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Citation for final published version:

Masson, Emmanuelle, Berthet, Stéphanie, Le Gac, Gerald, Le Rhun, Marc, Ka, Chandran, Autret, Sandrine, Gourlaouen, Isabelle, Cooper, David N. , Férec, Claude, Rebours, Vinciane and Chen, Jian-Min 2023. Identification of protease-sensitive but not misfolding PNLIP variants in familial and hereditary pancreatitis. Pancreatology 23 (5) , pp. 507-511. 10.1016/j.pan.2023.05.011

Publishers page: https://doi.org/10.1016/j.pan.2023.05.011

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Identification of protease-sensitive but not misfolding *PNLIP* variants in familial and hereditary pancreatitis

Short title: *PNLIP* variants and pancreatitis

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ABSTRACT

Mutations in the *PNLIP* gene have recently been implicated in chronic pancreatitis. Several *PNLIP* missense variants have been reported to cause protein misfolding and endoplasmic reticulum stress although genetic evidence supporting their association with chronic pancreatitis is currently lacking. Protease-sensitive *PNLIP* missense variants have also been associated with early-onset chronic pancreatitis although the underlying pathological mechanism remains enigmatic. Herein, we provide new evidence to support the association of protease-sensitive *PNLIP* variants (but not misfolding *PNLIP* variants) with pancreatitis. Specifically, we identified protease-sensitive *PNLIP* variants in 5 of 373 probands (1.3%) with a positive family history of pancreatitis. The protease-sensitive variants, p.F300L and p.I265R, were found to segregate with the disease in three families, including one exhibiting a classical autosomal dominant inheritance pattern. Consistent with previous findings, protease-sensitive variant-positive patients were often characterized by early-onset disease and invariably experienced recurrent acute pancreatitis, although none has yet developed chronic pancreatitis.

Keywords: Allele frequency; Exome sequencing; Hereditary pancreatitis; Pancreatic Lipase; Targeted next-generation sequencing

List of abbreviations

ER, endoplasmic reticulum

gnomAD, the Genome Aggregation Database

HGVS, Human Genome Variation Society

NGS, next-generation sequencing

PNLIP, pancreatic lipase

RAP, recurrent acute pancreatitis

1. Introduction

The *PNLIP* gene (OMIM# 246600) is located on chromosome 10q25.3 and contains 13 exons (the first is non-coding; NM_000936.4). It encodes pancreatic lipase, a 465 amino acid protein that is expressed abundantly and almost exclusively in the exocrine pancreas [1]. In 2019, a candidate gene study [2] found that a very limited number of heterozygous *PNLIP* missense variants (precisely five: p.P245A, p.I265R, p.F300L, p.S304F, and p.F314L), all of which rendered the mutant protein prone to proteolytic degradation by trypsin and chymotrypsin, were enriched in German and French nonalcoholic or idiopathic chronic pancreatitis patients (13/1163 cases (1.1%) *vs.* 3/3000 controls (0.1%); odds ratio 11.3, $P < 0.0001$). Of the 13 carrier patients, 7 harbored p.F300L, 3 harbored p.I265R whilst the remaining 3 possessed p.P245A, p.S304F and p.F314L variants, respectively. These 13 patients shared two noteworthy features. First, all were children or young adults. Second, all had experienced recurrent acute pancreatitis (RAP) but none had yet been diagnosed with chronic pancreatitis based upon morphological imaging. These features were compatible with the mechanistic definition of early chronic pancreatitis according to recommendations from an International Working Group [3]. However, no further examples of protease-sensitive *PNLIP* variants were found in non-European cohorts (n = 545) from the United States, Japan and India [2]. The reasons for this discrepancy may include varying definitions of what is termed "idiopathic" between the different cohorts [4].

Another interesting finding from the Lasher study [2] was that four heterozygous *PNLIP* missense variants, namely p.A174P, p.G233E, p.C254R and p.V454F, were characterized by significantly reduced protein secretion as compared to the wild-type [2]. These four variants, together with another naturally occurring *PNLIP* missense variant p.T221M, were shown to cause protein misfolding and endoplasmic reticulum (ER) stress in cell transfection experiments [5, 6]. Moreover, *Pnlip* p.T221M mice developed spontaneous and progressive chronic pancreatitis, which was attributable to Pnlip misfolding-induced ER stress [7]. These five variants may thus increase the risk of pancreatitis, a postulate that is particularly apposite when considered in the context of the increasingly appreciated role for the misfolding pathway in chronic pancreatitis [8-11]. However, of the aforementioned four variants, two (p.G233E and p.C254R) were found once each in patients whilst the other two (p.A174P and p.V454F) were found once each in controls [2]. The p.T221M variant was identified, in the homozygous state, in two brothers with congenital pancreatic lipase deficiency [12]. Whether or not the two brothers could have had subclinical chronic pancreatitis was unknown since no abdominal imaging data were available from either brother. This notwithstanding, their heterozygous parents were clinically unaffected [12].

