Functionally deficient *TRPV6* variants contribute to hereditary and familial chronic pancreatitis

Shin Hamada[1,#], Emmanuelle Masson[2,3,#], Jian-Min Chen[2], Reiko Sakaguchi[4,5], Vinciane Rebours[6], Louis Buscail[7], Ryotaro Matsumoto[1], Yu Tanaka[1], Kazuhiro Kikuta[1], Fumiya Kataoka[1], Akira Sasaki[1], Marc Le Rhun[8], Hela Audin[9], Alain Lachaux[10], Bernard Caumont[11], Diane Lorenzo[12], Kareen Billiemaz[13], Raphael Besnard[14], Stéphane Koch[15], Thierry Lamireau[16], Xavier De Koninck[17]; GREPAN (Genetic REsearch on PANcreatitis) Study Group[§], Emmanuelle Génin[2], David N. Cooper[18], Yasuo Mori[4], Atsushi Masamune[1,†], Claude Férec[2,3,†]

[1]Division of Gastroenterology, Tohoku University Graduate School of Medicine, Sendai, Japan

[2]Univ Brest, Inserm, EFS, UMR 1078, GGB, F-29200 Brest, France

[3]Service de Génétique Médicale et de Biologie de la Reproduction, CHRU Brest, F-29200 Brest, France

[3]Division of Gastroenterology, Tohoku University Graduate School of Medicine, Sendai, Japan

[4]Laboratory of Molecular Biology, Department of Synthetic Chemistry and Biological Chemistry, Graduate School of Engineering, Kyoto University, Kyoto, Japan

[5]Institute for Integrated Cell-Material Sciences, Kyoto University, Kyoto, Japan

[6]Department of Gastroenterology and Pancreatology, Beaujon Hospital, Assistance Publique-Hôpitaux de Paris, Clichy, Université de Paris, Paris, France

[7]Department of Gastroenterology and Pancreatology, CHU Rangueil and University of Toulouse, Toulouse, France

[8]Service d'Hépato-Gastroentérologie et Assistance Nutritionnelle, Institut des Maladies de l'Appareil Digestif (IMAD), Centre Hospitalo-Universitaire (CHU), Nantes, France

[9]Médecine 'Chauvet' à Orientation Gastro-Entérologique, CH Gabriel Martin, Saint Paul, France

[10]Hospices Civils de Lyon, Department of Pediatric Hepato-Gastroenterology and Nutrition, Hôpital Femme Mere Enfant HFME, 59 Bd Pinel, 69677, Bron, France

[11]Service de Médecine à Orientation Hépato-Gastro-Entérologique, CH Sud Gironde, Langon, France

[12]Department of Digestive Endoscopy, Beaujon Hospital, APHP, Clichy, and Paris-Diderot University, Paris, France

[13]Service de Réanimation Pédiatrique, CHU-Hôpital Nord, Saint-Étienne, France

[14]Service d'Hépato-Gastro-Entérologie et Oncologie Digestive, CHR Orléans, Orléans, France

[15]Department of Gastroenterology, University Hospital of Besançon, Besançon, France

[16]Pediatric Hepatology and Gastroenterology Unit, Bordeaux University Hospital, Pellegrin-Enfants Hospital, Bordeaux, France

[17]Division of Gastroenterology, Clinique Saint-Pierre, Avenue Reine Fabiola, 9, 1340, Ottignies, Belgium

[18]Institute of Medical Genetics, School of Medicine, Cardiff University, Cardiff, United Kingdom

Disclosure statement

The authors are unaware of any conflict of interest.

Funding information

Institut National de la Santé et de la Recherche Médicale (INSERM), France; and AMED (21ek0109550h0001 [to Atsushi Masamune]), Japan.

[#]**Authors share co-first authorship.**

[§]See Supplementary Table S1 for all members of the GREPAN study group.

[†]**Authors share co-senior authorship.**

Correspondence

Jian-Min Chen, INSERM UMR1078 – EFS – UBO, 22 avenue Camille Desmoulins, 29238 BREST, France.

Email: jian-min.chen@univ-brest.fr

Emmanuelle Masson, Service de Génétique Médicale et de Biologie de la Reproduction, CHRU Brest, F-29200 Brest, France.

Email: emmanuelle.masson@univ-brest.fr

Abstract

The recent discovery of *TRPV6* as a pancreatitis susceptibility gene served to identify a novel mechanism of chronic pancreatitis (CP) due to Ca^{2+} dysregulation. Herein, we analyzed *TRPV6* in 81 probands with hereditary CP (HCP), 204 probands with familial CP (FCP) and 462 patients with idiopathic CP (ICP) by targeted next-generation sequencing. We identified 25 rare nonsynonymous *TRPV6* variants, 18 of which had not been previously reported. All 18 variants were characterized by a Ca^{2+} imaging assay, with 8 being identified as functionally deficient. Evaluation of functionally deficient variants in the three CP cohorts revealed two novel findings: (i) functionally deficient *TRPV6* variants appear to occur more frequently in HCP/FCP patients than in ICP patients (3.2% *vs*. 1.5%) and (ii) functionally deficient *TRPV6* variants found in HCP and FCP probands appear to be more frequently co-inherited with known risk variants in *SPINK1*, *CTRC* and/or *CFTR* than those found in ICP patients (66.7% vs 28.6%). Additionally, genetic analysis of available HCP and FCP family members revealed complex patterns of inheritance in some families. Our findings confirm that functionally deficient *TRPV6* variants are an important contributor to CP. Moreover, functionally deficient *TRPV6* variants account for a significant proportion of cases of HCP/FCP.

KEYWORDS

chronic pancreatitis, complex disease, genetic variant, genotype-phenotype relationship, targeted next-generation sequencing, *TRPV6* gene

1. INTRODUCTION

Chronic pancreatitis (CP) is a long-standing inflammation of the pancreas that leads to irreversible structural and functional abnormalities of that organ (Beyer, Habtezion, Werner, Lerch, & Mayerle, 2020). It is a complex disease that is generally caused by a combination of genetic and environmental factors (Chen et al., 2021; Kleeff et al., 2017; Mayerle et al., 2019; Ru et al., 2021). Since the first identification of a gain-of-function missense variant, p.Arg122His, in the *PRSS1* gene (encoding cationic trypsinogen; MIM# 276000) as a cause of autosomal dominant hereditary pancreatitis (Whitcomb et al., 1996), a diverse array of inherited variants in more than 10 gene loci have been reported to cause, predispose to or protect against pancreatitis (see (Masson et al., 2021) for references). Most of the known CP genes encode pancreatic zymogens that are specifically expressed in the pancreatic acinar cells; the corresponding pathogenic variants operate either through a trypsin- or misfolding-dependent pathway (Hegyi & Sahin-Tóth, 2017; Sahin-Tóth, 2017).

