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ORIGINAL ARTICLE

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

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

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(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	CATTTACAGCAAATGCTTGCTAG	224
13	TAGATGACCAGGAAATAGAGA	ATGAGGCGGTGAGAAAAGGT	351
15	GGTGGCATGAAACTGTACTG	TGTATACATCCCCAAACTATCT	251
17	TCAGTAAGTACTTTGGCTGC	CCTATTGATGGTGGATCAGC	390
21	TGTGCCCTAGGAGAAGTGTG	TGACAGATACACAGTGACCCTC	335
23	TATGTCACAGAAGTGATCCC	TGAGTACAAGTATCAAATAGC	252
25	GCTTGAGTGTTTTTAACTCTGTGG	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 *PRSS1*, *SPINK1*, *CTRC*, and *CPA1* 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

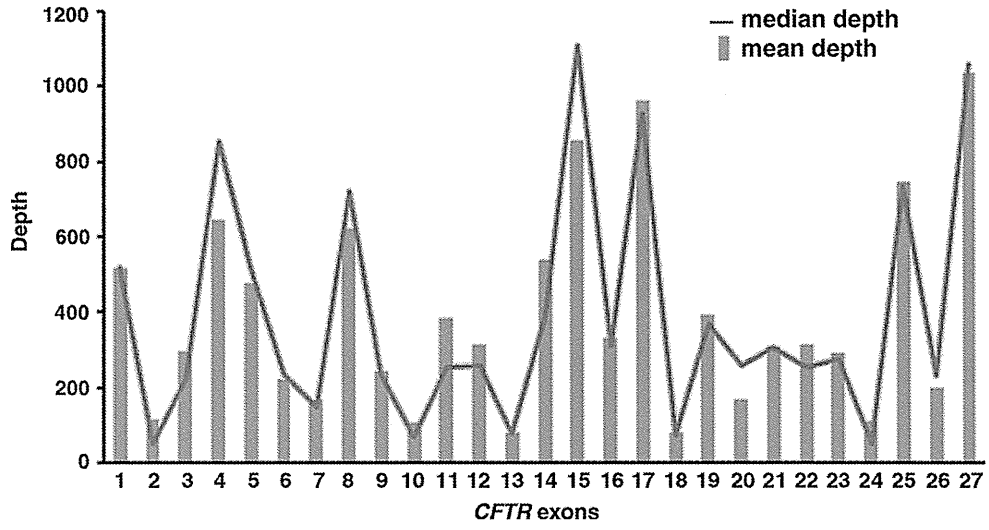

Fig. 1 Graph of the mean depth, median depth, and sequencing coverage for all the 27 exons in the *CFTR* gene. By MiSeq NGS, a high-quality sequence was obtained for 27 exons and the flanking sequences from the *CFTR* gene, including a mean depth of ×516 and a median depth of ×442. On average, 90.3 % 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)
				AA			5/46 (10.9)	19/121 (15.7)	1/26 (3.8)
12	c.1666A>G	p.I556V	rs75789129	AG	T (0.536)	B (0.334)	2/46 (4.3)	8/121 (6.6)	0/26 (0)
				GG			0/46 (0)	0/121 (0)	0/26 (0)
13	c.1753G>T	p.E585X	–	GT	–	–	1/46 (2.2)	0/121 (0)	0/26 (0)
17	c.2869delC	p.L957fs	–		–	–	0/46 (0)	1/121 (0.8)	0/26 (0)
21	c.3468G>T	p.L1156F	rs139729994	GT	T (0.163)	PD (0.994)	2/46 (4.3)	10/121 (8.3)	2/26 (7.7)
				TT			1/46 (2.2)	0/121 (0)	0/26 (0)
25	c.4045G>A	p.G1349S	rs201686600	GA	D (0)	PD (1)	1/46 (2.2)	0/121 (0)	0/26 (0)
25	c.4056G>C	p.Q1352H	rs113857788	GC	D (0)	PD (1)	5/46 (10.9)	11/121 (9.1)	4/26 (15.4)
				CC			0/46 (0)	0/121 (0)	0/26 (0)
27	c.4357C>T	p.R1453W	rs4148725	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 (%)	*P* 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, *HGVB* 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 *PRSS1*, *SPINK1*, *CTRC*, or *CPA1* 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	*CTRC* 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 *PRSS1*, *SPINK1*, *CTRC*, and *CPA1* 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 *PRSS1*, *SPINK1*, *CTRC*, and *CPA1* 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)

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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 Cl^-/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 *CPA1* mutations

Case#	Etiology	*CFTR* variants[a]	c.1210-34TG(9_13) c.1210-12T(5_9)	*SPINK1*	*PRSS1*	*CTRC*	*CPA1*
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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CLINICAL REPORT

AMERICAN JOURNAL OF medical genetics PART A

A Novel Heterozygous *MAP2K1* Mutation in a Patient with Noonan Syndrome with Multiple Lentigines