The above *PNLIP* genetic findings were made using a cohort of patients with idiopathic pancreatitis [2]. We wondered whether patients with a positive family history of pancreatitis might exhibit a similar pattern of *PNLIP* variants. We therefore analyzed the *PNLIP* gene in a large French cohort of patients with familial or hereditary pancreatitis.

2. Methods

2.1. Patients

A total of 373 probands with a positive family history of pancreatitis but without any known etiological factors such as alcohol abuse, trauma, medication or metabolic disorders, participated in this study. All probands had been previously screened and found not to harbor known disease causative mutations in the *PRSS1*, *SPINK1* and *TRPV6* genes or any *CFTR* genotypes comprising a severe allele plus a mild allele. The patients were divided into two groups for operational purposes, in line with our previous publications [13, 14]: familial pancreatitis (the proband had one symptomatic family member; n = 276) and hereditary pancreatitis (the proband had ≥2 symptomatic family members; n = 97). In addition, several family members of three probands harboring a protease-sensitive variant were also analyzed (see *Results*).

This study was approved by the Ethics Committee of the Brest University Hospital. Informed consent was obtained from all subjects studied.

2.2. Genetic analysis

Three patients from pedigree A (see *Results*) were initially analyzed by exome analysis whereas all other subjects were analyzed by targeted next-generation sequencing (NGS). Exome sequencing was performed by IntegraGen (Genopole® Evry, France) and variant data were interpreted on the SeqOne Genomics Platform (Montpellier, France). Targeted NGS included all *PNLIP* coding exons (i.e., exons 2-13) plus their flanking intronic regions (for primer sequences, see Supplementary Table 1). The NGS library was prepared with the Assess Array™ IFC system (Fluidigm, Les Ulis, France) according to the manufacturer's protocol. Library sequencing was performed using the Ion Torrent Sequencing System (Thermo Fisher Scientific). SeqNextsoftware (JSI Medical Systems) was used to identify variants from the NGS data. Only rare *PNLIP* variants (global allele frequency of <1% in accordance with the Genome Aggregation Database (gnomAD) [15]) were included for analysis. All reported variants were confirmed by Sanger sequencing. All variants were named in accordance with Human Genome Variation Society (HGVS) recommendations [16].

2.3. SpliceAI prediction and minigene splicing assay

SpliceAI [17] was used to predict the potential impact of all reported variants on splicing. One *PNLIP* variant, c.1-1G>A, was analyzed by means of a minigene splicing assay as previously described [18] (for details, see Supplementary Methods).

3. Results

Rare *PNLIP* variants identified in the probands (and co-inherited risk variants in other chronic pancreatitis-related genes wherever applicable) are summarized in Table 1. Of these, c.1-1G>A was an unequivocal loss-of-function variant. It was predicted by SpliceAI to cause the skipping of exon 2 (c.1-46), and this prediction was confirmed by minigene assay (Supplementary Fig. 1). The use of the nearest downstream translation initiation codon would predict the synthesis of a completely new and different protein of 66 amino acids (Supplementary Fig. 2). By contrast, the two other intronic variants detected (c.325-5A>G and c.325-11C>T), as well as all synonymous (n = 6) and missense (n = 3) variants, were not predicted by SpliceAI to affect splicing.

All three missense variants (p.I265R, p.F300L and p.M414K) have been previously reported [2]. Of these, p.M414K was identified only in a single proband in this study. Not only was the p.M414K substitution functionally neutral (i.e., no significant effect on protein secretion and activity) [2] but it also has a much higher global allele frequency than the two other missense variants, p.F300L and p.I265R (Table 1), respectively the top two protease-sensitive variants in terms of their frequencies in the previously analyzed German and French patient cohorts [2]. Moreover, it should be emphasized that the global allele frequency of p.M414K, 0.001320, is above the allele frequency threshold (i.e., 0.001) that has often been used as a filter to identify variants responsible for autosomal dominant diseases [19].

p.F300L and p.I265R were together identified in a total of 5/373 pancreatitis probands (1.3%). Specifically, p.F300L was identified in one proband with hereditary pancreatitis and two probands with familial pancreatitis whereas p.I265R was identified in two probands with familial pancreatitis (Table 1). We were able to analyze several family members of three variant-positive probands. The p.F300L and p.I265R variants were found to segregate with the disease in their respective families (Figure 1).