An important recent advance in pancreatitis genetics was the discovery of *TRPV6* (encoding transient receptor potential cation channel subfamily V member 6; MIM# 606680) as a new CP susceptibility gene (Masamune et al., 2020; Sahin-Tóth, 2020; Zou et al., 2020). Compared to other developments in the field of pancreatitis genetics over the last 10 years, which were either limited to specific populations or involved a small genetic effect (Burgos et al., 2021; Fjeld et al., 2015; Lasher et al., 2019; Moore et al., 2019; Rosendahl et al., 2018; Tang et al., 2018; Whitcomb et al., 2012; Witt et al., 2013; Wu et al., 2017; Zou et al., 2016), the *TRPV6* discovery was noteworthy in that aggregated functionally deficient *TRPV6* variants were found to exert a very strong genetic effect on CP across four populations; indeed, the corresponding odds ratios (OR) were 8.9 in the Chinese cohort (Zou et al., 2020), 48.3 in the Japanese cohort, and infinity in the French and German cohorts (Masamune et al., 2020). Unlike most other CP genes, *TRPV6* is expressed in several types of epithelial cell

including pancreatic ductal and acinar cells, and encodes a constitutively active Ca^{2+}-selective ion channel (Fecher-Trost, Wissenbach, & Weissgerber, 2017; Stoerger & Flockerzi, 2014). On the basis that (i) TRPV6 plays an important role in Ca^{2+} homeostasis (Saotome, Singh, Yelshanskaya, & Sobolevsky, 2016), (ii) calcium signaling is one of the main initiation events in experimental pancreatitis (Gerasimenko, Peng, Tsugorka, & Gerasimenko, 2018) and (iii) *Trpv6* knockin mice with a homozygous missense variant (i.e., p.Asp541Ala) that is known to block the ability of TRPV6 to conduct Ca^{2+}, developed more severe cerulein-induced pancreatitis (Masamune et al., 2020), a functional deficiency of *TRPV6* would appear to constitute a novel pathogenic mechanism of CP due to Ca^{2+} dysregulation (Masamune et al., 2020; Zou et al., 2020).

The *TRPV6* breakthrough was made essentially through the analysis of patients with idiopathic CP (ICP). Given the remarkably high ORs associated with the functionally deficient *TRPV6* variants (Masamune et al., 2020; Zou et al., 2020), we wondered whether some families presenting with a phenotype consistent with autosomal dominant hereditary pancreatitis might harbor genetic defects in the *TRPV6* gene. Herein, we describe our findings from the analysis of the *TRPV6* gene in three distinct CP cohorts, followed by functional analysis of all the newly identified rare *TRPV6* nonsynonymous variants.

2. MATERIALS AND METHODS

2.1. Ethical statement

This study was approved by the Ethics Committee of the Regional and University Hospital Center (Centre Hospitalier Régional et Universitaire (CHRU)), Brest, France. All participants (or parents/guardians when the participants were under the age of 18) gave their informed consent.

2.2. Disease subtype classifications

Three CP subtypes, namely hereditary CP (HCP), familial CP (FCP) and ICP, were defined in accordance with our previous publications (Chen & Férec, 2009; Le Maréchal et al., 2006; Masson, Chen, Scotet, Le Maréchal, & Férec, 2008). ICP is diagnosed when neither external causative risk factors (e.g., heavy drinking, infection, trauma and drug use) nor a positive family history of pancreatitis were reported; HCP is defined by having three or more affected family members spanning at least two generations whereas FCP is defined in terms of a positive family history but not satisfying the stricter diagnostic criteria for HCP. Additionally, ICP was further divided into two groups (≤20 years and >20 years) in terms of the age of onset of disease symptoms (in patients who did not experience prior symptoms, the age at diagnosis of CP) as previously described (Masson et al., 2008).

2.3. Patients

285 probands with a positive family history of pancreatitis (including 81 with HCP and 204 with FCP) and 462 ICP (including 108 of ≤20 years and 354 of >20 years) patients, all of whom were recruited by the Brest Genetics Laboratory, participated in this study. Here it should be noted that in (Masamune et al., 2020), all 470 of the participating French CP patients belonged to the ICP group of ≤20 years; the currently included 108 patients with ICP of ≤20 years were newly recruited. In addition, the HCP and FCP probands had been previously found to be negative for a causative variant in the *PRSS1* gene by established methods (Masson, Chen, Audrézet, Cooper, & Férec, 2013).

2.4. Targeted next generation sequencing (NGS)

The entire coding region and exon/intron boundaries of the *TRPV6* gene were analyzed by targeted NGS. Primer sequences used for this purpose are provided in Supplementary Table

S2. A targeted DNA sequencing library was prepared with the Assess Array™ IFC system by Fluidigm (Fluidigm, Les Ulis, France) according to the manufacturer's protocol. Library sequencing was performed using the Ion Torrent Sequencing System (Thermo Fisher Scientific, Waltham, US-MA). The SeqNext software (JSI Medical Systems, New York, NY) was used to detect variants in the NGS data. Rare nonsynonymous variants including (i) small deletions or insertions that affected canonical GT-AG splice sites and/or coding sequence and (ii) single nucleotide substitutions that altered either canonical GT-AG splice sites or resulted in missense or nonsense variants were included for analysis. Rare variants were defined as having an allele frequency of <0.005 in controls (Manolio et al., 2009). Global population data from the Genome Aggregation Database (gnomAD; http://gnomad.broadinstitute.org/) were used as controls (Lek et al., 2016).

