequenced in 1,027 healthy controls.

**Results:** In the exonic regions, 5 novel non-synonymous alterations were detected as well as 14 previously described alterations of which some were associated with ATP8B1 deficiency. However, allele frequencies for any of these variations did not significantly differ between patients and controls. Furthermore, several non-synonymous variants were exclusively detected in control subjects and multiple variants in the non-coding sequence were identified with similar frequencies in both groups.

**Conclusions:** We did not find an association between heterozygous ATP8B1 variants and chronic pancreatitis in our cohort of patients with hereditary and idiopathic chronic pancreatitis.

Introduction

Chronic pancreatitis (CP) is an inflammatory disease characterized by destruction of pancreatic parenchyma that can result in permanent impairment of both exocrine and endocrine pancreatic function [1]. CP might cluster in families, and in many of these affected subjects as well as young patients without a family history of pancreatitis, mutations in the genes coding for cationic trypsinogen (PRSS1), pancreatic secretory trypsin inhibitor (SPINK1) and chymotrypsinogen C (CTRC) can be identified [2–5]. In general, mutations in these genes disturb the protease-antiprotease equilibrium within the pancreas, either through enhanced activation of trypsinogen or a reduced inhibition of this activated protease. CFTR mutations too enhance the susceptibility for idiopathic chronic pancreatitis [6]. Despite the growing number of genes associated with CP, in many patients with pancreatitis and an inheritance pattern suggesting a genetic cause, no variant within these genes can be identified, suggesting that other still unidentified genes might exist [1].

ATP8B1 deficiency is an autosomal recessive disease characterized by mutations in ATP8B1 (formerly designated as FIC1) [7]. ATP8B1 deficiency can present with persistent cholestasis, usually at young age (progressive familial intrahepatic cholestasis; PFIC) or with episodic cholestasis at any age (benign recurrent intrahepatic cholestasis; BRIC). Occasionally the benign variant will progress to the more severe and permanent form of intrahepatic cholestasis, indicative of a clinical continuum [8]. Extrahepatic manifestations such as diarrhea, pancreatitis and hearing loss can be observed in patients with ATP8B1 deficiency [9]. ATP8B1 is thought to be essential for maintaining membrane lipid asymmetry by translocation of aminophospholipids from the

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outer to the inner leaflet of the plasmamembrane. Loss of this asymmetric distribution of phospholipids in cellular membranes is presumed to affect fundamental processes such as membrane transport. Therefore deficiency of ATP8B1 might result in dysfunction of transmembrane transporters such as ABCB11, the bile salt export pump, within the canalicular membrane of liver cells, causing intrahepatic cholestasis [8]. Similarly, the stability of the cellular membranes in cochlear hair cells is reduced in patients with ATP8B1 deficiency, resulting in progressive hearing loss [10]. Apart from liver and cochlear cells, ATP8B1 is also expressed in other tissues, especially the pancreas [7], [10], [11]. As the incidence of pancreatitis is higher in patients with ATP8B1 deficiency, we hypothesized that mutations in this gene might also be associated with CP [9], [12], [13]. Therefore, we investigated a large cohort of CP patients and control subjects for ATP8B1 mutations.

### Materials and Methods

#### Study subjects

The study was approved by the local ethics committee of the Technische Universität München and the ethical review committee of the Universität de Bretagne Occidentale. All study subjects gave their written informed consent for genetic analysis. For this study, 507 patients with hereditary or idiopathic chronic pancreatitis were included. The patients originated from Germany (n = 316) and France (n = 191). In the German patients, the diagnosis of CP was based on two or more of the following findings as described previously: presence of a typical history of recurrent pancreatitis, pancreatic calcifications and/or pancreatic ductal irregularities revealed by endoscopic retrograde pancreatectography or by magnetic resonance imaging of the pancreas, and pathological sonographic findings [5]. Hereditary pancreatitis was diagnosed when one first-degree relative or two or more second-degree relatives suffered from recurrent acute or chronic pancreatitis without apparent precipitating factors. Affected individuals were classified as having idiopathic chronic pancreatitis.