We reviewed clinical data from the p.F300L- and p.I265R-positive patients (n = 10) illustrated in Figure 1. All were reported to have RAP, their ages of onset for the first episode of pancreatitis being indicated in Figure 1. Data were available from six patients with respect to pancreas imaging and pancreatic exocrine functional testing; all had normal pancreas imaging findings and none exhibited pancreatic insufficiency or diabetes.

4. Discussion

Herein, we report our findings from the analysis of the *PNLIP* gene in 373 probands with a positive family history of pancreatitis. The currently most accurate prediction tool for splicing variants, SpliceAI, predicted that neither the c.325-5A>G and c.325-11C>T intronic variants nor the six synonymous exonic variants would have any effect on splicing. These variants were therefore considered to have no pathological relevance to pancreatitis.

Interestingly, the c.1-1G>A transition was found in two apparently unrelated probands with familial pancreatitis but was neither present in gnomAD nor was it co-inherited with any known risk variants in other chronic pancreatitis-related genes, suggesting its potential pathological relevance. However, since c.1-1G>A was shown by minigene assay to lead to the complete skipping of exon 2, the aberrant mutant transcript could not encode any protein with PNLIP activity. Were c.1-1G>A to constitute a straightforward loss-of-function variant, it would be most unlikely to predispose to pancreatitis. This is because two previous observations are at odds with a pathological role for *PNLIP* loss-of-function variants in chronic pancreatitis. First, a homozygous nonsense variant in the *PNLIP* gene, p.Trp419Ter, was found in a 3-year-old girl with steatorrhea that is typical for patients with congenital pancreatic lipase deficiency [20]. However, the patient did not exhibit exocrine pancreatic insufficiency; abdominal ultrasonography, computed tomography of the abdomen, and magnetic resonance cholangiopancreatography were all indicative of a normal pancreas; and her heterozygous parents and two heterozygous siblings were not reported to exhibit any clinical symptoms [20]. Second, three *PNLIP* heterozygous missense variants, p.H92N, p.C198Y and p.D264Y, are characterized by an almost complete loss of lipase activity whilst displaying normal secretion of functionally defective protein [2] but these three variants have invariably been found in the French control dataset [2]. We cannot however exclude the possibility (i) that c.1-1G>A occurs in *cis* with an as yet unidentified functional variant with pathological relevance or (ii) that it could lead to the production of a protein that is toxic to pancreatic cells.

We did not identify any misfolding *PNLIP* missense variants. By contrast, we found protease-sensitive *PNLIP* variants in 1.3% (5/373) of the analyzed probands; this detection rate was quite similar to that (i.e., 1.1%) previously reported in the German and French nonalcoholic chronic pancreatitis patients [2]. Most importantly, the identified protease-sensitive variants, p.F300L and p.I265R, were found to segregate with the disease in all three families in which additional family members were analyzed (Figure 1). One of these families, pedigree A,

exhibited a classical autosomal dominant mode of inheritance with respect to the putatively pathogenic p.F300L variant.

Apropos the abovementioned similar detection rates for the protease-sensitive *PNLIP* variants, two other findings concurred between the previous study [2] and our own. First, the protease-sensitive variants detected in both studies were predominantly associated with early onset pancreatitis. Second, all variant-positive patients were afflicted with RAP whereas none were known to have developed chronic pancreatitis.

In short, we provide new genetic evidence to support the emerging association between protease-sensitive *PNLIP* variants and RAP. Looking ahead, it will be important to follow patients harboring protease-sensitive *PNLIP* variants prospectively in order to ascertain whether or not chronic pancreatitis will eventually develop. Further, mouse studies of protease-sensitive *PNLIP* variants, particularly p.Phe300Leu, are warranted with a view to elucidating the enigmatic pathological mechanism underlying these *PNLIP* variants.