The four most extensively studied CP genes, *PRSS1*, *SPINK1* (encoding pancreatic secretory trypsin inhibitor; MIM# 167790), *CTRC* (encoding chymotrypsin C; MIM# 601405) and *CFTR* (encoding cystic fibrosis transmembrane conductance regulator; MIM# 602421) (Masson et al., 2013; Rosendahl et al., 2013; Zou et al., 2018), were analyzed simultaneously with the *TPRV6* gene by targeted NGS in all patients. Primer sequences pertaining to these four genes are available upon email request. All patients found to carry a rare *TRPV6* nonsynonymous variant were evaluated with respect to known CP risk variants in *PRSS1*, *SPINK1*, *CTRC* and/or *CFTR* (with the exception of those risk variants that represent common polymorphisms in the general population e.g. the common *PRSS1-PRSS2* haplotype tagged by rs10273639C (Herzig et al., 2020; Whitcomb et al., 2012)).

All reported variants were confirmed by Sanger sequencing.

2.5. Screening for copy number variants (CNVs) in the *TRPV6* gene by quantitative fluorescent multiplex PCR (QFM-PCR)

Fifteen primer pairs targeting the 15 exons of the *TRPV6* gene (Supplementary Table S3) were designed for this purpose. One primer of each pair was 5'-labeled with the HEX Fluorochrome. The targeted *TRPV6* regions and two control regions (i.e., exon 19 of the *EPHB6* gene located on chromosome 7, and exon 9 of the *DSC2* gene located on chromosome 18) were simultaneously amplified in a single reaction. QFM-PCR was performed using the Qiagen Multiplex PCR kit (Qiagen, Courtaboeuf, France), with 50 ng genomic DNA in a 10-µl reaction mixture. The PCR program comprised an initial denaturation step at 95°C for 15 min, 27 cycles of denaturation at 95°C for 30 s, annealing at 58°C for 1 min and extension at 72°C for 90 s, and a final extension at 72°C for 30 min.

Amplified DNA fragments were separated on a 3130XL Genetic Analyzer (Applied Biosystems, Foster City, CA), and data were analyzed with the GeneMarker® software (Softgenetics LLC, State College, PA). Peak heights of the 15 *TRPV6* amplicons in a given sample were first normalized against those of the co-amplified control *DSC2* and *EPHB6* amplicons. The normalized fluorescent profiles of a test sample were then superimposed upon those of a control sample. A reduction in peak height to 50% of normal was held to be suggestive of a deletion.

2.6. Ca^{2+} imaging assay

The Ca^{2+} imaging assay was performed at the Division of Gastroenterology, Tohoku University Graduate School of Medicine, Sendai, Japan, as previously reported (Masamune et al., 2020). We co-transfected HEK293 cells (American Type Culture Collection) with recombinant *TRPV6* expression plasmids and pEGFP-N1 or pEGFP-C1 (Clontech Laboratories) as a transfection marker, using the SuperFect Transfection Reagent (Qiagen). After transfection, cells were plated on coverslips and loaded with 1 µM Fura-2-acetoxymethyl ester (AM) (Dojindo Molecular Technologies Inc, Kumamoto, Japan).

Fluorescence images of the cells were recorded and analyzed with a video image analysis system (AQUACOSMOS; Hamamatsu Photonics, Hamamatsu, Japan). Fura-2-AM fluorescence at an emission wavelength of 510 nm was obtained by exciting Fura-2-AM sequentially at 340 nm and 380 nm. The increase in the fluorescence ratio *F340/F380* (*F340/F380*) evoked by the application of 2 mM Ca^{2+} was then determined. The Δ*F340/F380* value in the cells expressing wild-type (WT) TRPV6 was taken as 100% TRPV6 activity. We defined functionally-defective variants as those for which an increase of intracellular calcium concentration was significantly diminished compared to the WT (see below).

2.7. Statistical analysis

For Ca^{2+} imaging assays, data are shown as mean + SEM of the assays from the sum of two or three independent transfections. Differences between more than two groups in Ca^{2+} imaging assays were analyzed using the Tukey-Kramer method. A *p* value of <0.05 was considered significant.

Differences in carrier frequencies of the functionally-defective *TRPV6* variants between patients and controls were performed using a 2x2 Contingency Table available at http://vassarstats.net/odds2x2.html. A difference was regarded as being statistically significant when the *P* value was ≤0.05.

Meta-analysis of the association between functionally-defective *TRPV6* variants with CP across Japanese, Chinese, German and French cohorts was performed using the Review Manager 5.3 software (https://review-manager.software.informer.com/5.3/), as previously described (Herzig et al., 2020).

2.8. Reference mRNA sequences and variant nomenclature

NM_018646.6, NM_002769.5, NM_001379610.1, NM_007272.3 and NM_000492.4 were used as the reference *TPRV6*, *PRSS1*, *SPINK1*, *CTRC* and *CFTR* mRNA sequences, respectively. All variants were named in accordance with Human Genome Variation Society recommendations (http://varnomen.hgvs.org/; (den Dunnen et al., 2016)). Names for newly described variants were verified as recommended (Higgins et al., 2021). *TRPV6* variants that were not previously described in the human SNP databases have been deposited in GenBank (https://www.ncbi.nlm.nih.gov/genbank/).

3. RESULTS

3.1. Rare *TRPV6* variants identified in three CP cohorts by targeted NGS

All functionally deficient *TRPV6* variants previously reported in (Masamune et al., 2020; Zou et al., 2020) were 'rare variants' (defined as having an allele frequency of <0.005 in their respective control populations). We therefore focused our analysis here on rare nonsynonymous variants by reference to the global population data in gnomAD. As such, we found a total of 25 variants: 5 in HCP, 6 in FCP (Table 1), 4 in ICP of ≤20 years and 10 in ICP of >20 years (Table 2). Each variant was found once in a single patient. With the exception of c.1936C>T (p.Arg646Trp), which was found in an ICP individual of >20 years in the homozygous state (Table 2), all variants found were heterozygous. All variants were either absent from gnomAD or in the case of their presence, their allele frequencies were extremely low (Tables 1 and 2). Of the 25 variants, only 7 (6 in Table 1 and 1 in Table 2) were previously described in (Masamune et al., 2020; Zou et al., 2020). In other words, the other 18 variants were newly described in this study.

The p.Arg646Trp homozygote and all heterozygous HCP and FCP probands were also analyzed by QFM-PCR for the possible presence of CNVs but none were found. This served

to validate the authenticity of the homozygous p.Arg646Trp variant as well as to exclude the involvement of large deletions in these subjects.