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Noonan syndrome with multiple lentigines (NSML), formerly referred to as LEOPARD syndrome, is a rare autosomal-dominant condition, characterized by multiple lentigines, electrocardiographic conduction abnormalities, ocular hypertelorism, pulmonary stenosis, abnormal genitalia, growth retardation, and sensorineural deafness. To date, *PTPN11*, *RAF1*, and *BRAF* have been reported to be causal for NSML. We report on a 13-year-old Japanese boy, who was diagnosed with NSML. He was found to have a novel heterozygous missense variant (c.305A > G; p.E102G) in *MAP2K1*, a gene mostly causal for cardio-facio-cutaneous syndrome (CFCS). He manifested fetal macrosomia, and showed hypotonia and poor sucking in the neonatal period. He had mild developmental delay, and multiple lentigines appearing at approximately age 3 years, as well as flexion deformity of knees bilaterally, subtle facial characteristics including ocular hypertelorism, sensorineural hearing loss, and precocious puberty. He lacked congenital heart defects or hypertrophic cardiomyopathy, frequently observed in patients with NSML, mostly caused by *PTPN11* mutations. He also lacked congenital heart defects, characteristic facial features, or intellectual disability, frequently observed in those with CFCS caused by *MAP2K1* or *MAP2K2* mutations. This may be the first patient clinically diagnosed with NSML, caused by a mutation in *MAP2K1*.

Key words: Noonan syndrome with multiple lentigines (NSML); *MAP2K1*; cardio-facio-cutaneous syndrome (CFCS)

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INTRODUCTION

Noonan syndrome with multiple lentigines (NSML), formerly referred to as LEOPARD syndrome, is a rare autosomal-dominant multiple congenital anomaly condition, characterized by multiple lentigines, electrocardiographic (ECG) abnormalities, ocular hypertelorism, pulmonary stenosis, genital abnormalities, growth retardation, and sensorineural deafness [Sarkozy et al., 2008; Gelb and Tartaglia, 2010; Martínez-Quintana and Rodríguez-González, 2012]. The diagnosis of NSML is made on clinical grounds by observation of specific features. Standard diagnostic criteria for NSML, proposed by Voron et al. [1976]; included multiple lentigines and two other cardinal features.

Together with Noonan syndrome (NS), Costello syndrome, cardio-facio-cutaneous syndrome (CFCS), and neurofibromatosis type 1, NSML is classified as RASopathy, a disorder affecting the RAS-MAPK signal transduction pathway [Aoki and Matsubara, 2013]. NSML is genetically heterogeneous and three causative genes have been identified, accounting for approximately 95% of affected individuals [Martínez-Quintana and Rodríguez-González, 2012]. Approximately 85% of patients with NSML have heterozygous

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missense mutations in the protein-tyrosine phosphatase, non-receptor type 11 (*PTPN11*) gene (OMIM#151100). To date, 11 different *PTPN11* mutations, all localized in the protein-tyrosine phosphatase (PTP) domain, have been reported in NSML, two of which (p.T279C and p.T468M) constitute approximately 65% of the cases [Martínez-Quintana and Rodríguez-González, 2012]. Two unrelated patients with NSML were found to have heterozygous missense mutations in the v-Raf-1 murine leukemia viral oncogene homolog 1 (*RAF1*) gene (p.L613V and p.S257L) (OMIM#611554) [Pandit et al., 2007]. The p.L613V mutation increases kinase activity and enhances downstream ERK activation [Pandit et al., 2007]. Two unrelated patients with NSML had heterozygous missense mutations in the v-Raf murine sarcoma viral oncogene homolog B1 (*BRAF*) gene (p.T241P and p.L245F) (OMIM#613707) [Koudova et al., 2009; Sarkozy et al., 2009].

Mitogen-activated protein kinase 1 (MAP2K1) and MAP2K2 are dual-specificity protein kinases, which function as effectors of the serine/threonine kinase *RAF* family members by phosphorylating

