

Targeted Next-Generation Sequencing Effectively Analyzed the Cystic Fibrosis Transmembrane Conductance Regulator Gene in Pancreatitis

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Abstract

Background The cystic fibrosis transmembrane conductance regulator (*CFTR*) gene, responsible for the development of cystic fibrosis, is known as a pancreatitis susceptibility gene. Direct DNA sequencing of PCR-amplified *CFTR* gene segments is a first-line method to detect unknown mutations, but it is a tedious and labor-intensive endeavor given the large size of the gene (27 exons, 1,480 amino acids). Next-generation sequencing (NGS) is becoming standardized, reducing the cost of DNA sequencing, and enabling the generation of millions of reads per run. We here report a comprehensive analysis of *CFTR* variants in Japanese patients with chronic pancreatitis using NGS coupling with target capture.

Methods Exon sequences of the *CFTR* gene from 193 patients with chronic pancreatitis (121 idiopathic, 46 alcoholic, 17 hereditary, and nine familial) were captured by HaloPlex target enrichment technology, followed by NGS.

Results The sequencing data covered 91.6 % of the coding regions of the *CFTR* gene by ≥ 20 reads with a mean read depth of 449. We could identify 12 non-

synonymous variants including three novel ones [c.A1231G (p.K411E), c.1753G>T (p.E585X) and c.2869delC (p.L957fs)] and seven synonymous variants including three novel ones in the exonic regions. The frequencies of the c.4056G>C (p.Q1352H) and the c.3468G>T (p.L1156F) variants were higher in patients with chronic pancreatitis than those in controls.

Conclusions Target sequence capture combined with NGS is an effective method for the analysis of pancreatitis susceptibility genes.

Keywords Chloride channel · HaloPlex · In silico analysis · MiSeq · Target enrichment

Abbreviations

bp	Base pair
CF	Cystic fibrosis
CFTR	Cystic fibrosis transmembrane conductance regulator
CP	Chronic pancreatitis
ERCP	Endoscopic retrograde cholangiopancreatography
NGS	Next-generation sequencing
PCR	Polymerase chain reaction
RD	Related disorder
SIFT	Sorting Intolerant From Tolerant

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Introduction

Chronic pancreatitis (CP) is a progressive inflammatory disease that eventually leads to impairment of the exocrine and endocrine functions of the organ [1, 2]. Since the identification of mutations in the cationic trypsinogen

(protease, serine, 1; *PRSS1*) gene as a cause of hereditary pancreatitis in 1996 [3], several pancreatitis susceptibility genes have been identified [3–6]. Gain-of-function mutations in the *PRSS1* gene as well as loss-of-function variants in the serine protease inhibitor Kazal type 1 (*SPINK1*) gene and the trypsin-degrading enzyme chymotrypsin C (*CTRC*) increase the risk of CP [3–5]. In 2013, carboxypeptidase A1 (*CPA1*) gene was identified as a novel pancreatitis susceptibility gene [6]. These studies have been replicated in the Japanese population [7–10].

The cystic fibrosis transmembrane conductance regulator (*CFTR*) gene is another pancreatitis susceptibility gene [11, 12]. Acute recurrent pancreatitis and CP have been accepted as *CFTR*-related disorders (*CFTR*-RDs), a clinical entity associated with *CFTR* dysfunction that does not fulfill the diagnostic criteria for cystic fibrosis (CF; MIM# 219700) [13]. The *CFTR* gene, responsible for the development of CF, encodes for a cyclic adenosine monophosphate-dependent chloride channel that is located in the apical membrane of secretory and absorptive epithelial cells of the pancreas, intestine, liver, airways, vas deferens, and sweat glands [14]. In general, the clinical manifestations of CF arise from ductal and glandular obstruction because of an inability to hydrate macromolecules within the ductal lumens [15]. Until now, more than 1,900 variants have been identified in the Cystic Fibrosis Mutation Database (<http://www.genet.sickkids.on.ca/cftr>). The human *CFTR* gene spans 250 kb and contains 27 exons that encode for a protein with a total length of 1,480 amino acids [14]. Direct DNA sequencing of polymerase chain reaction (PCR)-amplified *CFTR* gene segments is a first-line method to detect unknown *CFTR* mutations [16], but this is a tedious and labor-intensive endeavor given the large size of the gene.

A new approach that uses massive parallel sequencing called next-generation sequencing (NGS) is becoming standardized, and the cost is rapidly dropping [17]. Using the ultimate platforms, such systems are able to perform billions of sequencing reactions with a read length of 150–250 nucleotides. For most research groups, whole-genome sequencing of many samples remains a costly endeavor, and targeted capture of selected regions of interest followed by sequencing provides a more efficient and cost-effective option. This strategy has allowed identification of causal variants in several Mendelian disorders, variants associated with complex diseases, and recurrently mutated cancer genes [18–20]. The HaloPlex target enrichment technology is a selective circularization-based method that is a further development of the principle of selector probes [21]. In the HaloPlex technology, genomic DNA is fragmented by restriction enzyme digestion and circularized by hybridization to probes whose ends are complementary to the target fragments. Compared to hybrid capture methods, the HaloPlex system requires

smaller amounts of starting DNA, has higher specificity (fraction of sequence reads in the region of interest), and provides more uniform genome coverage [22]. Using the bench-top Illumina MiSeq platform, comprehensive analysis of many samples can be easily done. We here report the comprehensive analysis of *CFTR* variants in Japanese patients with CP.