3.2. Eight of the 18 newly described rare nonsynonymous *TRPV6* variants were found to be functionally deficient by means of a Ca^{2+} imaging assay

All 18 of the newly described *TRPV6* variants were functionally analyzed by means of a Ca^{2+} imaging assay, with 8 being identified as functionally deficient (Figure 1; Tables 1 and 2). Herein two points should be noted. First, two variants previously reported by Zou and colleagues (Zou et al., 2020), namely c.1517T>C (p.Met506Thr) and c.1360C>T (p.Arg454Cys), were also assessed by Ca^{2+} imaging assay in the current study. Entirely consistent findings with respect to the functional annotations of the two variants — the former was functionally deficient (see Table 1) but the latter not (see Table 2) — were obtained from the two studies. Second, unlike the two previous studies (Masamune et al., 2020; Zou et al., 2020), we did not perform protein expression analysis by means of Western blotting. Nonetheless, based upon findings from the two previous studies, functional deficiency of *TRPV6* variants, as measured by the Ca^{2+} imaging assay, was considered to reflect reduced mutant protein expression.

Functional analytical data corresponding to the remaining 5 previously described variants were taken directly from (Masamune et al., 2020; Zou et al., 2020).

3.3. Functionally deficient *TRPV6* variants tend to be found more frequently in HCP/FCP than in ICP and more frequently in ICP of ≤20 years than in ICP of >20 years

The carrier frequencies of functionally deficient *TRPV6* variants in the HCP, FCP, ICP of ≤20 years and ICP of >20 years cohorts (Tables 1 and 2) were respectively 4.9% (4/81), 2.5% (5/204), 1.9% (2/108) and 0.8% (3/354), respectively. The carrier frequency (1.9%) of the

functionally deficient *TRPV6* variants in ICP of ≤20 years matched that of the 470 previously analyzed French ICP patients of ≤20 years [1.9% (9/470) (Masamune et al., 2020)]; thus, for ICP of ≤20 years, we employed the combined data from the current and previous studies (1.9% (11/578)) (Table 3). In addition, the carrier frequencies of the functionally deficient *TRPV6* variants in the combined HCP and FCP patients and in the combined ICP patients are also provided in Table 3.

The *P* values for the difference in carrier frequencies for the functionally-defective *TRPV6* variants between each of the French patient cohorts and the previously analyzed 570 French controls are provided in Table 3. It should be emphasized here that the corresponding ORs were invariably infinite due to the absence of functionally deficient *TRPV6* variants in the 570 French controls (Masamune et al., 2020).

Inspection of the data in Table 3 revealed two tendencies. First, functionally deficient *TRPV6* variants were found to occur more frequently in patients with a positive family history than those without (3.2% *vs*. 1.5%; $P = 0.0723$, Pearson's χ^2 test). Second, functionally deficient *TRPV6* variants were found to occur more frequently in patients with an early disease onset (≤20 years) than those with a late disease onset (1.9% *vs.* 0.8%; $P = 0.198$, Pearson's χ^2 test). The lack of a statistically significant difference in either context was considered to be attributable to small sample size.

3.4. Functionally deficient *TRPV6* variants found in HCP and FCP tend to be more frequently associated with known risk variants in *SPINK1*, *CTRC* and/or *CFTR* than those found in ICP

Patients with a rare *TRPV6* variant were evaluated for known CP risk variants in *PRSS1*, *SPINK1*, *CTRC* and *CFTR*, with the findings being presented in Tables 1 and 2. The frequency of functionally deficient *TRPV6* variants co-segregating with another CP risk

variant was calculated for each disease subtype as well as for a straight comparison between CP patients with a positive family history (HCP/FCP) and those without (ICP irrespective of age) and the results are summarized in Table 4. The data from the French ICP patients of ≤20 years in the current study were combined with those from (Masamune et al., 2020). In the previous study, of the nine ICP patients (≤20 years) carrying a functionally deficient *TRPV6* variant, two also carried the *SPINK1* p.Asn34ser variant whilst another carried the common *CFTR* p.Phe508del variant (Masamune et al., 2020). In the current study, the corresponding figure was 1/2 ICP patients ≤20 years carrying a functionally deficient *TRPV6* variant also carried a defective *CFTR* allele (see Table 2).

Notably, functionally deficient *TRPV6* variants found in CP patients with a positive family history (HCP and FCP) appeared to be more frequently associated with known risk variants in *SPINK1*, *CTRC* and/or CFTR than functionally deficient *TRPV6* variants found in ICP patients (66.7% vs 28.6%; Table 4). Again, the lack of a statistically significant difference in this particular context ($P = 0.102$, two-tailed Fisher's exact test) was considered to be attributable to small sample size.

3.5. Genetic analysis of available family members in HCP and FCP revealed complex patterns of inheritance

Only a few relatives of the HCP and FCP probands were available for variant analysis (Figure 2). Whereas *TRPV6* variants were invariably found to cosegregate with the CP phenotype in all informative subjects, this was not always the case for the other known CP-predisposing variants. Thus, in pedigree C, the father of the proband did not harbor the detected *CFTR* p.Phe508del variant and it was almost certainly inherited from the proband's mother (although in the absence of a maternal sample for analysis, we could not formally confirm it).

By contrast, in pedigree G, the proband inherited from his father both the *TRPV6* p.Arg345Cys and the *CFTR* p.Leu997Phe but not the father's *SPINK1* p.Arg67His variant.

3.6. Meta-analysis of the association of functionally deficient *TRPV6* variants with ICP across four populations

Finally, we performed a meta-analysis of the association of functionally deficient *TRPV6* variants with ICP across Japanese, Chinese, German and French populations. Data from the first three populations were obtained from (Masamune et al., 2020; Zou et al., 2020), with the term 'non-alcoholic CP' being regarded as equivalent to ICP in accordance with our previous practice (Chen et al., 2021). As for the corresponding French data, it was the combined ICP data as provided in Table 3 that were used for analysis.