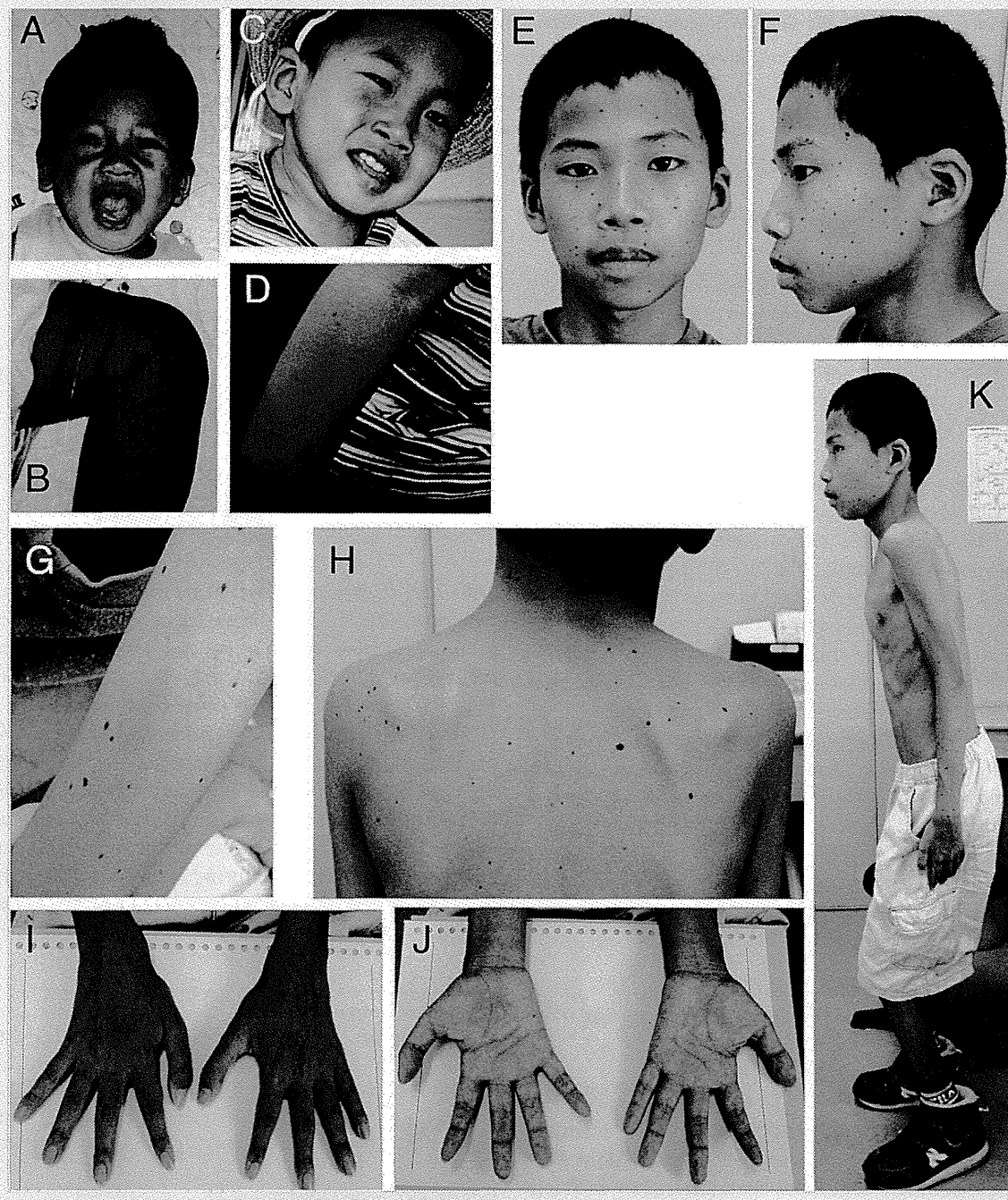

FIG. 1. Clinical photographs of the patient at the age 7 months (A, B), at 2 5/12 years (C, D), and at 11 years (E–K).

and activating ERK proteins. A heterozygous missense mutation in *MAP2K1* is known to be causal for CFCS or NS [Allanson and Roberts, 2011; Rauen, 2012]. To date, all published *MAP2K1* mutations occurred in exons 2, 3, and 6.

In this report, we present a patient clinically diagnosed with NSML, who had a de novo novel and heterozygous *MAP2K1* variant with probable pathogenicity.

CLINICAL REPORT

The patient, a 13-year-old Japanese boy, was the second child of a healthy 30-year-old mother and a healthy 35-year-old nonconsanguineous father. His two brothers were healthy. He was born by normal vaginal delivery at 41 weeks and 4 days of gestation after an uncomplicated pregnancy. His birth weight was 4,350 g (+3.2 SD), length was 51 cm (+1.0 SD), and OFC was 37 cm (+2.6 SD). He showed hypotonia and sucked poorly in the neonatal period. He raised his head at age 3 months, rolled over at 4 months, and sat unsupported at 7 months. He showed no distinctive facial features and only a few lentigines in infancy (Fig. 1A, B).

His growth was impaired with a weight of 8.25 kg (−2.1 SD), height of 76.9 cm (−1.6 SD), and OFC of 45.6 cm (−1.4 SD) at age 1 7/12 years. His weight was 11 kg (−2.5 SD), height was 90.0 cm (−2.4 SD), and OFC was 49 cm (−0.4 SD) at age 2 10/12 years. Lentigines increased on the face and the limbs (Fig. 1C, D). He walked unassisted at age 3 3/12 years, and spoke a two-word sentence at 3 years. His intellectual quotient was 60 at 4 years, and 82 at 7 years. He showed growth acceleration from age 8.5 years, accompanied by a change in voice, and was diagnosed as precocious puberty at 9 years with an advanced bone age of 11.5 years. At age 10 years, his weight was 22.1 kg (−1.5 SD), height was 130 cm (−1.2 SD), and OFC was 51.8 cm (−1.0 SD). He underwent surgical elongation of his hamstrings, which reduced the limitation of bilateral knee extension from −60° degrees to −20° degrees.