Materials and Methods

Subjects

One hundred and ninety-three patients with CP (121 idiopathic, 46 alcoholic, 17 hereditary, and nine familial) were enrolled in this study. Because we initially aimed to identify novel pancreatitis-associated genes using the HaloPlex system, majority of the patients recruited were nonalcoholic. To extend our findings, we additionally recruited patients with alcoholic CP who had developed CP at relatively younger ages (mean: 44.1 years old). The diagnosis of CP was based on at least two separate episodes of abdominal pain and radiological findings of pancreatic calcifications by computed tomography, endoscopic ultrasonography, and/or morphological findings such as pancreatic ductal irregularities and dilatations revealed by endoscopic retrograde cholangiopancreatography (ERCP) or by magnetic resonance imaging [23]. Hereditary pancreatitis was diagnosed when one first-degree relative or two or more second-degree relatives had recurrent acute pancreatitis or CP without any apparent predisposing factor [24]. Patients with CP in whom the criteria for hereditary pancreatitis were not met but who had at least two affected family members were classified as having familial pancreatitis. Idiopathic CP was diagnosed in the absence of a positive family history or possible predisposing factors such as alcohol abuse, trauma, medication, and anatomical abnormalities. Patients who consumed alcohol over 80 g/day (for men) or 60 g/day (for women) for more than 2 years were classified as alcoholic CP. All subjects were Japanese. This study was performed with the informed consent of the patients in accordance with the principles of the declaration of Helsinki. This study was approved by the Ethics Committee of Tohoku University School of Medicine (article#: 2013-1-498).

Peripheral Blood Collection and DNA Preparation

After written informed consent was obtained, 5–10 mL of peripheral blood was collected in disposable vacuum tubes for genetic testing. Genomic DNA was extracted from peripheral blood leukocytes using the Wizard genomic DNA purification kit (Promega, Madison, WI, USA).

Targeted Next-Generation Sequencing

We used the online design tool SureDesign to generate a customized HaloPlex target enrichment system (Agilent Technologies, Santa Clara, CA, USA) targeting the regions including *CFTR* exons and 50 base pairs (bp) of flanking introns. The expected coverage of the *CFTR* coding region based on the amplicon design was 99.6 %. The HaloPlex target enrichment system relies on a tailored cocktail of restriction enzymes and customized probes to capture genomic regions of interest, which are subsequently amplified by PCR. Sequencing libraries were prepared according to the manufacturer's instructions. Briefly, genomic DNA was digested with restriction enzymes, followed by hybridization to the biotinylated HaloPlex probe library in the presence of the indexing primer cassette. Hybridization results in the circularization of genomic DNA fragments and incorporation of indices and Illumina sequencing motifs. Hybridized probes were captured with streptavidin-coated magnetic beads. Subsequently, libraries were amplified by PCR to produce a sequencing-ready, target-enriched sample.

All libraries of target-enriched DNA were analyzed on a 2200 TapeStation (Agilent Technologies) to verify successful enrichment. All samples were sequenced on the Illumina MiSeq platform (Illumina Japan, Tokyo, Japan) with paired-end 151-bp reads according to the manufacturer's instruction.

Bioinformatic Analysis of Sequencing Data

The reads were trimmed with the utility program Trim Galore (http://www.bioinformatics.babraham.ac.uk/projects/trim_galore/) to remove possible adapter sequences, based on the Illumina TruSeq adapter index sequences. If either read from

a pair was shorter than 20 bp after trimming, that pair was removed from the analysis. The remaining quality reads were mapped to the GRCh37 primary assembly of the human genome (<http://ensembl.org/>) using the Burrows–Wheeler Alignment tool 0.6.1 (<http://bio-bwa.sourceforge.net/>). Further sequence data processing, assessment of coverage rates, variant calling, and filtration were performed with the Genome Analysis Toolkit, version 1.6 software (Broad Institute, Cambridge, MA, USA; <http://www.broadinstitute.org/gatk/>). SNPs and insertions/deletions (indels) were annotated using the ANNOVAR (<http://www.openbioinformatics.org/annovar/>; BIOBASE, Wolfenbüttel, Germany).