#### Table 1. Non-synonymous exonic ATP8B1 variations in CP patients and controls.

| Region | Nucleotide Change | Amino Acid Change | Genotype | Patients (%) | Controls (%) | P value |
|--------|-------------------|-------------------|----------|--------------|--------------|---------|
| Exon 2 | c.134A>C          | p.N45T            | CC       | 1/507 (0.2)  | 0/1027 (0)   | 0.33    |
|        |                   |                   | AC       | 7/507 (1.4)  | 17/1027 (1.7) | 0.83    |
| Exon 3 | c.208G>A          | p.D70N            | GA       | 8/507 (1.6)  | 9/1027 (0.9) | 0.3     |
|        |                   |                   | CG       | 1/507 (0.2)  | 0/1027 (0)   | 0.33    |
| Exon 7 | c.607A>G          | p.K203E           | AG       | 2/507 (0.4)  | 3/1027 (0.3) | 0.67    |
| Exon 10| c.913T>A          | p.F305I           | TA       | 1/507 (0.2)  | 8/1027 (0.8) | 0.29    |
| Exon 12| c.1046T>C         | p.I349T           | TC       | 0/507 (0)    | 1/1027 (0.1) | 0.33    |
|        | c.1102A>G         | p.N368D           | AG       | 0/507 (0)    | 1/1027 (0.1) | 0.33    |
|        |                   |                   | TG       | 5/507 (1.0)  | 6/1027 (0.6) | 0.52    |
| Exon 13| c.1286A>C         | p.E429A           | AC       | 1/507 (0.2)  | 0/1027 (0)   | 0.33    |
|        |                   |                   | CT       | 2/1027 (0.2) | 0.55        |
| Exon 15| c.1498T>C         | p.Y500H           | TC       | 1/507 (0.2)  | 3/1027 (0.3) | 1.0     |
| Exon 22| c.2442G>T         | p.K814N           | GT       | 1/507 (0.2)  | 0/1027 (0)   | 0.33    |
|        |                   |                   | CT       | 1/1027 (0.1) | 0.33        |
| Exon 23| c.2771A>G         | p.Y924C           | AG       | 0/507 (0)    | 1/1027 (0.1) | 0.33    |
|        |                   |                   | GA       | 1/507 (0.2)  | 0/1027 (0)   | 0.33    |
|        | c.2789G>A         | p.R930Q           | GC       | 1/507 (0.2)  | 0/1027 (0)   | 0.33    |
| Exon 27| c.3449T>C         | p.I1150T          | GC       | 1/507 (0.2)  | 0/1027 (0)   | 0.33    |
| Exon 28| c.3589G>T         | p.V1197L          | GT       | 1/507 (0.2)  | 0/1027 (0)   | 0.33    |
|        | c.3622_3628delGCCTACG | p.A1208fs    | TC       | 0/1027 (0)   | 0.33        |
| All exons | -                   | -                 | -       | 139/507 (27.4) | 264/1027 (25.7) | 0.5    |

CP = chronic pancreatitis.
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in the presence of such a family history and when known precipitating factors, such as alcohol abuse, trauma, medication, infection, metabolic disorders, were absent.

In the French patients, CP was diagnosed as described previously [3],[14]. Patients with hereditary pancreatitis had three or more affected family members involving at least two generations.

The control group consisted of 1,027 unrelated healthy individuals of German (n = 742) and French (n = 285) origin. Whenever a non-synonymous variation in the coding sequence was encountered all 1,027 controls were sequenced for the exon involved.

**Mutational analysis**

Genomic DNA of peripheral blood leukocytes was extracted routinely. Primer pairs for PCR were designed to amplify all 27 coding exons, with flanking intron-exon boundaries of ATP8B1 followed by uni-directional DNA sequencing. Primer sequences are available on request. PCR was performed using standard methodology and semi-automated sequence analysis using an ABI 3730 sequencer (Applied Biosystems).

To detect nucleotide sequence changes of potential relevance to clinical phenotypes, we analyzed the sequence output of the patient cohort for variants that resulted in amino acid changes, nonsense variants or deletions/insertions. Exons with one of these alterations were subsequently analyzed in the control population by DNA sequencing.

The reference sequence was derived from GenBank (http://www.ncbi.nlm.nih.gov/entrez, reference sequence NM_005603.4). The A of the ATG start codon was used as nucleotide +1. The mutations are described according to the nomenclature recommended by the Human Genome Variation Society (http://www.hgvs.org/mutnomen).

**Statistical analysis**

The significance of the differences between mutation frequencies between patients and controls was tested by two-tailed Fisher’s exact test. A p value of less than 0.05 was considered significant. Correction according to Bonferroni was carried out.