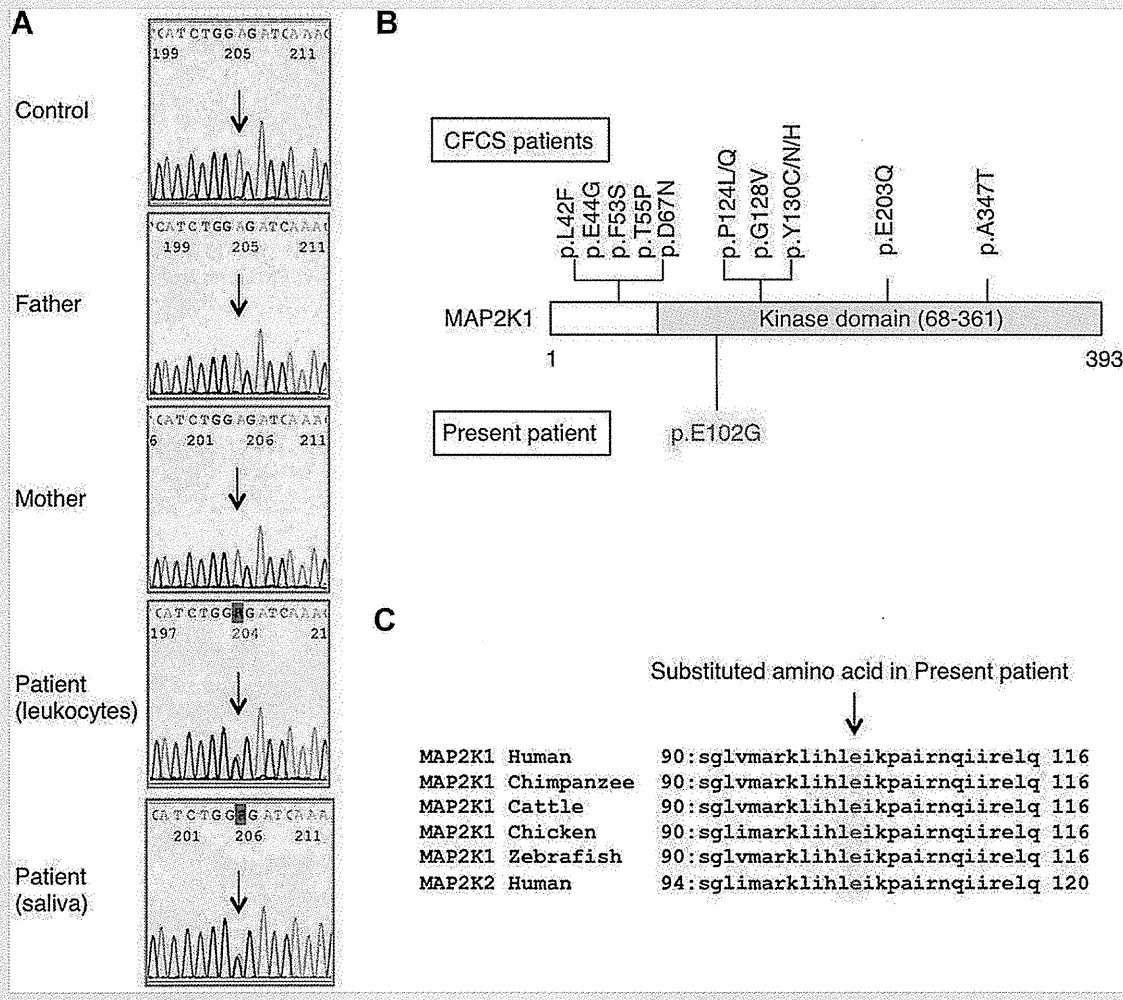

FIG. 2. A: Sanger sequencing of *MAP2K1*, showing an A→G substitution (c.305A > G, p.E102G) in exon 3, which was detected in the patient's DNA from leukocytes and saliva, but not detected in parental samples. B: MAP2K1 domain structure and location of residues altered in the present patient and previously reported patients with cardio-facio-cutaneous syndrome (CFCS). C: MAP2K1 amino acid alignment around the residue where the present amino acid change occurred. This residue is evolutionally conserved.

At age 11 years, his facial features included ocular hypertelorism, a long philtrum, thick upper and lower lip vermilions, and thickened ear helices (Fig. 1E, F). He had hyperextensible and dark skin with multiple lentigines all over the body, several café-au-lait spots, and fine wrinkles on the palms (Fig. 1G–J). He had a slender habitus with pectus carinatum, mild scoliosis, slender extremities, and limited extension of both elbows and knees (Fig. 1K). His weight was 23.0 kg (−1.8 SD) and height was 141 cm (−0.4 SD).

He had no abnormalities in the external genitalia. Resting or 24-hour ECG detected no conduction abnormalities. Echocardiography showed no congenital heart defects, pulmonary valve stenosis, or hypertrophic cardiomyopathy (HCM). Brain magnetic resonance imaging showed no structural abnormalities. He had bilateral mild sensorineural hearing loss with the threshold of 40 dB at approximately 2 kHz. G-banded chromosomes were normal.

