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ARTICLE

Functional analysis of pancreatitis-associated missense mutations in the pancreatic secretory trypsin inhibitor (*SPINK1*) gene

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Variations in the *SPINK1* gene (encoding pancreatic secretory trypsin inhibitor (PSTI)) are associated with chronic pancreatitis. We have recently determined the functional consequences of three missense mutations that occurred within the signal peptide sequence of PSTI by Western blotting analysis of wild-type and mutant PSTI expressed in Chinese hamster ovary cells. Here, this approach was extended to analyze seven missense mutations (p.N34S, p.G48E, p.D50E, p.Y54H, p.P55S, p.R65Q and p.R67C) occurring within the mature peptide of PSTI. This analysis enabled us to classify these missense mutations into three categories. The first category comprises the p.N34S and p.P55S polymorphisms, both of which occur in evolutionarily non-conserved residues, involve amino-acid substitutions with similar physicochemical properties, and do not cause any significant reduction in terms of PSTI mature peptide expression. The second category contains only the p.R65Q missense mutation, which occurs in a well-conserved residue, involves the substitution of a positively charged amino acid by a non-charged one, and causes a ~60% reduction of protein expression. The third category comprises p.G48E, p.D50E, p.Y54H, and p.R67C, all of which occur in strictly conserved residues, involve charged amino acids, and cause complete or nearly complete loss of PSTI expression. Having excluded the possibility that the reduced protein expression may have resulted from reduced transcription or unstable mRNA, we surmise that these missense mutations probably cause intracellular retention of their respective mutant proteins. This is suggestive of a potential unifying pathological mechanism underlying both the signal peptide and mature peptide mutations.
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Introduction

Chronic pancreatitis is a relapsing inflammatory disease of the pancreas. Since the mapping[1–3] and cloning[4] of one gene for hereditary pancreatitis (MIM no. 167800) in 1996, four lines of complementary evidence have pointed to the pivotal role of a *gain* of trypsin within the pancreas in causing the disease. First, 'gain of function' missense mutations such as the three activation peptide mutations

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(ie p.D19A, p.D22G and p.K23R),[5] p.N29I/T[6] and p.R122H[7] in the cationic trypsinogen gene (*PRSS1*; MIM no. 276 000) have been found to cause chronic pancreatitis (for a recent review, see Teich *et al*[8]). Second, 'loss of function' *PRSS1* mutations[9] and a degradation-sensitive *PRSS2* (encoding anionic trypsinogen, the second major isoform of trypsinogen (for a recent review on human trypsinogen family, see Chen and Ferec[10]); MIM no. 601564) variant (ie p.G191R)[11] have been shown to act as protective factors against the disease. Third, triplication of the trypsinogen locus on chromosome 7, which appears to cause a *gain* of trypsin through a gene dosage effect, has recently been detected in five French families with hereditary pancreatitis.[12] Last, a diverse range of variations including apparent 'loss of function' mutations (ie c.2T>C (p.M1?),[13] c.98_99insA,[14] c.27delC,[15] c.87+1G>A,[15] c.194+2T>C,[13] and a large genomic deletion[16]) in the *SPINK1* gene (encoding pancreatic secretory trypsin inhibitor (PSTI); MIM no. 167 790) have also been identified in subjects with chronic pancreatitis (for an up to date list of variations, see http://www.uni-leipzig.de/pancreasmutation/db.html). The above notwithstanding, a *loss* of trypsin was also hypothesized to cause chronic pancreatitis, based upon the biochemical characterization of the R122C missense mutation.[17] However, as already pointed out by Teich *et al,*[8] no evidence exists to support this hypothesis from a genetic point of view, and clear 'loss of function' *PRSS1* mutations (eg nonsense, splice site, or frameshift) have never been reported to cause the disease. In addition, although the E79K missense mutation was found to result in decreased autoactivation of cationic trypsinogen, E79K-trypsin proved to cause an increased transactivation of anionic trypsinogen.[18]