Sanger Sequencing

Sanger sequencing was performed to analyze the DNA sequences which included any nucleotide variant identified by NGS. Exons and adjacent intronic regions of the *CFTR* gene containing the nucleotide variants were amplified by PCR using the primer sets (Table 1). The cycle conditions were as follows: preheating at 95 °C for 5 min, followed by 40 cycles of 95 °C for 30 s, 60 °C for 30 s, and 72 °C for 30 s; and then a final extension at 72 °C for 5 min. PCR products were cleaned up using the Illustra ExoProStar S (GE Healthcare Life Sciences; Little Chalfont, UK). The PCR products were sequenced using an ABI Prism BigDye Terminator Cycle Sequencing Kit, version 3.1 on ABI3730xl DNA Analyzer (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. The results were compared with the reference sequence derived from the GenBank (<http://www.ncbi.nlm.nih.gov/genbank>, reference sequence NM_000492) to identify nucleotide substitutions. The A of the ATG start codon was used as nucleotide +1. The mutations are described according to the nomenclature recommended by

Table 1 Primers used for direct sequencing

Exon	Forward	Reverse	Size of PCR product (bp)
2	CCAGAAAAGTTGAATAGTATCA	AAGCAATCCTCTCATCTTGG	369
4	AATTCTCAGGGTATTTTATGAG	CCAGCTCACTACCTAATTTATG	363
10	AGCATCTATTGAAAATATCTGACAAAC	AAAGAGACATGGACACCAAATTAAG	315
11	GGAGGCAAGTGAATCCTGAG	AACCGATTGAATATGGAGCC	343
12	CAGATTGAGCATACTAAAAGTG	CATTACAGCAAATGCTTGCTAG	224
13	TAGATGACCAGGAAATAGAGA	ATGAGGCGGTGAGAAAAGGT	351
15	GGTGGCATGAACTGTACTG	TGTATACATCCCCAACTATCT	251
17	TCAGTAAGTACTTTGGCTGC	CCTATTGATGGTGGATCAGC	390
21	TGTGCCCTAGGAGAAGTGTG	TGACAGATACACAGTGACCCTC	335
23	TATGTACAGAAGTGATCCC	TGAGTACAAGTATCAAATAGC	252
25	GCTTGAGTGTTTTAACTCTGTGG	AGACCCCCACACGCAGAC	335
27	CTCTGGTCTGACCTGCCTTC	AGCTCCAATTCCATGAGGTG	334

bp base pairs

the Human Genome Variation Society (<http://www.hgvs.org/mutnomen>).

In addition, all exons and adjacent intronic regions of the *PRSSI*, *SPINK1*, *CTRC*, and *CPAI* genes were amplified by PCR and directly sequenced as previously reported [5, 6, 8, 25].

In Silico Prediction

SIFT (Sorting Intolerant From Tolerant; <http://sift.jcvi.org/>) and PolyPhen-2 (<http://genetics.bwh.harvard.edu/pph2/>) were used to predict whether an amino acid substitution would affect the structure and function of a protein. SIFT uses sequence homology, whereas PolyPhen-2 offers predictions based on conservation, protein folding, and crystal structure [26, 27]. The SIFT scores range from zero to one, with zero predicted to be the most deleterious mutation and one the least deleterious. The PolyPhen-2 scoring predicts three outcomes for mutations: “benign” (most likely lacking any phenotypic effect), “possibly damaging” (may affect protein structure or function), and “probably damaging” (high degree of confidence that protein structure function will be affected).

Statistical Analysis

The variant frequencies in the Japanese population were obtained from the Human Genetic Variation Database (www.genome.med.kyoto-u.ac.jp/SnpDB/). The significance of the differences in variant frequencies between patients and controls was tested by two-tailed Fisher’s exact test. A *P* value <0.05 was considered significant. All statistical analyses were performed using the SPSS version 17.0 statistical analysis software (SPSS Inc., Chicago, IL, USA).

Results

Approximately 10 kb of the coding regions and the adjacent noncoding regions of the *CFTR* gene were analyzed in this study. On average, 98.8, 97.0, and 95.1 % of the coding regions of the *CFTR* gene were covered by at least one, five, and 10 sequence reads, respectively. The sequencing data covered 91.6 % of the coding regions of the *CFTR* gene by ≥ 20 reads with a mean read depth of 449 and a median depth of 412 (Fig. 1). These results indicate a high-resolution capability for the identification of variants, such as mutations.

In our cohort of 193 CP patients, we identified 12 non-synonymous and seven synonymous variants in the exons of the *CFTR* gene by targeted NGS (Tables 2, 3, 4, 5). The presence of these variants was confirmed by Sanger sequencing. Based on the presence in dbSNP137, Exome Variant Server (NHLBI GO Exome Sequencing Project, Seattle, WA, USA; URL: <http://evs.gs.washington.edu/EVS/>), and the Human Genetic Variation Database, three non-synonymous variants [c.1231A>G (p.K411E), c.1753G>T (p.E585X) and c.2869delC (p.L957fs)] and three synonymous variants (c.372C>T, c.3975A>G and c.4254G>A) were novel. The frameshift variant c.2869delC (p.L957fs) leads to a stop codon afterward at amino acid 967, to premature termination of translation and a heavily truncated protein missing more than one-third of its amino acids. This variant was found in a 22-year-old female with idiopathic CP. She was admitted due to a pancreatitis attack and diagnosed as having CP. She had suffered from back and abdominal pain since 20 years old. The value of the *n*-benzoyl-*l*-tyrosyl-*p*-aminobenzoic acid test was 52 % (normal: >70 %), suggesting pancreatic exocrine dysfunction. The patient also had the c.4056G>C (p.Q1352H) variant in a