MOLECULAR INVESTIGATION

Genomic DNA was isolated from the patient's leukocytes and saliva and his parents' leukocytes after appropriate informed consent. All coding exons and flanking introns in *PTPN11*, *KRAS*, *HRAS*, and *SOS1*, exons 6 and 11–16 in *BRAF*, exons 7, 14, and 17 in *RAF1*, exons 2 and 3 in *MAP2K1/2*, and exon 1 in *SHOC2* were amplified by polymerase chain reaction (PCR) with primers based on GenBank sequences. The primer sequences are available on request. PCR amplification was performed under standard condition using Taq DNA polymerase. After amplification, the PCR products were gel-purified and sequenced on the ABI 3500xL automated DNA sequencer (Applied Biosystems, Carlsbad, CA). A heterozygous missense variant (c.305A > G, p.E102G) was identified in exon 3 of *MAP2K1* in the patient's DNA extracted from his leukocytes and saliva. The variant was not detected in the parental samples (Fig. 2A). No mutation, other than c.305A > G in *MAP2K1*, was identified by the analysis using custom HaloPlex panel (Agilent Technologies, Santa Clara, CA) designed to identify mutations in exons and exon-intron boundaries of the following RASopathy-related genes: *PTPN11*, *HRAS*, *KRAS*, *NRAS*, *BRAF*, *RAF1*, *MAP2K1/2*, *SOS1*, *SHOC2*, *CBL*, *RIT1*, *NF1*, *SPRED1*, and *RRAS*.

DISCUSSION

The present patient had multiple lentigines, café-au-lait spots, ocular hypertelorism, growth impairment, sensorineural hearing loss, hypotonia, low average intelligence, and skeletal abnormalities.

TABLE I. Clinical Features of the Present Patient, Patients With Noonan Syndrome With Multiple Lentigines (NSML), and Patients With Cardio-Facio-Cutaneous Syndrome (CFCS) Caused by *MAP2K1* or *MAP2K2* Mutations

	Present patient	Patients with NSML [Gelb and Tartaglia, 2010]	Patients with CFCS caused by *MAP2K1* or *MAP2K2* mutations [Dentici et al., 2009]	
Causative gene	*MAP2K1*	*PTPN11* (90%) *RAF1* (n = 2) *BRAF* (n = 2)	*MAP2K1* (n = 41)	*MAP2K2* (n = 20)
Sex	Male	Male > Female	Male:Female = 9:14	
Nevi/lentigines	+	<100%	11/34 (32%)	
Café-au-lait spots	+	70–80%	5/30 (17%)	
Congenital heart defects	−	85%	25/39 (64%)	
HCM	−	70%	14/42 (33%)	
ECG abnormalities	−	23%	2/28 (7%)	
Pulmonary valve stenosis	−	25%	17/42 (40%)	
Polyhydramnios	−		20/32 (63%)	
Fetal macrosomia	+		13/25 (52%)	
Short stature	+	<50%	30/38 (79%)	
Macrocephaly	+		26/34 (76%)	
Hypertelorism	+		23/30 (77%)	
Thickened helix	+		27/30 (90%)	
Sparse hair	−		33/49 (67%)	
Sparse eyebrow	−		35/38 (92%)	
Palpebral ptosis	−		18/27 (67%)	
Flat nasal bridge	−		10/12 (83%)	
Joint limitation	+			
Failure to thrive	+		29/35 (83%)	
Intellectual disability	−	30%	43/46 (93%)	
Development delay	+		43/45 (96%)	
Hypotonia	+		40/45 (89%)	
Sensorineural hearing loss	+	<20%		
Seizures	−		16/44 (36%)	

CFCS, cardio-facio-cutaneous syndrome; ECG, electrocardiograph; HCM, hypertrophic cardiomyopathy , NSML, Noonan syndrome with multiple lentigines.

He lacked ECG conduction abnormalities, pulmonary stenosis, or abnormal genitalia. These findings were compatible with the standard diagnosis of NSML by Voron et al. [1976]. The variant c.305A > G, p.E102G was found de novo and not detected in db SNP Release 137 (http://www.ncbi.nlm.nih.gov/projects/SNP/), the Exome Sequencing Project (NHLBI-ESP) database (ESP6500SI-V2) (http://evs.gs.washington.edu/EVS/), the 1000 Genomes Project (1KGP) (http://www.1000genomes.org/), or the Human Gene Mutation Database (http://www.hgmd.cf.ac.uk/ac/index.php). In the COSMIC database, c.302_307delTGGAGA, resulting in an in-frame deletion (p.E102_I103delEI), has been identified in two samples with malignant melanoma and lung cancer (http://cancer.sanger.ac.uk/cancergenome/projects/cosmic/). The glutamine residue at codon 102 is located in the kinase domain (residues 68–361) of *MAP2A1* (Fig. 2B) and is conserved in higher organisms (Fig. 2C). Polymorphism Phenotyping v2 (PolyPhen-2) (http://genetics.bwh.harvard.edu/pph2/) predicts the variant to be possibly damaging, with a score of 0.711. In view of this evidence, the variant p.E102G may be causal for various clinical features consistent with NSML in the patient. However, no functional characterization of the variant was available and Sorting Intolerant From Tolerant (SIFT) (http://sift.jcvi.org) predicts the variant to be tolerated, with a score of 0.09.