As encountered in many disease-associated genes, a major challenge is interpretation of the identified missense mutations in terms of their likely clinical consequences, especially when they are found to be rare or even 'private'.[19] In this regard, most of the currently reported *PRSS1* and *PRSS2* missense mutations have been functionally analyzed *in vitro* (ie p.A16V, p.D19A, p.D22G, p.K23R, p.N29I, p.N29T, p.E79K, p.R122H, and p.R122C in *PRSS1*; p.G191R in *PRSS2*; see http://www.uni-leipzig.de/pancreasmutation/db.html for references). By contrast, of the nine *SPINK1* missense mutations currently reported in the literature (http://www.uni-leipzig.de/pancreasmutation/db.html; as of 14 December 2006), only the p.N34S and p.R67C have been subjected to *in vitro* analysis, using recombinant proteins expressed in non-mammalian cells.[20,21] Very recently, we have successfully expressed the *SPINK1* gene in two mammalian cell lines and analyzed three missense mutations that have occurred within the signal peptide of PSTI.[22] This study went further in analyzing a total of seven missense mutations (including one novel one) that have occurred in the mature peptide of PSTI, using one of the above-mentioned model systems.

Materials and methods

Mutation detection

Mutation screening for single-nucleotide substitutions or micro-deletions/-insertions in subjects with chronic pancreatitis was performed using our previously established denaturing high-performance liquid chromatography (DHPLC) technique.[15]

Mutation nomenclature

The newly found missense mutation was named in accordance with the standard nomenclature guidelines proposed by the Human Genome Variation Society (http://www.hgvs.org/)[23] in the context of reference sequence NM_003122.2.

Plasmid construction

The *SPINK1* cDNA sequence was amplified from the clone IMGCLO5225693 (http://www.geneservice.co.uk/public-cgi/Tools/ProductInfo?prod_id=IMGCLO5225693) using forward primer 5′-GCCATGAAGGTAACAGGCAT-3′ and reverse primer 5′-GTTCTCAGCAAGGCCCAGAT-3′. PCR was performed in a 50 μl reaction mixture containing 250 μM each dNTP, 2 mM $MgCl_2$, 1 U AmpliTaq DNA polymerase (PE Applied Biosystems, Foster City, CA, USA), and 1 μM each primer. The PCR program consisted of an initial denaturation at 95°C for 3 min, followed by 35 cycles denaturation at 95°C for 30 s, annealing at 58°C for 30 s, and extension at 72°C for 30 s. The PCR product was purified using Qiaquick Gel Extraction Kit (Qiagen, Courtabœuf, France). After addition of 3′-A overhangs, the PCR product was cloned into pcDNA 3.1/V5-His-TOPO (Invitrogen, Cergy-Pontoise, France). Missense mutations were introduced by directed mutagenesis, using a Quick Change Site Directed Mutagenesis Kit (Stratagene, Amsterdam, Netherlands).

Cell culture and transfection

Chinese hamster ovary (CHO) cells were maintained in F-12K nutrient mixture (Kaighn's Modification), supplemented with 10% fetal calf serum (Cambrex, Verviers, Belgium) and 1% penicillin/streptomycin. Transfection was performed using Lipofectamine 2000 Reagent (Invitrogen) in accordance with the manufacturer's instructions.

Western blotting

Conditioned media from transfected cells were collected and filtered on Centricon Ultracel YM3 (Millipore, Molsheim, France). The concentrated samples were diluted with 20 μl buffer containing protease inhibitors. A sample containing 80 μg protein was used for electrophoresis on a 16% SDS–PAGE gel and transferred onto a PVDF membrane. The membrane was blocked with 5% milk, 0.1% Tween overnight and then hybridized with anti-hPSTI antibody/monoclonal mouse IgG (MoBiTec, Göttingen, Germany) at a dilution of 1:250 for 2 h at room tempera-

ture. After incubation with the second antibody ECL Anti-mouse IgG, peroxidase/linked species-specific whole antibody (Amersham Bioscience, Orsay, France) at a dilution of 1:10 000 for 2 h, the blot was revealed using the ECL Plus Western Blotting Detection Reagents (Amersham). The film was photographed with the Infinity Capt software (Vilbert-Lourmat, Torcy, France); each band was quantified with the BIO 1D++ software (Vilbert-Lourmat). In the meantime, the membrane was stained with Coomassie blue and each lane was quantified in the same way as above; this was used to normalize protein loading between different samples. At least three independent transfections/Western blotting were performed for each mutant.