Fig. 1 Graph of the mean depth, median depth, and sequencing coverage for all the 27 exons in the *CFTR* gene. By MiSeq NGS, a high-quality sequence was obtained for 27 exons and the flanking sequences from the *CFTR* gene, including a mean depth of $\times 516$ and a median depth of $\times 442$. On average, 90.3 % of the coding region was successfully covered by ≥ 20 reads

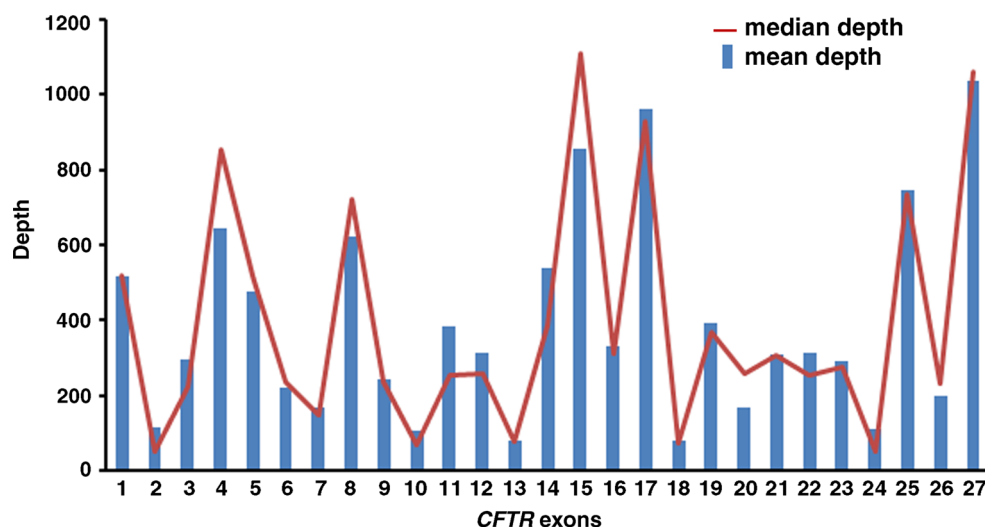


Table 2 Non-synonymous *CFTR* variants detected in this study

Exon	Non-synonymous variant	Amino acid change	dbSNP135	Genotype	SIFT (score)	PolyPhen-2 (score)	Alcoholic CP (%)	Idiopathic CP (%)	Hereditary/familial CP (%)
2	c.91C>T	p.R31C	rs1800073	CT	D (0.012)	PD (0.989)	0/46 (0)	3/121 (2.5)	0/26 (0)
2	c.92G>A	p.R31H	rs149353983	GA	T (0.183)	B (0.003)	0/46 (0)	1/121 (0.8)	0/26 (0)
4	c.374T>C	p.I125T	rs141723617	TC	D (0.005)	B (0.17)	0/46 (0)	2/121 (1.6)	1/26 (3.8)
10	c.1231A>G	p.K411E	-	AG	D (0.015)	B (0.233)	0/46 (0)	1/121 (0.8)	0/26 (0)
11	c.1408G>A	p.V470M	rs213950	GA	T (1)	B (0)	21/46 (45.7)	65/121 (53.7)	11/26 (42.3)
12	c.1666A>G	p.I556V	rs75789129	AA	T (0.536)	B (0.334)	5/46 (10.9)	19/121 (15.7)	1/26 (3.8)
13	c.1753G>T	p.E585X	-	GG	-	-	0/46 (0)	0/121 (0)	0/26 (0)
17	c.2869delC	p.L957fs	-	GT	-	-	1/46 (2.2)	0/121 (0)	0/26 (0)
21	c.3468G>T	p.L1156F	rs139729994	GT	T (0.163)	PD (0.994)	0/46 (0)	1/121 (0.8)	0/26 (0)
25	c.4045G>A	p.G1349S	rs201686600	TT	-	-	2/46 (4.3)	10/121 (8.3)	2/26 (7.7)
25	c.4056G>C	p.Q1352H	rs113857788	GA	D (0)	PD (1)	1/46 (2.2)	0/121 (0)	0/26 (0)
27	c.4357C>T	p.R1453W	rs4148725	GC	D (0)	PD (1)	5/46 (10.9)	11/121 (9.1)	4/26 (15.4)
				CC	D (0)	PD (0.999)	0/46 (0)	0/121 (0)	0/26 (0)
				CT	D (0)	PD (0.999)	3/46 (6.5)	6/121 (5.0)	1/26 (3.8)

B benign, *CP* chronic pancreatitis, *D* damaging, *PD* probably damaging, *T* tolerated, *SIFT* Sorting Intolerant From Tolerant

Table 3 Comparison of the non-synonymous variant frequencies between the patients with CP and controls