Acknowledgements

Members of the French GREPAN Study Group: Amandine ABRANTES (CH Cornouaille, Quimper, France), Lina AGUILERA MUNOZ (Beaujon Hospital, Clichy, France), Jérémie ALBOUYS (Dupuytren University Hospital, Limoges, France), Laurent ALRIC (CHU Rangueil, Toulouse, France), Xavier AMIOT (Hôpital Tenon (AP-HP), Paris, France), Isabelle ARCHAMBEAUD (CHU Nantes, Nantes, France), Solène AUDIAU (CH Cholet, Cholet, France), Laetitia BASTIDE (Hospital of Saint-Etienne, Saint-Etienne, France), Julien BAUDON (CH Cholet, Cholet, France), Guy BELLAICHE (CHI d'Aulnay-sous-Bois, Aulnay-sous-Bois, France), Serge BELLON (Avignon Hospital, Avignon, France), Valérie BERTRAND (Le Havre Hospital, Le Havre, France), Karine BIDEAU (CH Cornouaille, Quimper, France), Kareen BILLIEMAZ (Hospital of Saint-Etienne, St-Etienne, France), Claire BILLIOUD (University Hospital Croix Rousse, Lyon, France), Sabine BONNEFOY (HIA Clermont-Tonnerre, Brest, France), Corinne BORDERON (CHU Estaing, Clermont Ferrand, France), Barbara BOURNET (CHU Rangueil, Toulouse, France), Estelle BRETON (CH Saint Brieuc, Saint-Brieuc, France), Mathias BRUGEL (CHU Reims, Reims, France), Louis BUSCAIL (CHU Rangueil, Toulouse, France), Guillaume CADIOT (CHU Reims, Reims, France), Marine CAMUS (Saint Antoine Hospital, Paris, France), Xavier CAUSSE (CHR Orléans, Orléans, France), Patrick CHAMOUARD (CHRU Strasbourg, Strasbourg, France), Ulriikka CHAPUT (Saint Antoine Hospital, Paris, France), Jian-Min CHEN (EFS, Brest, France), Franck CHOLET (CHU Brest, Brest, France), Dragos Marius CIOCAN (Hôpital Antoine-Béclère, Clamart, France), Christine CLAVEL (CHU Reims, Reims, France), Benoit COFFIN (Hôpital Louis Mourier,

Colombes, France), Laura COIMET-BERGER (CH Cahors, Cahors, France), Isabelle CREVEAUX (CHU Clermont-Ferrand, Clermont-Ferrand, France), Adrian CULETTO (CHU Rangueil, Toulouse, France), Oussama DABOUSSI (CH Chartres, Chartres, France), Véronique DALSTEIN (CHU Reims, Reims, France), Louis DE MESTIER (Beaujon Hospital, Clichy, France), Thibault DEGAND (CHU Dijon, Dijon, France), Christelle D'ENGREMONT (CHU Grenoble, Grenoble, France), Bernard DENIS (Hôpital Pasteur, Colmar, France), Solène DERMINE (Beaujon Hospital, Clichy, France), Romain DESGRIPPES (CH Saint-Malo, Saint-Malo, France), Augustin DROUET D'AUBIGNY (CH Cornouaille, Quimper, France), Raphaël ENAUD (Pellegrin-Enfants Hospital, Bordeaux, France), Alexandre FABRE (Hôpital de la Timone Enfants, Marseille, France), Claude FEREC (CHU Brest, Brest, France), Dany GARGOT (CH Blois, Blois, France), Eve GELSI (Archet II Hospital, Nice, France), Elena GENTILCORE (CH des Escartons, Briançon, France), Rodica GINCUL (Hôpital privé Jean Mermoz, Lyon, France), Emmanuelle GINGLINGER-FAVRE (Hôpital de Mulhouse, Mulhouse, France), Marc GIOVANNINI (Institut Paoli Calmettes, Marseille, France), Cécile GOMERCIC (Archet II Hospital, Nice, France), Hannah GONDRAN (CHU Nantes, Nantes, France), Thomas GRAINVILLE (CHU Rennes, Rennes, France), Philippe GRANDVAL (Timone University Hospital, Marseille, France), Denis GRASSET (CH Bretagne Atlantique, Vannes, France), Stéphane GRIMALDI (CH Dax-Côte d'Argent, Dax, France), Sylvie GRIMBERT (GH Diaconesses, Paris, France), Hervé HAGEGE (CHI Villeneuve Saint Georges, Créteil, France), Sophie HEISSAT (Hôpital Femme Mère Enfant, Bron, France), Olivia HENTIC (Beaujon Hospital, Clichy, France), Anne HERBER-MAYNE (CH Chartres, Le Coudray, France), Marc HERVOUET (Hôpital d'instruction des armées Percy, Clamart, France), Solene HOIBIAN (Institut Paoli Calmettes, Marseille, France), Jérémie JACQUES (Dupuytren University Hospital, Limoges, France), Bénédicte JAIS (Beaujon Hospital, Paris, France), Mehdi KAASSIS (CH Cholet, Cholet, France), Stéphane KOCH (University Hospital of Besançon, Besançon, France), Elodie LACAZE (Le Havre Hospital, Le Havre, France), Joël LACROUTE (Clinique Sainte Barbe, Strasbourg, France), Thierry LAMIREAU (Pellegrin-Enfants Hospital, Bordeaux, France), Lucie LAURENT (Beaujon Hospital, Clichy, France), Xavier LE GUILLOU (CHU Poitiers, Poitiers, France), Marc LE RHUN (CHU Nantes, Nantes, France), Sarah LEBLANC (Hôpital privé Jean Mermoz, Lyon, France), Philippe LEVY (Beaujon Hospital, Clichy, France), Astrid LIEVRE (CHU Rennes, Rennes, France), Diane LORENZO (Beaujon Hospital, Paris, France), Frédérique MAIRE (Beaujon Hospital, Clichy, France), Kévin MARCEL (CH Bretagne Atlantique, Vannes, France), Emmanuelle MASSON (CHU Brest, Brest, France), Clément MATIAS (CHU Amiens, Amiens), Jacques MAUILLON (Rouen University Hospital, Rouen, France), Stéphanie MORGANT (Beaujon Hospital, Clichy, France), Driffa MOUSSATA (Trousseau Hospital,