Quantitative RT-PCR

Total RNA was extracted from transfected cells using the RNeasy Mini Kit (Qiagen). The quality of the extracted RNA was controlled by calculating the ratio of 28S/18S with the RNA 6000 Nano Kit (Agilent, Santa Clara, CA, USA). The concentration and purity of RNA was determined by measuring OD at 260 and 280 nm. About 500 ng of RNA was used for reverse transcription, by means of the Reverse Transcriptase Kit (Qiagen). Real-time quantitative RT-PCR was performed in accordance with the method of Livak and Schmittgen.[24] Briefly, while the expression of the target *SPINK1* gene was measured by RT-PCR using forward primer 5′-GCTGAGAACAAGGGCAATTC-3′ (spanning the TGA stop codon of the cloned *SPINK1* cDNA) and reverse primer 5′-AGACCGAGGAGAGGGTTAGG-3′ (located 5′ to the polyadenylation signal AATAAA in the vector), the expression of the reference neomycin gene (also located within the expression vector) was measured using forward primer 5′-ATGACTGGGCACAACAGACA-3′ and reverse primer 5′-CTCGTCCTGCAGTTCATTCA-3′. RT-PCR was performed in a 50 μl reaction mixture containing 2 × Quantitect SYBR Green Master MIX, 0.3 μM of each primer, and 5 μl of template DNA derived from the above reverse transcription reaction, employing the Quantitect Sybr Green PCR Kit (Qiagen). The PCR program consisted of an initial denaturation at 95°C for 15 min, followed by 45 cycles denaturation at 94°C for 15 s, annealing at 55°C for 30 s, and extension at 72°C for 30 s. For each mutant, quantitative RT-PCR was performed in parallel with Western blotting from three independent transfections. Data were read in Chromo4 (BIORAD, Marnes-la-coquette, France) and the mean fold-change in the expression level of the mutant in relation to the wild-type molecule was calculated as described previously.[24]

Statistical analysis

Both mRNA and protein expression levels of each mutant in relation to the wild-type PSTI was compared with Student's *t*-test. A *P*-value of lower than 0.05 was considered as significant.

Results and discussion

Identification of a novel missense mutation that occurred within the mature peptide of PSTI

As part of our routine analysis to identify pancreatitis-associated variations in the *SPINK1* gene using DHPLC,[15] we found a heterozygous G>A transition at position c.143 (cDNA-based numbering with the A of the ATG translational initiation codon as +1 in the context of NM_003122.2) in a 9-year-old French boy. This point mutation, which is predicted to result in a substitution of glycine by glutamic acid at amino acid position 48 (p.G48E; Figure 1), has not been reported previously. The boy has suffered from several attacks of acute pancreatitis since he was 3-years old, but has not developed chronic pancreatitis to date. As shown in Figure 1, the patient is a compound heterozygote for two *SPINK1* variations: while the p.N34S polymorphism[25] was inherited from the asymptomatic father, the p.G48E mutation (absent in 100 control chromosomes) was inherited from the asymptomatic mother. As demonstrated below, expression of the p.G48E mutant was not detectable by Western blotting.