Functionally deficient *TRPV6* variants were found to be significantly associated with an increased risk for ICP in the context of each population, as the corresponding 95% CIs did not overlap with 1 (Table 5). Since the test for heterogeneity did not reveal significant heterogeneity across the four populations ($\chi^2 = 1.56$, $P = 0.67$; $I^2 = 0\%$; Supp. Figure S1), the Mantel-Haenszel fixed-effect model was used to compute the pooled OR of the association across the four populations. The pooled OR was 21.65 [95% CI 6.56–71.44; $P < 0.00001$) (Table 5; Supp. Figure S1).

4. DISCUSSION

In the present study, we have for the first time analyzed the *TRPV6* gene in three distinct CP cohorts, HCP, FCP and ICP, by means of targeted NGS. We found a total of 25 rare nonsynonymous variants, of which 18 had not been previously reported in (Masamune et al., 2020; Zou et al., 2020). We performed functional analysis on all 18 variants, resulting in the

identification of 8 new functionally deficient *TRPV6* variants. Comparison of findings in the different CP cohorts revealed several novel observations;

First, functionally deficient *TRPV6* variants were previously reported to be more frequently found in patients with early-onset disease (i.e., age at symptom onset, ≤20 years) than in patients who developed symptoms after the age of 20 (Masamune et al., 2020). We confirmed this finding by analyzing further ICP patients of ≤20 years and >20 years. Importantly, we made a new observation; functionally deficient *TRPV6* variants were found to occur more frequently in HCP/FCP patients than in ICP patients. The biological reason underlying these trends would appear to lie in the strong genetic effect of functionally deficient *TRPV6* variants (Masamune et al., 2020; Zou et al., 2020), which not only predispose subjects to early-onset disease but also increase the probability of the disease manifesting in families. In this regard, it is pertinent to make a comparison with the rs10273639C-tagged common *PRSS1-PRSS2* haplotype, which has a very small genetic effect on CP (pooled OR of 1.28 from a meta-analysis (Herzig et al., 2020)) and was more frequently found in CP patients of >20 years than in CP patients of ≤20 years (Derikx et al., 2015).

Second, some 20% of the previously reported functionally deficient *TRPV6* variants were associated with a known risk variant in *SPINK1*, *CTRC* or *CFTR* (Masamune et al., 2020). We confirmed this in the context of our combined ICP patients (Table 4). Interestingly, we found that up to 66.7% of the functionally deficient *TRPV6* variants detected in the HCP/FCP probands carried a known risk variant in *SPINK1*, *CTRC* and/or *CFTR*. This new observation is readily interpretable as follows: two variants, irrespective of whether their effects were additive or synergistic, when inherited together, would normally confer a higher penetrance as compared to either variant inherited alone.

Third, we were able to analyze a few family members, which revealed complex patterns of inheritance in some families. In pedigree C, one may speculate that the co-inherited *TRPV6* p.Arg345His and *CFTR* Phe508del variants could have advanced the age of disease onset in the proband as compared to the father who harbored a single *TRPV6* p.Arg345His variant (Figure 2). However, in pedigree G, the father had a much later age of disease onset than the proband even although the former also carried *SPINK1* p.Arg67His. It should be noted that *SPINK1* p.Arg67His has previously been described in multiple pancreatitis patients (Boulling, Keiles, Masson, Chen, & Férec, 2012; Giefer et al., 2017; Jalaly et al., 2017; Werlin et al., 2015) and has been shown by *in vitro* transfection experiments to abolish SPINK1 protein expression (Boulling et al., 2012).

Finally, we have performed a meta-analysis on the association of functionally deficient *TRPV6* variants with ICP using data from the two previous studies (Masamune et al., 2020; Zou et al., 2020) and the current one, obtaining a pooled OR of 21.65 ($P < 0.00001$).

Our study has its limitations. For example, the sample size was small in the context of HCP and FCP cohorts. This, together with the rare occurrence of functionally deficient *TRPV6* variants, invalidated formal statistical analysis. Moreover, we did not explore the possibility that some missense variants might affect splicing or mRNA stability. However, we do not consider that these limitations would have changed the main conclusions of the study.

In summary, our findings confirm that functionally deficient *TRPV6* variants are an important contributor to CP. Moreover, we have for the first time demonstrated that functionally deficient *TRPV6* variants account for a significant proportion of cases of HCP and FCP.

ACKNOWLEDGMENT

The authors are grateful to Masaharu Nakamichi for excellent technical assistance.

DATA AVAILABILITY STATEMENT

The data that support the findings reported in this study are either included in the text of the article or have been uploaded as Supplementary information. Methodology pertaining to the NGS of the *PRSS1*, *SPINK1*, *CTRC* and *CFTR* genes is available from the corresponding author upon reasonable request.