Tours, France), Nelly MULLER (Beaujon Hospital, Clichy, France), Sophie NAMBOT (CHU Dijon, Dijon, France), Bertrand NAPOLEON (Hôpital privé Jean Mermoz, Lyon, France), Anne OLIVIER (Angers University Hospital, Angers, France), Maël PAGENAULT (CHU Rennes, Rennes, France), Anne-laure PELLETIER (Bichat Hospital, Paris France), Olivier PENNEC (CH Cornouaille, Quimper, France), Fabien PINARD (CH Cornouaille, Quimper, France), Mathieu PIOCHE (Edouard Herriot Hospital, Lyon, France), Bénédicte PROST (CH Saint Joseph Saint Luc, Lyon, France), Lucille QUENEHERVE (CHU Brest, Brest, France), Vinciane REBOURS (Beaujon Hospital, Clichy, France), Noemi REBOUX (CHU Brest, Brest, France), Samia REKIK (Bichat Hospital, Paris, France), Ghassan RIACHI (Rouen University Hospital, Rouen, France), Barbara ROHMER (Hôpital Femme Mère Enfant, Bron, France), Bertrand ROQUELAURE (Hôpital de la Timone Enfants, Marseille, France), Isabelle ROSA HEZODE (CHI de Créteil, Créteil, France), Florian ROSTAIN (Edouard Herriot Hospital, Lyon, France), Jean-Christophe SAURIN (Edouard Herriot Hospital, Lyon, France), Laure SERVAIS (Clinique Charcot, Saint Foy les Lyon, France), Roxana STAN-IUGA (Hôpital Dreux, Dreux, France), Clément SUBTIL (CHU Bordeaux, Bordeaux, France), Charles TEXIER (CH Cholet, Cholet, France), Lucie THOMASSIN (Rouen University Hospital, Rouen, France), David TOUGERON (Poitiers University Hospital, Poitiers, France), Laurent TSAKIRIS (CH Melun, Melun, France), Jean-Christophe VALATS (Hôpital Saint-Eloi, Montpellier, France), Lucine VUITTON (University Hospital of Besançon, Besançon, France), Timothée WALLENHORST (CHU Rennes, Rennes, France), Marc WANGERME (Poitiers University Hospital, Poitiers, France), Hélène ZANALDI (CH Bastia, Bastia, France), Frank ZERBIB (CHU Bordeaux, Bordeaux, France).

Financial support

This work was supported by the Institut National de la Santé et de la Recherche Médicale (INSERM), the Association des Pancréatites Chroniques Héréditaires and the Association Gaétan Saleün, France. The funding sources did not play any role in the study design, collection, analysis or interpretation of the data or in the writing of this report.

Author contributions

E.M. designed the study, performed NGS and contributed to the writing of the manuscript. S.E. and V.R. provided clinical data and revised the manuscript. G.L.G., C.K. and S.A. performed the minigene assay and revised the manuscript. D.N.C. critically revised the manuscript with important intellectual input. C.F. designed

and supervised the study. J.M.C. designed the study and drafted the manuscript. All authors approved the final manuscript submitted.

Declaration of competing interest

The authors are unaware of any conflict of interest.