We reviewed clinical features of the present patient, previously reported patients with NSML caused by *PTPN11* mutations in most (including two caused by *RAF1* mutations and two caused by *BRAF* mutations), and patients with CFCS caused by *MAP2K1* or *MAP2K2* mutations (Table I) [Pandit et al., 2007; Dentici et al., 2009; Koudova et al., 2009; Sarkozy et al., 2009]. Patients with NSML frequently had congenital heart defects and/or HCM, and sometimes had pulmonary valve stenosis and/or ECG abnormalities [Wakabayashi et al., 2011; Martínez-Quintana and Rodríguez-González, 2012], none of which were found in the present patient. Both patients with NSML caused by *RAF1* mutations had HCM, additionally, one had pulmonary valve stenosis, and the other had a mitral valve anomaly [Pandit et al., 2007]. One of the two patients with NSML caused by *BRAF* mutations had tetralogy of Fallot and the other had mitral and aortic valve dysplasia [Koudova et al., 2009; Sarkozy et al., 2009]. Patients with CFCS caused by *MAP2K1* or *MAP2K2* mutations frequently had congenital heart defects, polyhydramnios, characteristic facial "coarseness" (sparse hair/eyebrows, palpebral ptosis, and flat nasal bridge), and intellectual disability [Dentici et al., 2009], which were not found in the present patient. They rarely or sometimes had nevi, café-au-lait spots, or sensorineural hearing loss [Dentici et al., 2009], which were found in the present patient. Fetal macrosomia, postnatal failure to thrive/growth impairment, macrocephaly, hypotonia, developmental delay, and facial features including hypertelorism and thickened helices were shared by the present patient and over half of the patients with CFCS caused by *MAP2K1* or *MAP2K2* mutations.

In conclusion, the present patient may be the first to fit the standard clinical diagnostic criteria for NSML by Voron et al. [1976]; associated with a *MAP2K1* mutation. He lacked congenital heart defects or HCM, frequently observed in those with NSML, mostly caused by *PTPN11* mutations. He had fetal macrosomia, postnatal failure to thrive/growth impairment, macrocephaly, hypotonia, developmental delay, and hypertelorism but lacked congenital heart defect, characteristic facial features, or intellectual disability; which are frequently observed features in CFCS caused by *MAP2K1* or *MAP2K2* mutations. These observations could offer new insight into the phenotypic spectrum of RASopathies.

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RESEARCH REPORT

Seven Novel Mutations in Bulgarian Patients with Acute Hepatic Porphyrias (AHP)

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Abstract Acute intermittent porphyria (AIP), variegate porphyria (VP), and hereditary coproporphyria (HCP) are caused by mutations in the hydroxymethylbilane synthase (HMBS), protoporphyrinogen oxidase (PPOX), and coproporphyrinogen oxidase (CPOX) genes, respectively. This study aimed to identify mutations in seven Bulgarian families with AIP, six with VP, and one with HCP. A total of 33 subjects, both symptomatic ($n = 21$) and asymptomatic ($n = 12$), were included in this study. The identification of mutations was performed by direct sequencing of all the coding exons of the corresponding enzymes in the probands. The available relatives were screened for the possible mutations. A total of six different mutations in HMBS were detected in all seven families with AIP, three of which were previously described: c.76C>T [p.R26C] in exon 3, c.287C>T [p.S96F] in exon 7, and c.445C>T [p.R149X] in exon 9. The following three novel HMBS mutations were found: c.345-2A>C in intron 7–8, c.279-280insAT in exon 7, and c.887delC in exon 15. A total of three different novel mutations were identified in the PPOX gene in the VP families: c.441-442delCA in exon 5, c.917T>C [p.L306P] in exon 9, and c.1252T>C [p.C418R] in exon 12. A novel nonsense mutation, c.364G>T [p.E122X], in exon 1 of the CPOX gene was identified in the HCP family. This study, which identified mutations in Bulgarian families with AHP for the first time, established seven novel mutation sites. Seven latent carriers were also diagnosed and, therefore, were able to receive crucial counseling to prevent attacks.

Abbreviations

AHP	Acute hepatic porphyrias
AIP	Acute intermittent porphyria
ALA	δ-Aminolevulinic acid
CPOX	Coproporphyrinogen oxidase
HCP	Hereditary coproporphyria
HMBS	Hydroxymethylbilane synthase
PBG	Porphobilinogen
PPOX	Protoporphyrinogen oxidase
VP	Variegate porphyria

Introduction

Acute intermittent porphyria (AIP) (OMIM 176000), variegate porphyria (VP) (OMIM 176200), and hereditary coproporphyria (HCP) (OMIM 121300) are autosomal dominant, low-penetrant inborn errors of the heme biosynthesis pathway that result in the decreased activity of hydroxymethylbilane synthase (HMBS) (EC 4.3.1.8), protoporphyrinogen oxidase (PPOX) (EC 1.3.3.4), and coproporphyrinogen oxidase (CPOX) (EC 1.3.3.3), respectively.