Functional analysis of missense mutations that have occurred within the mature peptide of PSTI

Before this work was initiated, six missense mutations located within the mature peptide of PSTI had been reported in the literature (Figure 2): p.N34S is a polymorphism[25] but its frequency is increased in subjects with chronic pancreatitis (see Kiraly *et al*[22] and references therein); p.P55S is also a polymorphism but appears not to be disease-associated.[25,26] By contrast, all four remaining missense mutations (ie p.D50E,[26] p.Y54H,[27] p.R65Q,[28] and p.R67C[29,30]) were found in isolated patients or families. Of the six variations, only p.N34S and p.R67C were subjected to functional characterization, whereas the former one was shown to be fully active,[20] the latter was suggested to significantly affect PSTI's conformation.[21] However, it should be emphasized that both studies were performed in non-mammalian expression systems. Obviously, there is an urgent need to functionally characterize these missense mutations, ideally in a mammalian expression system, with a view to clarify their possible role in the etiology of pancreatitis.

Very recently, through a close collaboration with Sahin-Toth's group, we have elucidated the functional consequences of three missense mutations that have occurred within the signal peptide sequence of PSTI (ie p.L12F, p.L14R, and p.L14P; see also Figure 2) in two mammalian expression model systems, in which comparable results were independently obtained.[22] In this study, we further analyzed the above-mentioned six mature peptide missense mutations as well as the newly found p.G48E in the CHO expression system. Interestingly, functional consequences of these missense

mutations as determined by the relative expression level of each mutant peptide in relation to the wild-type peptide correlate well with the conservation status of the involved residues in the context of the mammalian PSTI family (Figures 2, 3a and b). On the basis of this observation, the seven missense mutations can be divided into three categories. The first category consists of the p.N34S and p.P55S polymorphisms, both of which occur in non-conservative residues, involve amino-acid substitutions with similar physicochemical properties and cause no significant reduction in terms of PSTI mature peptide expression. The second category comprises only the p.R65Q missense mutation, which occurs in a well-conserved residue, involves the substitution of a positively charged amino acid by a non-charged one, and causes about 60% reduction of protein expression. The third category comprises p.G48E, p.D50E, p.Y54H and p.R67C, all of which occur in strictly conserved residues, involve charged amino acids, and cause complete or nearly complete loss of PSTI expression (Figure 3a and b).

To exclude the possibility that the reduced protein expression may have resulted from reduced transcription or unstable mRNA, we performed quantitative RT-PCR analysis of RNA prepared from transfected cells in parallel with the Western blotting analysis. As shown in Figure 3c, except for p.P55S, comparable mRNA levels expressed from the wild type and the p.N34S, G48E, D50E, Y54H, p.R65Q and p.R67C mutants were obtained. The reason for the slightly reduced mRNA expression of the p.P55S mutant in relation to the wild-type molecule is unclear. Nevertheless, this does not represent a major issue, given that p.P55S is a neutral variant and no apparent reduction in protein expression was observed. In addition, the reduced expression occurred at the level of translation also seems very unlikely given that these missense mutations are far downstream of the translational initiation codon. Therefore, the reduced expression associated with the p.G48E, p.D50E, p.Y54H, p.R65Q and p.R67C mutants, as determined by Western blotting, must have occurred post-translationally. The above notwithstanding, several issues warrant further clarification.