Amino acid change	Genotype	All CP (%)	HGVD (%)	<i>P</i> value (vs. HGVD)				
				All CP	Alcoholic CP	Nonalcoholic CP	Idiopathic CP	Hereditary/familial CP
p.R31C	CT	3/193 (1.6)	12/1102 (1.1)	0.48	>0.99	0.41	0.18	>0.99
p.R31H	GA	1/193 (0.5)	0	–	–	–	–	–
p.I125T	TC	3/193 (1.6)	5/1102 (0.5)	0.11	>0.99	0.057	0.15	0.13
p.K411E	AG	1/193 (0.5)	0	–	–	–	–	–
p.V470M	GA	97/193 (50.3)	573/1199 (47.8)	0.66	0.57	0.68	0.38	0.12
	AA	25/193 (13.0)	185/1199 (15.4)					
p.I556V	AG	10/193 (5.2)	78/1150 (6.8)	0.70	0.79	0.81	>0.99	0.45
	GG	0/193 (0)	3/1150 (0.3)					
p.E585X	GT	1/193 (0.5)	0	–	–	–	–	–
p.L957fs		1/193 (0.5)	0	–	–	–	–	–
p.L1156F	GT	14/193 (7.3)	45/1136 (4.0)	0.04	0.06	0.07	0.11	0.30
	TT	1/193 (0.5)	1/1136 (0.1)					
p.G1349S	GA	1/193 (0.5)	4/1094 (0.4)	0.56	0.19	>0.99	>0.99	>0.99
p.Q1352H	GC	20/193 (10.4)	57/1153 (4.9)	0.009	0.12	0.037	0.17	0.062
	CC	0/193 (0)	1/1153 (0.1)					
p.R1453W	CT	10/193 (5.2)	42/1144 (3.7)	0.32	0.25	0.49	0.45	>0.99

CP chronic pancreatitis, HGVD Human Genetic Variation Database

P values were determined versus HGVD by the Fisher's exact test

Table 4 Synonymous variants in the exons of the *CFTR* gene detected in this study

Exon	Synonymous variant	Amino acid change	dbSNP135	Genotype	Alcoholic CP (%)	Idiopathic CP (%)	Hereditary/familial CP (%)
4	c.372C>T	p.G124=	–	CT	0/46 (0)	1/121 (0.8)	0/26 (0)
13	c.1731C>T	p.Y577=	rs55928397	CT	0/46 (0)	1/121 (0.8)	0/26 (0)
15	c.2562T>G	p.T854=	rs1042077	TG	20/46 (43.5)	69/121 (57.0)	12/26 (46.2)
				GG	6/46 (13.0)	18/121 (14.9)	0/26 (0)
23	c.3723C>A	p.G1241=	rs185065886	CA	1/46 (2.2)	0/121 (0)	0/26 (0)
25	c.3975A>G	p.R1325=	–	AG	0/46 (0)	1/121 (0.8)	0/26 (0)
27	c.4254G>A	p.E1418=	–	GA	0/46 (0)	1/121 (0.8)	0/26 (0)
27	c.4389G>A	p.Q1463=	rs1800136	GA	1/46 (2.2)	3/121 (2.5)	0/26 (0)

CP chronic pancreatitis

heterozygous form (Table 6). The nonsense variant c.1753G>T (p.E585X) was found in a patient with alcoholic CP. He was diagnosed as having alcoholic CP at 28 years old. The c.1231A>G (p.411E) variant was found in a 19-year-old male with idiopathic CP. He had suffered from pancreatitis attacks since 12 years old. ERCP showed multiple stones in the main pancreatic duct. He underwent extracorporeal shock wave lithotripsy for the treatment of pancreatic stones. The patient also had the c.3468G>T (p.L1156F) variant in a heterozygous form. None of these three patients had known pancreatitis susceptibility mutations in the *PRSSI*, *SPINK1*, *CTRC*, or *CPAI* genes

(Table 6). All of the patients carrying the novel synonymous variants were idiopathic CP (Table 4).

The frequency of the c.4056G>C (p.Q1352H) variant was higher in all patients with CP than that in controls ($P = 0.009$; Table 3). Stratification based on the etiologies showed that the association was significant in patients with nonalcoholic CP (combination of cases with idiopathic, hereditary, and familial CP) ($P = 0.037$). The frequency of the c.3468G>T (p.L1156F) variant was also higher in patients with CP than that in controls ($P = 0.04$). There were no significant difference for any other non-synonymous or synonymous variants detected in the exons

Table 5 Comparison of the synonymous variant frequencies between the patients with CP and controls