AIP, VP, and HCP present with clinically identical recurrent neurovisceral attacks. Additionally, erosive bullous cutaneous lesions and hyperpigmentation on sun-exposed areas are more common in VP than in HCP (Sassa 2006). The acute attacks include three major classes of symptoms: gastrointestinal, neurological, and psychiatric. These symptoms are represented by severe abdominal pains, motor neuropathy, depression, and psychosis. The most common factor trigger-

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ing these attacks is the use of numerous porphyrinogenic drugs. Infections, alcohol, a low-calorie diet, and natural sex hormones fluctuations in women, related to menstrual cycle and pregnancy can also provoke attacks (Kappas et al. 1995).

The diagnosis of acute porphyric attack is based on both clinical manifestations and typical biochemical abnormalities. The main laboratory finding is the dramatic increase of the porphyrin precursors porphobilinogen (PBG) and δ-aminolevulinic acid (ALA) in urine. The exact distinction between the three different diseases requires measuring the urinary and fecal porphyrin excretion patterns, which are characteristic for each enzymatic defect. A fluorescence scan of native plasma is also an important diagnostic criterion, presenting (or not) a characteristic peak for each entity (Sassa 2006; Hift et al. 2004). Decreased levels of HMBS activity in AIP, PPOX activity in PV, and CPOX activity in HCP clarify the diagnosis in the proband and establish the enzymatic defect in the latent carriers (Meyer et al. 1972; Deybach et al. 1981). Unfortunately, when measuring enzymatic activities, results similar to the reference values may bring in uncertainty in the precise diagnosis of the latent carriers (Mustajoki 1981). Thus, the optimal strategy for the detection of these individuals includes the implementation of molecular genetic methods (Whatley et al. 2009).

At least 600 different mutations in the HMBS, PPOX, and CPOX genes have been identified so far (www.hgmd.cf.ac.uk). Most of these mutations are specific to one or a few families, although a founder effect has been clearly demonstrated for both AIP and VP (Thunnel et al. 2006; Meissner et al. 1996). Most AIP, VP, and HCP carriers are heterozygotes. Mutations are heterogeneous and are comprised of single nucleotide substitutions, small insertions and deletions. Recently, large insertions/deletions have been described in the HMBS, PPOX, and CPOX genes (Whatley et al. 2009; Barbaro et al. 2013). In Bulgaria, during a 50-year period as the sole porphyria service at University Hospital "Saint Ivan Rislki" Sofia, 35 families with AIP, 20 with VP, and 2 with HCP have been diagnosed, treated, and followed up. However, the molecular analysis of the patients with AIP, VP, and HCP has not yet been performed. The aim of this study was to identify mutations in the HMBS, PPOX, and CPOX genes in Bulgarian families with acute hepatic porphyrias.

Materials and Methods

Patients

Seven independent index cases with AIP were included. The diagnosis was based on clinical symptoms and increased urinary PBG and ALA values. Decreased levels of HMBS activity in erythrocytes and the absence of a plasma fluorescence peak at 624–627 nm confirmed the diagnosis of AIP. Six independent index cases with VP were also studied. The diagnosis of these patients included the evaluation of the clinical symptoms, a typical plasma fluorescence peak at 624–627 nm, elevated urinary PBG and ALA levels during acute attacks and increased stool porphyrins, with a predominance of protoporphyrin over coproporphyrin in the cases with cutaneous symptoms. One patient with HCP was included. The diagnosis was based on the symptoms that occurred during acute attack, increased urinary PBG and ALA levels, markedly increased total porphyrins in the urine, and a plasma fluorescence peak at 618 nm. Molecular analysis of the HMBS, PPOX, and CPOX genes confirmed the precise diagnosis. These 14 probands were diagnosed, treated, and followed up in the Porphyria Unit of "Saint Ivan Rilski" University Hospital Sofia. The precise places of birth and the pedigree trees of the patients were determined, with no apparent signs of consanguinity. Once the diagnosis of AIP was confirmed, the HMBS activity was evaluated in the available asymptomatic family members. A total of 33 individuals, including the probands and asymptomatic relatives, from 15 families with AIP, VP, and HCP gave their written consent to participate in this study, which was approved by the Ethics Committee.

Methods

Biochemical Measurements

PBG and ALA levels in the urine were measured according to the method described by Mauzarell and Granick (1956). Urinary and fecal porphyrins were assessed according to the method of Rimington (1971). Total fecal total porphyrin levels were measured according to the method of Lockwood et al. (1985). Total porphyrin levels in urine were evaluated according to our modification and optimization of the method described by Deacon and Elder (2001). HMBS activity in the erythrocytes was determined according to the method described by Adjarov et al. (1994). Plasma fluorescence scanning was performed on a Perkin–Elmer fluorescence spectrophotometer MPF 43, with an excitation wavelength of 398 nm and an emission spectrum from 580 to 700 nm.