WEB RESOURCES

2x2 Contingency Table: http://vassarstats.net/odds2x2.html

GenBank: https://www.ncbi.nlm.nih.gov/genbank/

gnomAD: http://gnomad.broadinstitute.org/

HGVS Variant Nomenclature: http://varnomen.hgvs.org/

Review Manager 5.3 software: https://review-manager.software.informer.com/5.3/

FIGURE LEGENDS

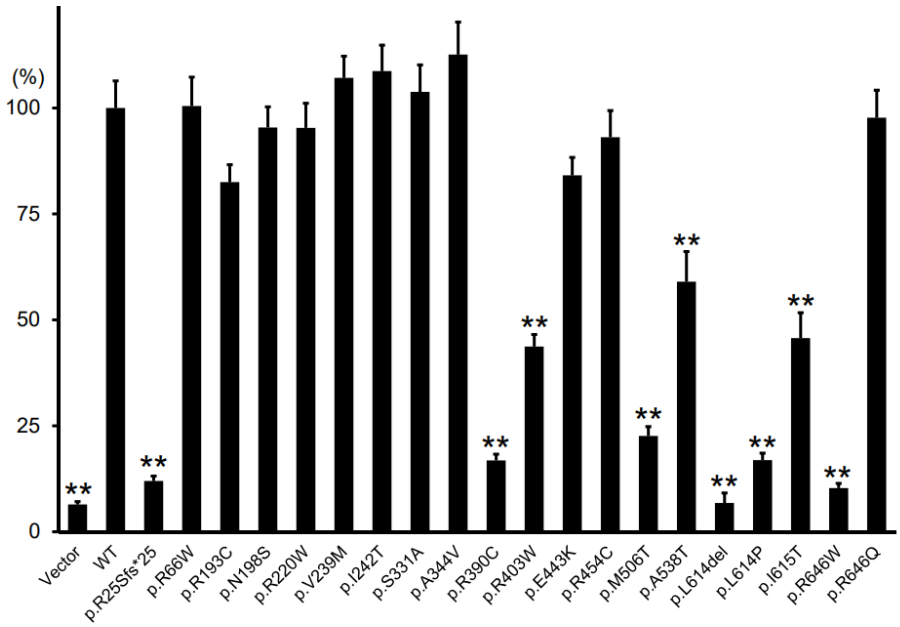

Figure 1. Functional analysis of the 18 newly described *TRPV6* variants by means of a Ca^{2+} imaging assay. Two variants previously reported by (Zou et al., 2020), p.Arg454Cys and p.Met506Thr, were additionally included for analysis. Intracellular Ca^{2+} concentration in wild-type (WT) *TRPV6*-transfected HEK293 cells was set as 100% TRPV6 activity. **, $P < 0.01$.

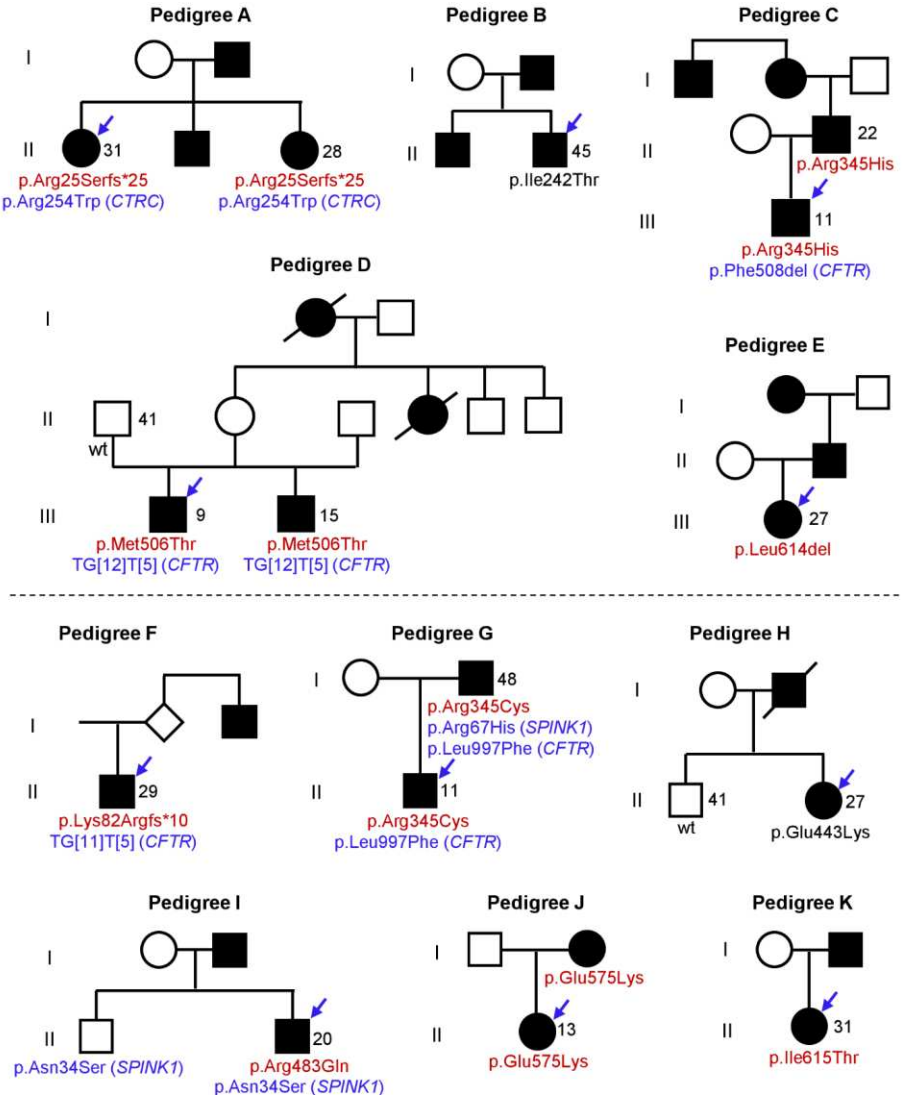

Figure 2. Rare nonsynonymous *TRPV6* variants found in five families with hereditary chronic pancreatitis (A to E) and six families with familial chronic pancreatitis (F to K). Probands are indicated by arrows. Numbers to the right of patient symbols refer to the age at onset of pancreatitis symptoms. Variants identified in *TRPV6,* as well as in the pancreatitis-relevant genes *SPINK1*, *CTRC* and/or *CFTR,* are indicated below the individuals subjected to genetic analysis. Functionally deficient *TRPV6* variants are given in red type whereas functionally sufficient *TRPV6* variants are given in normal type. Pancreatitis-relevant variants in *SPINK1*, *CTRC* and/or *CFTR* are given in blue type. All variants were found in the heterozygous state.

REFERENCES

Beyer, G., Habtezion, A., Werner, J., Lerch, M. M., & Mayerle, J. (2020). Chronic pancreatitis. *Lancet, 396*(10249), 499-512. doi:10.1016/S0140-6736(20)31318-0

Boulling, A., Keiles, S., Masson, E., Chen, J. M., & Férec, C. (2012). Functional analysis of eight missense mutations in the *SPINK1* gene. *Pancreas, 41*(2), 329-330. doi:10.1097/MPA.0b013e3182277b83

Burgos, M., Philippe, R., Antigny, F., Buscaglia, P., Masson, E., Mukherjee, S., . . . Mignen, O. (2021). The p.E152K-STIM1 mutation deregulates Ca^{2+} signaling contributing to chronic pancreatitis. *J Cell Sci, 134*(3), jcs244012. doi:10.1242/jcs.244012

Chen, J. M., & Férec, C. (2009). Chronic pancreatitis: genetics and pathogenesis. *Annu Rev Genomics Hum Genet, 10*, 63-87. doi:10.1146/annurev-genom-082908-150009