Synonymous variant	Genotype	All CP (%)	HGVD (%)	P value (vs. HGVD)				
				All CP	Alcoholic CP	Nonalcoholic CP	Idiopathic CP	Hereditary/familial CP
c.C372T	CT	1/193 (0.5)	0	–	–	–	–	–
c.1731C>T	CT	1/193 (0.5)	0	–	–	–	–	–
c.2562T>G	TG	101/193 (52.3)	528/1154 (45.8)	0.22	0.81	0.11	0.045	0.033
	GG	24/193 (12.4)	181/1154 (15.7)					
c.3723C>A	CA	1/193 (0.5)	3/671 (4.5)	>0.99	0.23	>0.99	>0.99	>0.99
c.3975A>G	AG	1/193 (0.5)	0	–	–	–	–	–
c.4254G>A	GA	1/193 (0.5)	0	–	–	–	–	–
c.4389G>A	GA	4/193 (2.1)	40/1112 (3.6)	0.48	>0.99	0.53	0.81	>0.99
	AA	0/193 (0)	1/1112 (0.1)					

CP chronic pancreatitis, HGVD Human Genetic Variation Database
 P values were determined against HGVD by the Fisher’s exact test

Table 6 Total *CFTR* sequencing results of patients carrying rare non-synonymous *CFTR* variants

Case#	Etiology	Age at onset	Rare variant	Additional non-synonymous variants	c.1210-34TG(9_13) c.1210-12T(5_9)	Mutation in other pancreatitis susceptibility genes ^a
A1	Idiopathic	34	p.R31C/-	p.R1453W/-	TG11/TG11, 7T/7T	–
A2	Idiopathic	8	p.R31C/-	–	TG11/TG12, 7T/7T	–
A3	Idiopathic	16	p.R31C/-	–	TG11/TG12, 7T/7T	–
A4	Idiopathic	10	p.R31H/-	–	TG11/TG12, 7T/7T	–
A5	Idiopathic	16	p.I125T/-	p.L1156F/-	TG11/TG12, 7T/7T	<i>CTRC</i> p.R29Q/-
A6	Idiopathic	2	p.I125T/-	–	TG11/TG12, 7T/7T	–
A7	Hereditary	28	p.I125T/-	p.R1453W/-	TG11/TG12, 7T/7T	–
A8	Idiopathic	19	p.K411E/-	p.L1156F/-	TG11/TG12, 7T/7T	–
A9	Alcoholic	28	p.E585X/-	p.I556V/-	TG11/TG11, 7T/7T	–
A10	Idiopathic	21	p.L957fs/-	p.Q1352H/-	TG11/TG12, 7T/7T	–
A11	Alcoholic	40	p.G1349S/-	–	TG11/TG11, 7T/7T	–

^a Pancreatitis-associated mutations in the *PRSSI*, *SPINK1*, *CTRC*, and *CPAI* genes

between all patients with CP and controls (Tables 3, 5). The frequency of the c.2562T>G variant was different between the controls and the patients with idiopathic or hereditary/familial CP.

The 5T and, more rarely, 3T splicing variants of the intron 9 acceptor splice site [c.1210-12T(5_9)] are considered to be variants associated with CFTR-RD [16]. The 5T or 3T allele is a polymorphic variant with variable penetrance, causing less efficient exon 10 splicing and a lower *CFTR* transcript level [28]. The splicing efficiency of exon 10 is further affected by the length of the adjacent TG repeat [c.1210-34TG(9_13)]. The distribution of the c.1210-34TG(9_13) and c.1210-12T(5_9) variants is shown in Table 7. In our cohort, nine patients with CP had the 5T allele, all in a heterozygous form. Four patients (two alcoholic, one idiopathic, one hereditary) had the 5T-TG13. No patient had the haplotype TG10-7T-M470, which was reported to increase the risk of idiopathic CP [28].

It has been increasingly recognized that compound and trans-heterozygosity in the pancreatitis susceptibility genes are an overt risk factor for idiopathic CP [29–32]. Among the 193 patients with CP enrolled in this study, 29 patients had pancreatitis-associated mutations in the *PRSSI*, *SPINK1*, *CTRC*, and *CPAI* genes. Among these, nine patients had the non-synonymous *CFTR* variants, which are probably damaging based on the SIFT and/or the PolyPhen-2 prediction (Table 8).

Discussion

In this study, we performed comprehensive analysis of the variants in the *CFTR* gene by targeted NGS. To our knowledge, this is the first study to analyze pancreatitis susceptibility genes by targeted NGS. Comprehensive analysis by targeted NGS enabled us to identify novel and

Table 7 Distribution of the c.1210-34TG(9_13) and c.1210-12T(5_9) variants in patients with CP

	c.1210-34TG(9_13), c.1210-12T(5_9)	All CP (%)	Alcoholic CP (%)	Idiopathic CP (%)	Hereditary/ familial CP (%)
TG10/TG11, 7T/9T		1/193 (0.5)	0/46 (0)	1/121 (0.8)	0/26 (0)
TG11/TG11, 7T/7T		46/193 (23.8)	15/46 (32.6)	23/121 (19.0)	8/26 (30.8)
TG11/TG11, 7T/9T		4/193 (2.1)	1/46 (2.2)	3/121 (2.5)	0/26 (0)
TG11/TG12, 5T/7T		5/193 (2.6)	0/46 (0)	4/121 (3.3)	1/26 (3.8)
TG11/TG12, 6T/7T		1/193 (0.5)	0/46 (0)	1/121 (0.8)	0/26 (0)
TG11/TG12, 7T/7T		124/193 (64.2)	27/46 (58.7)	81/121 (66.9)	16/26 (61.5)
TG11/TG13, 6T/7T		1/193 (0.5)	1/46 (2.2)	0/121 (0)	0/26 (0)
TG11/TG13, 7T/7T		1/193 (0.5)	0/46 (0)	1/121 (0.8)	0/26 (0)
TG12/TG12, 7T/7T		6/193 (3.1)	0/46 (0)	6/121 (5.0)	0/26 (0)
TG12/TG13, 5T/7T		4/193 (2.1)	2/46 (4.3)	1/121 (0.8)	1/26 (3.8)