Appendix A. Supplementary data

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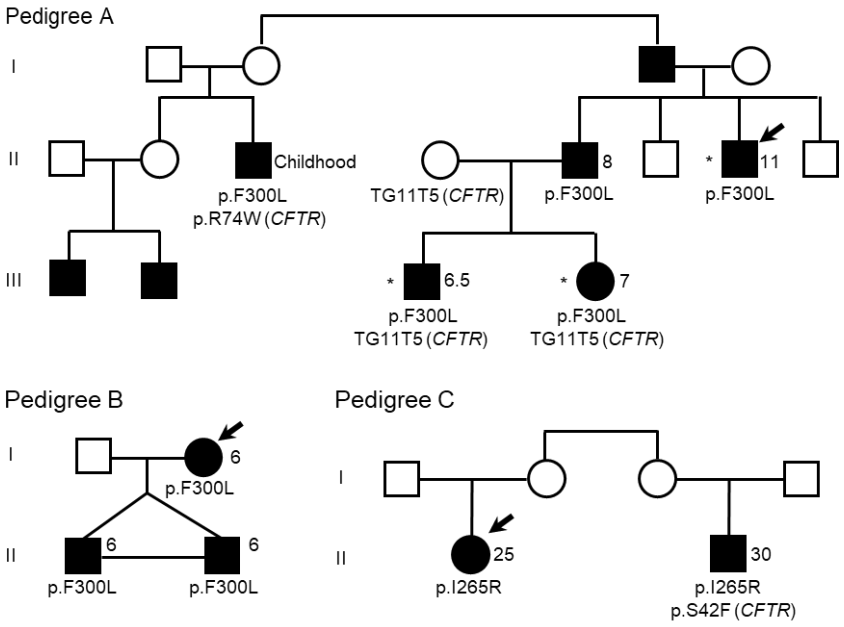


Fig. 1. Pedigrees of the three pancreatitis families with several analyzed subjects in addition to the probands. Probands are indicated by arrows. The three patients subjected to exome sequencing are indicated by asterisks. Variants identified in the *PNLIP* gene (as well as risk variants in other chronic pancreatitis-related genes wherever applicable) are indicated below the individuals subjected to genetic analysis. Arabic numbers to the right of the genetically analyzed patients refer to the age of onset of the first episode of pancreatitis. All variants were found in the heterozygous state. The standard HGVS name for *CFTR* TG11T5 is c.1210-34_1210-6TG[11]T[5].

Table 1
Rare *PNLIP* variants identified in the studied probands with a positive family history of pancreatitis.

Location	Variant		Familial +/n	Hereditary +/n	Global allele frequency in gnomAD	Co-inherited variant(s) in other CP-related gene(s)
	Nucleotide change (NM_000936.4)	Amino acid change				
Intron 1	c.1-1G>A	p.?	2/276	0/97	Absent	#1: none #2: none
Exon 3; Intron 4	c.96A>C(;)325-11C>T	p.(Pro32=)(;)p.?	1/276	0/97	0.004101; 0.000004953	*CFTR* TG11T5[a]
Intron 4	c.325-5A>G	p.?	1/276	0/97	0.0004501	None
Exon 5	c.420C>T	p.(Ile140=)	2/276	0/97	0.002563	#1: *SPINK1* p.Asn34Ser #2: none
Exon 6; Exon 8; Exon 8; Exon 12	c.468C>T(;)699A>T(;)711C>T(;)1241T>A	p.(Phe156=)(;)(Gly233=)(;)(Val237=)(;)Met414Lys	1/276	0/97	0.0005923; 0.00009548; 0.001323; 0.001320	None
Exon 8	**c.794T>G**	**p.Ile265Arg**	2/276	0/97	0.00004245	#1 (pedigree C)[b]: none #2: none
Exon 8	c.798C>T	p.(Asp266=)	3/276	0/97	0.004285	#1: *SPINK1* p.Asn34Ser #2: *CFTR* TG11T5 #3: none
Exon 9	**c.900C>A**	**p.Phe300Leu**	2/276	1/97	Absent	#1 (pedigree A)[b]: none #2 (pedigree B)[b]: None #3: *CFTR* TG11T5