Chen, J. M., Herzig, A. F., Genin, E., Masson, E., Cooper, D. N., & Férec, C. (2021). Scale and scope of gene-alcohol interactions in chronic pancreatitis: a systematic review. *Genes (Basel), 12*(4), 471. doi:10.3390/genes12040471

den Dunnen, J. T., Dalgleish, R., Maglott, D. R., Hart, R. K., Greenblatt, M. S., McGowan-Jordan, J., . . . Taschner, P. E. (2016). HGVS recommendations for the description of sequence variants: 2016 update. *Hum Mutat, 37*(6), 564-569. doi:10.1002/humu.22981

Derikx, M. H., Kovacs, P., Scholz, M., Masson, E., Chen, J. M., Ruffert, C., . . . Collaborators. (2015). Polymorphisms at *PRSS1-PRSS2* and *CLDN2-MORC4* loci associate with alcoholic and non-alcoholic chronic pancreatitis in a European replication study. *Gut, 64*(9), 1426-1433. doi:10.1136/gutjnl-2014-307453

Fecher-Trost, C., Wissenbach, U., & Weissgerber, P. (2017). TRPV6: From identification to function. *Cell Calcium, 67*, 116-122. doi:10.1016/j.ceca.2017.04.006

Fjeld, K., Weiss, F. U., Lasher, D., Rosendahl, J., Chen, J. M., Johansson, B. B., . . . Molven, A. (2015). A recombined allele of the lipase gene *CEL* and its pseudogene *CELP* confers susceptibility to chronic pancreatitis. *Nat Genet, 47*(5), 518-522. doi:10.1038/ng.3249

Gerasimenko, J. V., Peng, S., Tsugorka, T., & Gerasimenko, O. V. (2018). Ca^{2+} signalling underlying pancreatitis. *Cell Calcium, 70*, 95-101. doi:10.1016/j.ceca.2017.05.010

Giefer, M. J., Lowe, M. E., Werlin, S. L., Zimmerman, B., Wilschanski, M., Troendle, D., . . . Uc, A. (2017). Early-onset acute recurrent and chronic pancreatitis is associated with *PRSS1* or *CTRC* gene mutations. *J Pediatr, 186*, 95-100. doi:10.1016/j.jpeds.2017.03.063

Hegyi, E., & Sahin-Tóth, M. (2017). Genetic risk in chronic pancreatitis: the trypsin-dependent pathway. *Dig Dis Sci, 62*(7), 1692-1701. doi:10.1007/s10620-017-4601-3

Herzig, A. F., Genin, E., Cooper, D. N., Masson, E., Férec, C., & Chen, J. M. (2020). Role of the common *PRSS1-PRSS2* haplotype in alcoholic and non-alcoholic chronic pancreatitis: Meta- and re-analyses. *Genes (Basel), 11*(11), 1349. doi:10.3390/genes11111349

Higgins, J., Dalgleish, R., den Dunnen, J. T., Barsh, G., Freeman, P. J., Cooper, D. N., . . . Cutting, G. R. (2021). Verifying nomenclature of DNA variants in submitted manuscripts: Guidance for journals. *Hum Mutat, 42*(1), 3-7. doi:10.1002/humu.24144

Jalaly, N. Y., Moran, R. A., Fargahi, F., Khashab, M. A., Kamal, A., Lennon, A. M., . . . Singh, V. K. (2017). An evaluation of factors associated with pathogenic *PRSS1*, *SPINK1*, *CTFR*, and/or *CTRC* genetic variants in patients with idiopathic pancreatitis. *Am J Gastroenterol, 112*(8), 1320-1329. doi:10.1038/ajg.2017.106

Kleeff, J., Whitcomb, D. C., Shimosegawa, T., Esposito, I., Lerch, M. M., Gress, T., . . . Neoptolemos, J. P. (2017). Chronic pancreatitis. *Nat Rev Dis Primers, 3*, 17060. doi:10.1038/nrdp.2017.60

Lasher, D., Szabo, A., Masamune, A., Chen, J. M., Xiao, X., Whitcomb, D. C., . . . Sahin-Toth, M. (2019). Protease-sensitive pancreatic lipase variants are associated with early onset chronic pancreatitis. *Am J Gastroenterol, 114*(6), 974-983. doi:10.14309/ajg.0000000000000051

Le Maréchal, C., Masson, E., Chen, J. M., Morel, F., Ruszniewski, P., Levy, P., & Férec, C. (2006). Hereditary pancreatitis caused by triplication of the trypsinogen locus. *Nat Genet, 38*(12), 1372-1374. doi:10.1038/ng1904

Lek, M., Karczewski, K. J., Minikel, E. V., Samocha, K. E., Banks, E., Fennell, T., . . . Exome Aggregation, C. (2016). Analysis of protein-coding genetic variation in 60,706 humans. *Nature, 536*(7616), 285-291. doi:10.1038/nature19057

Manolio, T. A., Collins, F. S., Cox, N. J., Goldstein, D. B., Hindorff, L. A., Hunter, D. J., . . . Visscher, P. M. (2009). Finding the missing heritability of complex diseases. *Nature, 461*(7265), 747-753. doi:10.1038/nature08494

Masamune, A., Kotani, H., Sorgel, F. L., Chen, J. M., Hamada, S., Sakaguchi, R., . . . Shimosegawa, T. (2020). Variants that affect function of calcium channel TRPV6 are associated with early-onset chronic pancreatitis. *Gastroenterology, 158*(6), 1626-1641.e1628. doi:10.1053/j.gastro.2020.01.005

Masson, E., Chen, J. M., Audrézet, M. P., Cooper, D. N., & Férec, C. (2013). A conservative assessment of the major genetic causes of idiopathic chronic pancreatitis: data from a comprehensive analysis of *PRSS1*, *SPINK1*, *CTRC* and *CFTR* genes in 253 young French patients. *PLoS One, 8*(8), e73522. doi:10.1371/journal.pone.0073522

Masson, E., Chen, J. M., Scotet, V., Le Maréchal, C., & Férec, C. (2008). Association of rare chymotrypsinogen C (*CTRC*) gene variations in patients with idiopathic chronic pancreatitis. *Hum Genet, 123*(1), 83-91. doi:10.1007/s00439-007-0459-3