CP chronic pancreatitis

rare variants in the *CFTR* gene. The c.1753G>T (p.E585X) variant is a nonsense variant, and the c.2869delC (p.L957fs) variant leads to a stop codon afterward at amino acid 967. These variants result in a heavily truncated protein missing nearly two-thirds (p.E585X) or more than one-third (p.L957fs) of its amino acids. Because we did not perform functional assays, we do not have direct evidence that these two variants cause loss of the *CFTR* expression and/or function. However, a general acknowledgment has been agreed that mutations of this type, called class I mutations, are associated with complete loss or near complete loss of the *CFTR* function (<3 % of wild-type *CFTR* function) [33, 34]. The pathogenic potential of another novel variant, c.1231A>G (p.K411E), is currently unknown, but the in silico analyses suggest that this variant is deleterious. Importantly, the clinical phenotype of this patient might be complicated by the presence of another variant, p.L1156F. Noone et al. [30] reported that pancreatitis risk was increased approximately 40-fold by having two *CFTR* mutations. This is also the case with the c.374T>C (p.I125T) variant. Two of the three patients carrying this variant had other non-synonymous variants (p.I556V and p.R1453W). This p.I125T variant was originally reported in Chinese patients with idiopathic bronchiectasis and considered to be associated with *CFTR*-RD [35].

There are considerable regional and ethnic variations in the spectrum of the *CFTR* mutations [15]. Approximately 70 % of individuals with CF in the Caucasian population are homozygous for the F508del mutation, and almost 90 % of the patients have at least one F508del allele [36]. This mutation is extremely rare in the Japanese population, accounting for the rare presentation of classical CF in this region (approximately 1/350,000 live births) [37]. It is not surprising that the CF-causing mutations are frequently found in Caucasians, but very rarely in East Asia. Audrézet et al. [29] reported from France that at least 20 % of the patients with idiopathic CP carried one of the most

common *CFTR* mutations. Fujiki et al. [38] reported from Japan that none of the 20 common CF-causing mutations was found in 65 Japanese patients with CP (51 alcoholic and 14 idiopathic). Wang et al. [39] reported comprehensive screening of pancreatitis susceptibility genes including *CFTR* in 75 pediatric patients with idiopathic CP from China. They identified a novel 8-bp deletion in exon 4, but not the common CF-causing mutations. In this study, we found no common severe CF-causing mutations, in agreement with these previous studies from East Asia.

We found a significant association between the p.Q1352H variant and CP. This finding confirms the previous reports from Japan and Korea showing that this variant was over-presented in patients with CP compared to controls [38, 40]. Fujiki et al. [38] from Japan reported that the frequency of this variant was higher in patients with CP (8/65, 12.3 %) than in controls (6/162, 3.7 %). Lee et al. [40] reported from Korea that 14.3 % (4/28) of the patients with CP had this variant, whereas only 0.9 % (1/117) of the controls did. Glutamine at 1,352 is located in the second nucleotide-binding fold of *CFTR*, and its change to histidine (p.Q1352H) causes reductions in both the protein expression and channel activity of *CFTR* [40]. Similarly, we found that the p.L1156F variant was overexpressed in patients with CP. A functional study reported reduced $\text{Cl}^-/\text{HCO}_3^-$ permeability in the presence of the p.L1156F variant [41].

Gene–gene interactions of known pancreatitis susceptibility genes, especially between the *CFTR* and *SPINK1* genes, have been increasingly recognized. Indeed, seven out of 25 patients carrying the *SPINK1* variant(s) had the *CFTR* p.Q1352H and/or p.L1156F variants. One patient was trans-heterozygous for the *CTRC* p.R29Q and *CFTR* p.I125T/p.L1156F variants. Noone et al. [30] reported that pancreatitis risk was increased approximately 40-fold by having two *CFTR* mutations, 20-fold by having the *SPINK1* p.N34S variant, and 900-fold by having both. Trans-heterozygosity of the *SPINK1* p.N34S with the

Table 8 Total *CFTR* sequencing results of patients with *SPINK1*, *PRSS1*, *CTRC*, or *CPAI* mutations