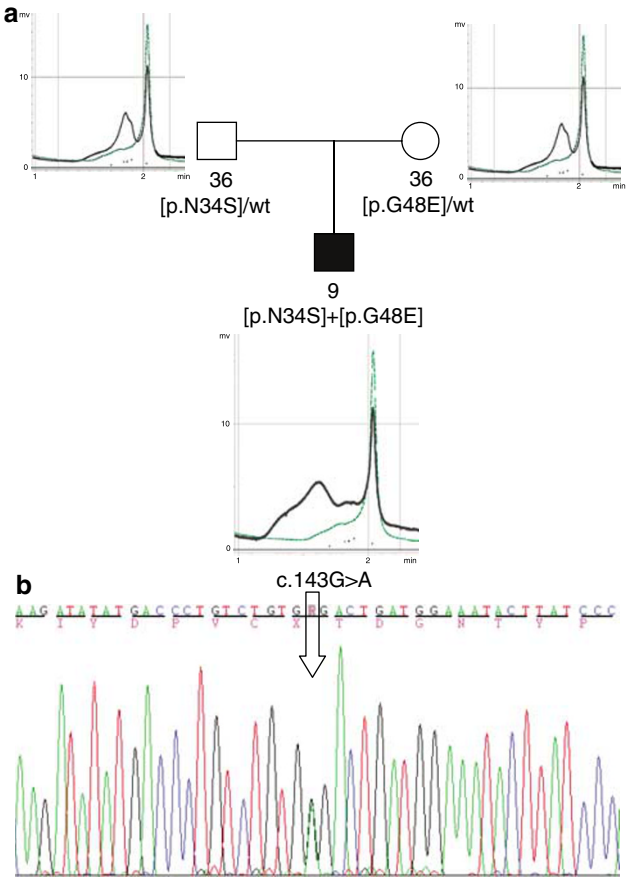

Figure 1 Identification of a novel single-nucleotide substitution, c.143G>A, in exon 3 of the *SPINK1* gene, in a 9-year-old boy with acute pancreatitis. (**a**) DHPLC profiles of the index patient and his asymptomatic patients (black line) as well as a normal control (green dotted line). Note the different DHPLC patterns in the three subjects. Current age and genotype for each subject are also indicated. (**b**) Direct sequencing showing the c.143G>A point mutation (indicated by a vertical arrow) that would result in a p.Gly48Glu missense mutation.

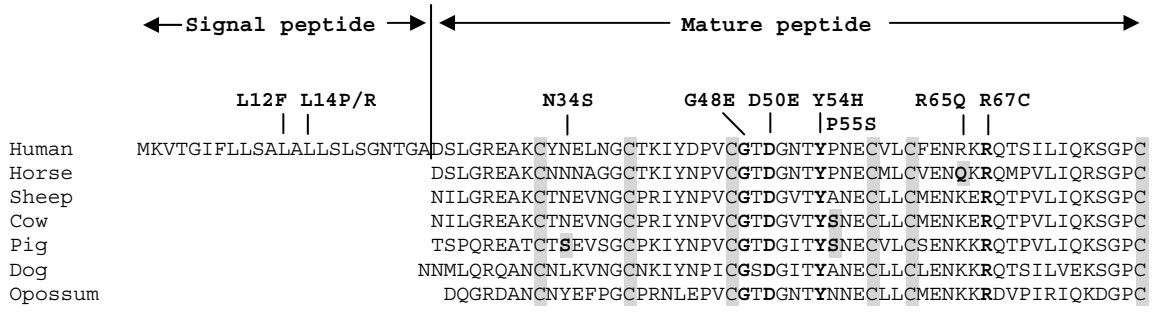

Figure 2 Survey of *SPINK1* missense mutations in the context of the mammalian PSTI family. Signal peptide: sequence is not available for horse, sheep, cow, pig, dog and opossum. The three signal peptide missense mutations – all functionally analyzed in a recent study[22] are indicated. Mature peptide: all the missense mutations (note that p.G48E is a novel one) analyzed in this study are positioned within the sequence. The strictly conserved six cysteine residues forming three intra-chain disulfide bridges are shaded in grey.