Masson, E., Rebours, V., Buscail, L., Frete, F., Pagenault, M., Lachaux, A., . . . Chen, J. M. (2021). The reversion variant (p.Arg90Leu) at the evolutionarily adaptive p.Arg90 site in *CELA3B* predisposes to chronic pancreatitis. *Hum Mutat, 42*(4), 385-391. doi:10.1002/humu.24178

Mayerle, J., Sendler, M., Hegyi, E., Beyer, G., Lerch, M. M., & Sahin-Toth, M. (2019). Genetics, cell biology, and pathophysiology of pancreatitis. *Gastroenterology, 156*(7), 1951-1968.e1951. doi:10.1053/j.gastro.2018.11.081

Moore, P. C., Cortez, J. T., Chamberlain, C. E., Alba, D., Berger, A. C., Quandt, Z., . . . Oakes, S. A. (2019). Elastase 3B mutation links to familial pancreatitis with diabetes and pancreatic adenocarcinoma. *J Clin Invest, 129*(11), 4676-4681. doi:10.1172/JCI129961

Rosendahl, J., Kirsten, H., Hegyi, E., Kovacs, P., Weiss, F. U., Laumen, H., . . . all members of the PanEuropean Working group on, A. C. P. (2018). Genome-wide association study identifies inversion in the *CTRB1-CTRB2* locus to modify risk for alcoholic and non-alcoholic chronic pancreatitis. *Gut, 67*(10), 1855-1863. doi:10.1136/gutjnl-2017-314454

Rosendahl, J., Landt, O., Bernadova, J., Kovacs, P., Teich, N., Bodeker, H., . . . Witt, H. (2013). *CFTR, SPINK1, CTRC* and *PRSS1* variants in chronic pancreatitis: is the role of mutated *CFTR* overestimated? *Gut, 62*(4), 582-592. doi:10.1136/gutjnl-2011-300645

Ru, N., Xu, X. N., Cao, Y., Zhu, J. H., Hu, L. H., Wu, S. Y., . . . Liao, Z. (2021). The impacts of genetic and environmental factors on the progression of chronic pancreatitis. *Clin Gastroenterol Hepatol*. doi:10.1016/j.cgh.2021.08.033

Sahin-Tóth, M. (2017). Genetic risk in chronic pancreatitis: the misfolding-dependent pathway. *Curr Opin Gastroenterol, 33*(5), 390-395. doi:10.1097/MOG.0000000000000380

Sahin-Tóth, M. (2020). Channelopathy of the pancreas causes chronic pancreatitis. *Gastroenterology, 158*(6), 1538-1540. doi:10.1053/j.gastro.2020.03.027

Saotome, K., Singh, A. K., Yelshanskaya, M. V., & Sobolevsky, A. I. (2016). Crystal structure of the epithelial calcium channel TRPV6. *Nature, 534*(7608), 506-511. doi:10.1038/nature17975

Stoerger, C., & Flockerzi, V. (2014). The transient receptor potential cation channel subfamily V member 6 (TRPV6): genetics, biochemical properties, and functions of exceptional calcium channel proteins. *Biochem Cell Biol, 92*(6), 441-448. doi:10.1139/bcb-2014-0063

Tang, X. Y., Zou, W. B., Masson, E., Hu, L. H., Férec, C., Chen, J. M., . . . Liao, Z. (2018). The *CTRB1-CTRB2* risk allele for chronic pancreatitis discovered in European populations does not contribute to disease risk variation in the Chinese population due to near allele fixation. *Gut, 67*(7), 1368-1369. doi:10.1136/gutjnl-2017-315180

Werlin, S., Konikoff, F. M., Halpern, Z., Barkay, O., Yerushalmi, B., Broide, E., . . . Wilschanski, M. (2015). Genetic and electrophysiological characteristics of recurrent acute pancreatitis. *J Pediatr Gastroenterol Nutr, 60*(5), 675-679. doi:10.1097/MPG.0000000000000623

Whitcomb, D. C., Gorry, M. C., Preston, R. A., Furey, W., Sossenheimer, M. J., Ulrich, C. D., . . . Ehrlich, G. D. (1996). Hereditary pancreatitis is caused by a mutation in the cationic trypsinogen gene. *Nat Genet, 14*(2), 141-145. doi:10.1038/ng1096-141

Whitcomb, D. C., LaRusch, J., Krasinskas, A. M., Klei, L., Smith, J. P., Brand, R. E., . . . Devlin, B. (2012). Common genetic variants in the *CLDN2* and *PRSS1-PRSS2* loci alter risk for alcohol-related and sporadic pancreatitis. *Nat Genet, 44*(12), 1349-1354. doi:10.1038/ng.2466

Witt, H., Beer, S., Rosendahl, J., Chen, J. M., Chandak, G. R., Masamune, A., . . . Sahin-Toth, M. (2013). Variants in *CPA1* are strongly associated with early onset chronic pancreatitis. *Nat Genet, 45*(10), 1216-1220. doi:10.1038/ng.2730

Wu, H., Zhou, D. Z., Berki, D., Geisz, A., Zou, W. B., Sun, X. T., . . . Liao, Z. (2017). No significant enrichment of rare functionally defective *CPA1* variants in a large Chinese idiopathic chronic pancreatitis cohort. *Hum Mutat, 38*(8), 959-963. doi:10.1002/humu.23254

Zou, W. B., Boulling, A., Masamune, A., Issarapu, P., Masson, E., Wu, H., . . . Liao, Z. (2016). No association between CEL-HYB hybrid allele and chronic pancreatitis in Asian populations. *Gastroenterology, 150*(7), 1558-1560.e1555. doi:10.1053/j.gastro.2016.02.071

Zou, W. B., Tang, X. Y., Zhou, D. Z., Qian, Y. Y., Hu, L. H., Yu, F. F., . . . Liao, Z. (2018). *SPINK1*, *PRSS1*, *CTRC*, and *CFTR* genotypes influence disease onset and clinical outcomes in chronic pancreatitis. *Clin Transl Gastroenterol, 9*(11), 204. doi:10.1038/s41424-018-0069-5

Zou, W. B., Wang, Y. C., Ren, X. L., Wang, L., Deng, S. J., Mao, X. T., . . . Liao, Z. (2020). *TRPV6* variants confer susceptibility to chronic pancreatitis in the Chinese population. *Hum Mutat, 41*(8), 1351-1357. doi:10.1002/humu.24032