Case#	Etiology	<i>CFTR</i> variants ^a	c.1210-34TG(9_13) c.1210-12T(5_9)	<i>SPINK1</i>	<i>PRSS1</i>	<i>CTRC</i>	<i>CPAI</i>
B1	Familial	p.Q1352H/-	TG11/TG12, 7T/7T	p.N34S/p.N34S			
B2	Idiopathic	–	TG12/TG12, 7T/7T	p.N34S/p.N34S			
B3	Idiopathic	–	TG11/TG12, 7T/7T	p.N34S/p.N34S			
B4	Idiopathic	p.L1156F/-, p.Q1352H/-	TG11/TG11, 7T/7T	p.N34S/-			
B5	Idiopathic	p.Q1352H/-	TG11/TG12, 7T/7T	p.N34S/-			
B6	Idiopathic	p.Q1352H/-	TG11/TG12, 7T/7T	p.N34S/-			
B7	Idiopathic	–	TG11/TG12, 7T/7T	p.N34S/-			
B8	Idiopathic	–	TG11/TG12, 7T/7T	p.N34S/-			
B9	Idiopathic	–	TG11/TG12, 7T/7T	p.N34S/-			
B10	Idiopathic	–	TG11/TG12, 7T/7T	p.N34S/-			
B11	Idiopathic	–	TG11/TG12, 7T/7T	p.N34S/-			
B12	Idiopathic	–	TG11/TG12, 7T/7T	p.N34S/-			
B13	Idiopathic	–	TG11/TG12, 7T/7T	p.N34S/-			
B14	Alcoholic	–	TG12/TG13, 5T/7T	p.N34S/-			
B15	Idiopathic	–	TG11/TG12, 7T/7T	p.N34S/IVS3+2T>C			
B16	Idiopathic	p.R1453W/-	TG11/TG11, 7T/7T	p.N34S/IVS3+2T>C			
B17	Idiopathic	–	TG11/TG12, 7T/7T	IVS3+2T>C/IVS3+2T>C			
B18	Idiopathic	–	TG11/TG12, 7T/7T	IVS3+2T>C/IVS3+2T>C			
B19	Hereditary	p.I125T/-, p.L1156F/-	TG11/TG12, 5T/7T	IVS3+2T>C/-			
B20	Familial	p.L1156F/-	TG11/TG12, 7T/7T	IVS3+2T>C/-			
B21	Idiopathic	–	TG11/TG12, 7T/7T	IVS3+2T>C/-			
B22	Alcoholic	p.Q1352H/-	TG11/TG12, 7T/7T	IVS3+2T>C/-			
B23	Alcoholic	–	TG11/TG12, 7T/7T	IVS3+2T>C/-			
B24	Idiopathic	–	TG11/TG12, 7T/7T	p.P45S/-			
B25	Idiopathic	–	TG12/TG12, 7T/7T	IVS3+2T>C/-	p.R122H/-		
B26	Hereditary	–	TG11/TG12, 7T/7T		p.R122H/-		
B27	Idiopathic	p.I556V/-	TG11/TG12, 7T/7T		p.N29I/-		
B28	Idiopathic	p.I125T/-, p.L1156F/-	TG11/TG12, 7T/7T			p.R29Q/-	
B29	Idiopathic	–	TG11/TG12, 7T/7T				p.T368_Y369ins20/-

Nine patients had the non-synonymous *CFTR* variants, which are probably damaging based on the SIFT or the PolyPhen-2 prediction

The p.I556V variant appeared to be benign based on the SIFT or the PolyPhen-2 prediction

Case B28 is the same as A5 in Table 6

^a We excluded the p.V470M variant from the list because of its similar frequencies in patients and controls

CFTR p.R75Q was reported to increase CP risk [31]. 6.5 % of the patients with idiopathic or hereditary CP carried variants in at least two pancreatitis susceptibility genes [32]. Whether the coinheritance of variants/mutations in pancreatitis susceptibility gene is a *bona fide* example of digenic inheritance or interaction between a disease-causing gene and a genetic modifier is unclear in most cases [42].

We used targeted sequence capture and high-throughput NGS to detect variants in the *CFTR* gene. Due to the large

size (27 exons, 1,480 amino acids), traditional technologies, such as PCR and capillary sequencing, are time- and cost-consuming. A major advantage of the HaloPlex-targeted enrichment system is the convenient workflow, integrating both capture and library preparation. The protocol allows one person to prepare a set of finished libraries within two working days and requires no larger specialized instruments. Sequence capture eliminates the necessity of setting up hundreds of PCR, instead allowing for parallel

enrichment of target regions in a single experiment. A weakness of this method is that the detection of larger copy number variations would require different methods. We have designed the HaloPlex platform for more than 70 genes, including the known pancreatitis susceptibility genes such as *CFTR*, *PRSS1*, *SPINK1*, *CTRC*, and *CPA1*. This system has allowed us to perform rapid screening of the known susceptibility genes simultaneously and gives an overview of potentially pathogenic variants in patients with pancreatitis. In addition, our HaloPlex platform includes candidates of novel pancreatitis susceptibility genes such as pancreatic digestive enzymes, those highly expressed in the pancreas and those related to autophagy and endoplasmic reticulum stress. This system might contribute to the identification of novel pancreatitis susceptibility genes in the future.

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Conflict of interest None.

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