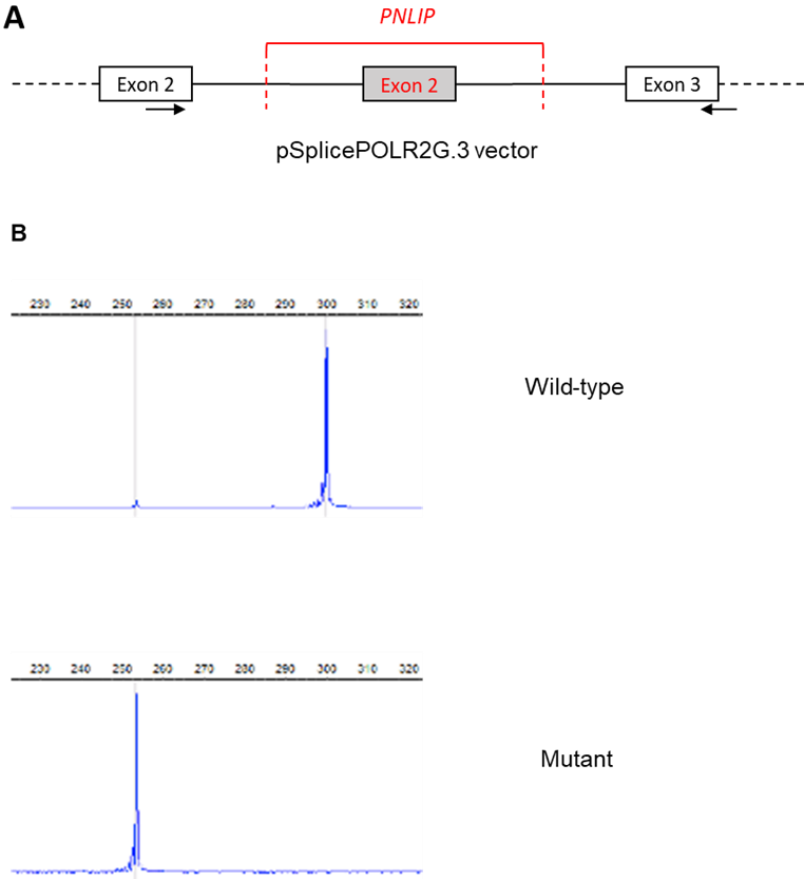
All variants were found in the heterozygous state. Previously described protease-sensitive *PNLIP* variants are highlighted in bold.
[a]Legacy name. Standard HGVS name, c.1210-34_1210-6TG[11]T[5].
[b]See Figure 1.

Supplementary methods

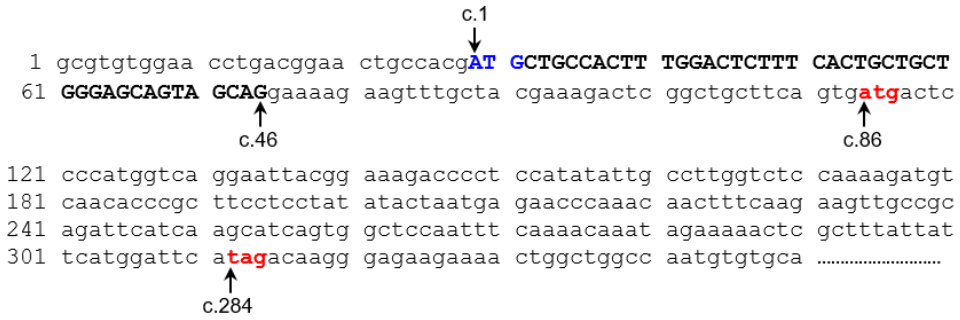
Minigene splicing assay of the *PNLIP* c.1-1G>A variant

A genomic fragment including *PNLIP* exon 2 and its flanking intronic regions (115 bp of intron 1 and 229 bp of intron 2) were PCR amplified using the forward primer 5'-aggaacatgcgaattcTCCAGGCCTAATTGATCAGAA-3' and the reverse primer 5'-ggaggctcaggaattcTGTTTGTGGGAAATATTTGCTGT-3' (underlined are sequences homologous to the vector ends, which were required for the in-fusion cloning reaction (Clontech)). The wild-type and mutant inserts were generated from the DNA of a *PNLIP* c.1-1G>A heterozygous patient; they were respectively cloned into our in-house splicing vector (pSplicePOLR2G.3), and the resulting *PNLIP* cassettes were confirmed by Sanger sequencing (Applied Biosystems).

Wild-type and mutant minigene constructs respectively were transiently transfected into HEK293T cells for 48 h. Total RNAs were extracted by means of a spin column procedure including a genomic DNA removal step. After DNAseI treatment, cDNAs were produced using an oligo-dT(22) primer and the Superscript III enzyme (Invitrogen) following the manufacturer's recommendations. The *PNLIP/POLR2G* exogenous transcripts were PCR amplified using the following primers: forward 5'-caacttgctcaacacggtgaag-3' and reverse 5'-gaattgcccttcttgttgacctg-3'.



Supplementary Figure 1. Minigene splicing assay of the *PNLIP* c.1-1G>A variant. (**A**) Illustration of the *PNLIP* minigene expression vector, in which *PNLIP* exon 2 and its proximal flanking intronic sequences were inserted into the pSplicePOLR2G.3 vector. Arrows indicate the approximate positions of the primers used for RT-PCR analysis. (**B**) Splicing outcomes of the wild-type and mutant variants. The ~300 bp band corresponds to the normally spliced wild-type transcript that contains *PNLIP* exon 2 whereas the ~254 bp band corresponds to the aberrantly spliced transcript lacking *PNLIP* exon 2.