Identification of Mutations

Genomic DNA was isolated from peripheral whole blood samples using the innuPREP Blood DNA Midi kit (Analytik Jena Life Science, Germany) according to the manufacturer's protocol. PCR amplification of exons 1 to

Table 1 Clinical and biochemical data and mutations detected in the HMBS gene in families with acute intermittent porphyria

Fam	Pts	Sex	Age/age at first symptoms	Nucleotide change	Amino acid change	Symptoms	HMBS	No. of acute attacks	Urine		TF	TT
									PBG	ALA		
I	P1	F	40/19	c.76C>T	p.R26C	+	18.7	1	715	95	MC	Glu
	Fa	M	65/44	c.76C>T	p.R26C	+	20.1	–	129	122	Inf + M	–
II	P2	F	47/20	c.279-280insAT[a]	Frameshift	+	14.1	2	153	42.7	–	C + Glu
	Sc1	F	45/26	c.279-280insAT[a]	Frameshift	+	10	2	189	–	P	Glu
	Sc2	F	37/30	c.279-280insAT[a]	Frameshift	+	14.3	1	304	–	Inf + M	Glu
	Mo	F	69	c.279-280insAT[a]	Frameshift	–	14	–	–	–	–	–
III	P3	F	44/24	c.887delC[a]	Frameshift	+	20.6	1	320	19.4	MC	Hem
	So	M	20	Neg		–	34.3	–	–	–	–	–
IV	P4	F	27/22	c.445C>T	p.R149X	+	ND	2	955	100	MC	Glu
	Mo	F	50	c.445C>T	p.R149X	+	ND	–	350	78	MC	–
	Si	F	28	Neg	–	–	ND	–	–	–	–	–
V	P5	F	59/39	c.345-2A>C[a]	Splice acceptor site	+	18.7	1	341	214	Inf + M	C + Glu
VI	P6	F	43/25	c.287C>T	p.S96F	+	25.1	1	421	45	MC	Glu
	D	F	21	c.287C>T	p.S96F	–	ND	–	–	–	–	–
VII	P7	F	42/28	c.287C>T	p.S96F	+	ND	1	384	130	MC	Glu
	D	F	15	c.287C>T	p.S96F	–	ND	–	18.2	–	–	–

Fam family, *Pts* patients, *P* proband number, *Fa* father, *Sc* second cousin, *Mo* mother, *So* son, *Si* sister, *D* daughter, *F* female, *M* male, *HMBS* hydroxymethylbilane synthase activity in the erythrocytes, normal range: 25–45 pkat/gHb, *PBG* porphobilinogen, normal value: <15 μmol/24 h, *ALA* δ-aminolevulinic acid, normal range: 11.4–57.2 μmol/24 h, *TF* triggering factor, *Inf* infection, *M* medication, *MC* menstrual cycle, *P* pregnancy, *TT* treatment, Glu, 10% glucose i.v. infusion, *C* cimetidine, *Hem* Hem-arginate (Normosang), *ND* not determined
[a] Novel mutations identified

15 for HMBS, exons 1 to 13 for PPOX, and exons 1 to 7 for CPOX, with corresponding flanking intron–exon boundaries, was performed; the primers and PCR conditions are available upon request. The PCR products were automatically sequenced using the BigDye Terminator v3.1 Cycle Sequencing Kit and a 3500xL Genetic Analyzer (Applied Biosystems, Foster City, USA).

To prove the rarity of the identified novel missense mutations, the corresponding exons of the HMBS and PPOX genes were screened in 96 control DNA samples. In silico prediction of the pathogenicity of these mutations was determined by the HumVar score using the PolyPhen-2 tool (http://genetics.bwh.harvard.edu/pph2/).

Results and Discussion

Detailed clinical, biochemical, and genetic data are presented in Table 1 for the AIP families and in Table 2 for the VP and HCP families. In three of our female AIP patients, the attacks were related to the patient's menstrual cycle. Infections and/or medications played a triggering role in three AIP and four VP cases, as well as in the HCP patient. Nine subjects with AIP manifested with one or two acute attacks. Some patients (family I-F, family II-P2 and II-Sc1, family IV-P4 and family V-P5) suffered chronic symptoms, including fatigue, lower back pain, paresthesia in the lower limbs, and depression. The proband P2 had suffered from two unrecognized acute attacks and had residual paresis at the time of presentation in our clinic. She also had chronic neurological symptoms when Cimetidine treatment was applied. During the 6 months course of Cimetidine administration, a reduction in porphyrin precursors levels and clinical improvement was achieved. Family V-P5 suffered from one acute attack following infection and antibiotic treatment after surgery. She also had chronic neurological symptoms. Cimetidine was administered after the acute onset, but over the first weeks the pains worsened and no biochemical improvement was noticed. Both acute and cutaneous symptoms were present in four symptomatic VP patients, and only acute symptoms were present in one patient and only cutaneous symptoms in four patients. All VP patients manifested with a single acute attack, and only family III-So exhibited chronic symptoms similar to those observed in AIP. The majority of our VP patients presented with severe photodermatosis alone or accompanying acute