**Results**

In our cohort of 507 CP patients we identified 19 different ATP8B1 variants leading to an amino acid change (Table 1). Five (p.R933W, p.K885T, p.R946T, p.I1150T, p.V1197L) were not described before, neither in the published literature nor in the Human Genome Mutation Database. Fourteen variants were described earlier; three of these variants (p.Y500H, p.E665X, p.A1208fs) are associated with the progressive form of ATP8B1 deficiency and six with the episodic form (p.N45T, p.H780Q, p.D70N, p.E429A, p.I577V, p.M674T). Four were mentioned in the literature for their possible association with intrahepatic cholestasis of pregnancy (p.N45T, p.D70N, p.K203E, p.F305I). In two patients more than one ATP8B1 variant was detected. The first patient had three non-synonymous variants (p.H780Q, p.I577V, p.M674T), all possibly associated in literature with episodic ATP8B1 deficiency. The second patient had two variants (p.E665X, p.A1208fs), both described in patients with the progressive form of ATP8B1 deficiency. Interestingly both these patients presented with chronic pancreatitis and had no signs of liver disease whatsoever.

Those exons in which a non-synonymous variant was detected in CP patients (13 exons) were also sequenced in our control cohort of 1,027 subjects. No alteration was significantly overrepresented in the patient group. Also the combined frequency of these non-synonymous exonic ATP8B1 variations in CP patients did not significantly differ from that in controls (p = 0.5). Furthermore, in the control population we detected 8 additional non-synonymous variations, of which 5 had not been described before (Table 1). In addition, synonymous or non-coding sequence variations were detected in both groups (Table 2 and 3). There was no significant difference for any of these variants between the CP patients and control group except for the SNP c.2097+89T>G. However, after using the Bonferroni correction for multiple testing, this significance disappeared.

**Discussion**

At the outset of our investigations, ATP8B1 seemed a plausible candidate gene for chronic pancreatitis due to its high expression...
in the pancreas, its supposed general function in membrane organization and the finding that 2 out of 10 individuals affected with ATP8B1 deficiency had chronic pancreatitis [12]. However, we did not find an association between heterozygous ATP8B1 variants and hereditary or idiopathic chronic pancreatitis when comparing 507 patients and 1,027 controls.

We did identify two CP patients with two or three non-synonymous ATP8B1 variants. We could not experimentally verify independent inheritance as no genomic material was available from parents or unaffected family members. However if these patients were indeed compound heterozygous, these genotypes are predicted to result in an ATP8B1 deficiency phenotype. Especially the p.E665X and p.A1208fs mutations change the structure of ATP8B1 significantly and can cause PFIC. Yet these two CP patients did not have any signs of liver disease or extrahepatic features of ATP8B1 deficiency other than pancreatitis. ATP8B1 deficiency without liver disease has been described before, suggesting that reduced penetrance of the liver phenotype can