Supplementary Figure 2. Illustration of the predicted functional consequences of the *PNLIP* c.1-1G>A variant. Sequence shown is the partial mRNA-encoding sequence of the *PNLIP* gene (NM_000936.4), with exon 2 highlighted in bold and capital letters. The normal translation initiation codon ATG is highlighted in blue. c.1-1G>A was predicted by SpliceAI to cause the skipping of exon 2 (c.1-46), thereby removing the normal translation initiation codon. The mutant *PNLIP* transcript is predicted to encode a completely new and different protein of 66 amino acids (N.B. the normal length of the PNLIP protein is 465 amino acids]. The predicted translation initiation codon and translation termination codon of this mutant transcript are highlighted in red.

Supplementary Table 1

Primers used for analyzing the 12 coding exons of the *PNLIP* gene by targeted next-generation sequencing.

Exon	Primer sequence (5' to 3')	Amplicon size (bp)
2	Forward: ACACTGACGACATGGTTCTACAAGTCGGGAACATGTTTTCCAG Reverse: TACGGTAGCAGAGACTTGGTCTCTGAATTCAGCTGCCCTAGC	300
3	Forward: ACACTGACGACATGGTTCTACATACCACACAGTGTAATTGAACTCATA Reverse: TACGGTAGCAGAGACTTGGTCTAACACAGTGATAGTTCTTACTTGAAAG Forward: ACACTGACGACATGGTTCTACAGCTGCTTCAGTGATGACTCC Reverse: TACGGTAGCAGAGACTTGGTCTTCACCATGTTAGCCAGCATG	310 271
4	Forward: ACACTGACGACATGGTTCTACACATGTACAAATGGTTCTATTGGC Reverse: TACGGTAGCAGAGACTTGGTCTCCAGTGCCTTACCGTTTGTTA	301
5	Forward: ACACTGACGACATGGTTCTACAAGGGAATTTATCCTAGTCCTCCAG Reverse: TACGGTAGCAGAGACTTGGTCTACCTGAAGAAATTCAACAAAATATG Forward: ACACTGACGACATGGTTCTACACAGAATCTGTTCAAGGTGGAAAG Reverse: TACGGTAGCAGAGACTTGGTCTGCCAAGAGAAACATCATATCTGAG	270 317
6	Forward: ACACTGACGACATGGTTCTACAGTAACGTATCCCTGTTGTTGAGC Reverse: TACGGTAGCAGAGACTTGGTCTGCCTCAAGAGATCATCTTGCCT	286
7	Forward: ACACTGACGACATGGTTCTACACATCCCTTTCCATGCATAACTC Reverse: TACGGTAGCAGAGACTTGGTCTTCAGACACTATAACACCCTTTGG	296
8	Forward: ACACTGACGACATGGTTCTACACCCCAAAGGGTGTTATAGTGTC Reverse: TACGGTAGCAGAGACTTGGTCTGCTTTCTCCCAAGAAGATCTCT	278
9	Forward: ACACTGACGACATGGTTCTACAATTCTGAATATGACGTTAACTTGG Reverse: TACGGTAGCAGAGACTTGGTCTAGGAAAGCACACCAGTCATGAG	301
10	Forward: ACACTGACGACATGGTTCTACAGACAACATGTAGGAAATATGGTACAC Reverse: TACGGTAGCAGAGACTTGGTCTCTTACGTGCAAAATTACTGGCAT Forward: ACACTGACGACATGGTTCTACAGATGTGGGCCAGAAATTTTATCTAG Reverse: TACGGTAGCAGAGACTTGGTCTGCCACCCTAATTAGCTATAATTACCT	253 266
11	Forward: ACACTGACGACATGGTTCTACAGTAGTGGGATGCAAATTAACTTG Reverse: TACGGTAGCAGAGACTTGGTCTACAGAGCAAGACTCCAGCCC	321
12	Forward: ACACTGACGACATGGTTCTACAACCTTAGCCAGAAATGCATTG Reverse: TACGGTAGCAGAGACTTGGTCTCACTCTAGGTAAAGTTGGGTTGAT Forward: ACACTGACGACATGGTTCTACATGGATGTTGGGGACTTGCA Reverse: TACGGTAGCAGAGACTTGGTCTGAAGCAACTGCAACTAGGATGT	312 286
13	Forward: ACACTGACGACATGGTTCTACAACTAGGAGGTTGGGGGCATAG Reverse: TACGGTAGCAGAGACTTGGTCTCAAAAGAAACAGGCATGTAATGC	278