| Table 3. ATP8B1 variations in non-coding regions in CP patients and controls. |
|------------------|------------------|------------------|------------------|
| Region           | Nucleotide Change | Genotype         | Patients (%)     | Controls (%)     | P value |
| Promotor variation | c.-4C>G           | CG               | 0/507 (0)        | 1/1027 (0.1)     | 0.33    |
| Intron 2          | c.162-5T>A        | TA               | 1/507 (0.2)      | 0/1027 (0)       | 0.33    |
|                  | c.182-72G>A       | GA               | 80/507 (15.8)    | 184/1027 (17.9)  | 0.31    |
|                  |                   | AA               | 3/507 (0.6)      | 19/1027 (1.9)    | 0.07    |
| Intron 6          | c.555-3T>C        | TC               | 0/507 (0)        | 1/1027 (0.1)     | 0.33    |
| Intron 7          | c.628-30G>A       | GA               | 0/507 (0)        | 2/564 (0.4)      | 0.5     |
|                  | c.628-31C>T       | CT               | 1/507 (0.2)      | 0/564 (0)        | 0.47    |
| Intron 8          | c.698+20C>T       | CT               | 251/507 (49.5)   | 263/564 (46.6)   | 0.36    |
|                  |                   | TT               | 118/507 (23.3)   | 110/564 (19.5)   | 0.14    |
| Intron 9          | c.782-34G>A       | GA               | 1/507 (0.2)      | 0/1027 (0)       | 0.33    |
| Intron 12         | c.1221-8C>G       | CG               | 1/507 (0.2)      | 0/1027 (0)       | 0.33    |
| Intron 13         | c.1429+49G>A      | GA               | 1/507 (0.2)      | 2/1027 (0.2)     | 1.0     |
|                  | c.1430-42A>G      | AG               | 135/507 (26.6)   | NS -             |
|                  |                   | GG               | 17/507 (3.4)     | NS -             |
| Intron 15         | c.1631-10T>A      | TA               | 0/507 (0)        | 1/1027 (0.1)     | 0.33    |
|                  | c.1637-37T>C      | TC               | 2/507 (0.4)      | 6/1027 (0.6)     | 1.0     |
| Intron 16         | c.1820-27G>A      | GA               | 1/507 (0.2)      | NS -             |
| Intron 18         | c.2097+60T>G      | TG               | 0/507 (0)        | 3/1027 (0.3)     | 0.56    |
|                  | c.2097+89T>C      | TC               | 30/507 (5.9)     | 32/1027 (3.1)    | 0.01    |
|                  |                   | CC               | 3/507 (0.6)      | 0/1027 (0)       | 0.04    |
|                  | c.2097+97T>G      | TG               | 0/507 (0)        | 1/1027 (0.1)     | 0.33    |
| Intron 20         | c.2285+29C>T      | CT               | 206/507 (40.6)   | NS -             |
|                  |                   | TT               | 31/507 (6.1)     | NS -             |
|                  | c.2285+32A>G      | AG               | 3/507 (0.6)      | NS -             |
| Intron 22         | c.2707+9T>G       | TG               | 0/507 (0)        | 1/1027 (0.1)     | 0.33    |
|                  | c.2707+43A>G      | AG               | 0/507 (0)        | 1/1027 (0.1)     | 0.33    |
|                  | c.2709-59T>C      | TC               | 0/507 (0)        | 2/1027 (0.2)     | 0.55    |
| Intron 23         | c.2931+9A>G       | AG               | 0/507 (0)        | 1/1027 (0.1)     | 0.33    |
|                  | c.2931+59T>A      | TA               | 179/507 (35.3)   | 412/1027 (40.1)  | 0.07    |
|                  |                   | AA               | 43/507 (8.5)     | 90/1027 (8.8)    | 0.92    |
| Intron 24         | c.3016-9C>A       | CA               | 3/507 (0.6)      | NS -             |
| Intron 27         | c.3531+8G>T       | GT               | 137/507 (27.0)   | 282/1027 (27.5)  | 0.9     |
|                  |                   | TT               | 17/507 (3.4)     | 33/1027 (3.2)    | 0.88    |
|                  | c.3532-15C>T      | CT               | 186/507 (36.7)   | 422/1027 (41.1)  | 0.11    |
|                  |                   | TT               | 41/507 (8.1)     | 84/1027 (8.2)    | 1.0     |
| 3' flanking region (3'FR) | c.*11C>T             | CT               | 13/507 (2.6)     | 25/1027 (2.4)    | 0.86    |

CP = chronic pancreatitis; NS = no sequence data available.
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Indeed, other factors as modifier genes and environmental factors may also contribute to this phenomenon. Our findings are compatible with a model in which CP can be caused by mutations in ATP8B1 on both alleles, which is in line with the frequent occurrence of pancreatitis in patients with ATP8B1 deficiency. Pancreatitis might even be the only symptom in patients with ATP8B1 deficiency.

In addition, our data do contribute to a better understanding of the role of rare heterozygous ATP8B1 variants in health and disease. For example, p.D70N was previously suggested to contribute to the etiology of intrahepatic cholestasis of pregnancy (ICP) as 3/182 ICP patients harbored this variant and none of 120 controls [16]. Similarly p.N45T and p.K203E were each found in one ICP patient and in none of 100 controls [17]. Our current data, giving a frequency of respectively 0.9% for p.D70N, 1.7% for p.N45T and 0.3% for p.K203E in a cohort of over 1,000 healthy controls, suggest that these earlier findings might very well have been caused by statistical variation in a relatively small control cohort.

In conclusion, our investigation did not reveal an association between heterozygous ATP8B1 variants and hereditary or idiopathic chronic pancreatitis. However, it suggests that pancreatitis might be the first or sole symptom of ATP8B1 deficiency. Furthermore, earlier suggestions of involvement of ATP8B1 variants in ICP might have been due to a chance effect.

Author Contributions
Conceived and designed the experiments: JS RH. Performed the experiments: WW DH HW. Analyzed the data: WW HW RH. Contributed reagents/materials/analysis tools: SG CF EM PB HW. Wrote the paper: WW RH.

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