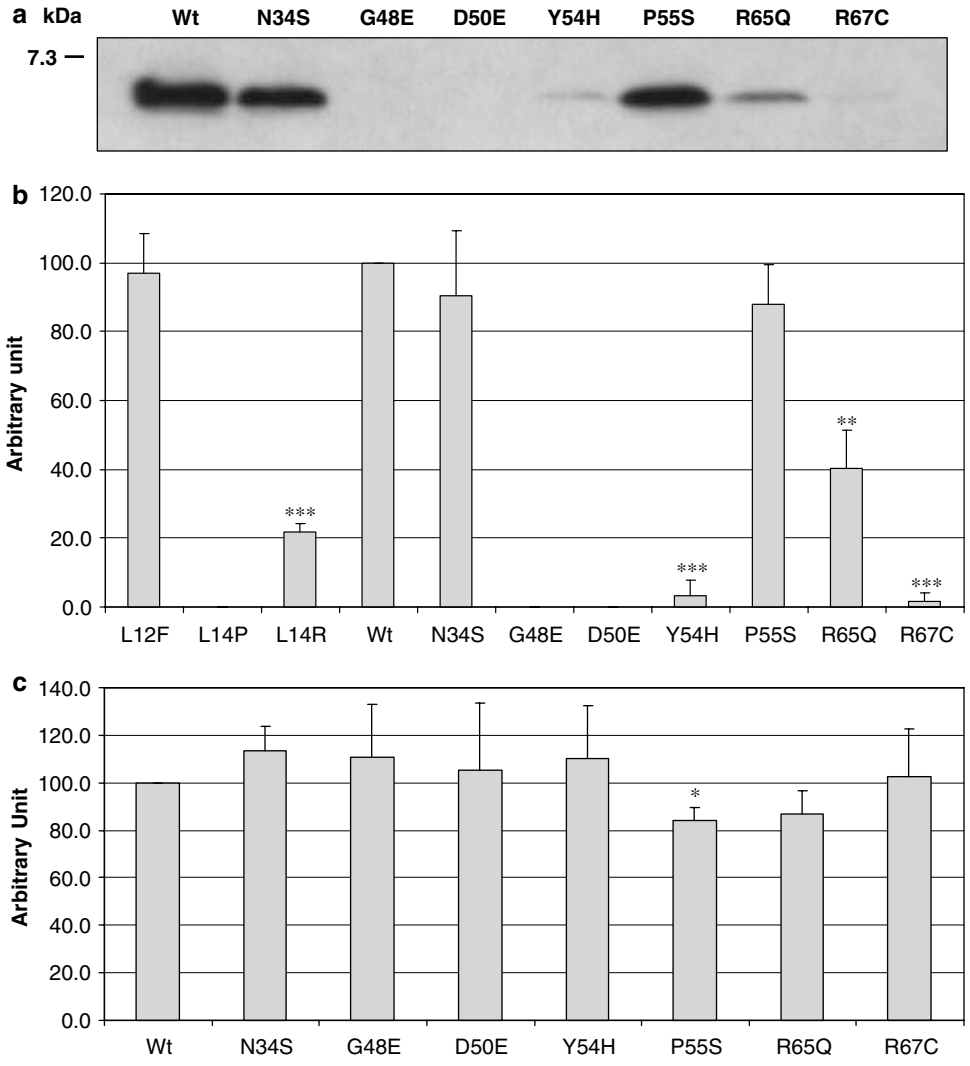

Figure 3 Functional analysis of seven missense mutations that have occurred within the mature peptide of PSTI. (**a**) A representative Western blot. (**b**) Mean expression level (column) of each mutant in relation to the wild-type peptide, as determined by at least three independent transfection/Western blotting analyzes. Bars, SD; $^{**}P<0.01$; $^{***}P<0.001$. Note that results obtained from the three signal peptide missense mutations (ie p.L12F, p.L14P and p.L14R)[22] were also included for comparison. (**c**) Mean mRNA expression level (column) of each mutant in relation to the wild-type molecule, as determined by quantitative RT-PCRs performed in parallel with three independent transfection/Western blotting analyzes. Bars, SD; $^{*}P<0.05$.

Can the constitutive secretory pathway of CHO cells adequately model the regulated secretory pathway of human pancreatic acinar cells?

This issue has been addressed in our previous study.[22] Here, we mention three points: (i) the CHO cell line is one of the most widely used model systems to characterize human secretory proteins (eg see two recent publications[31,32]), (ii) Western blotting analysis of the three missense mutations (ie p.L12F, p.L14P and p.L14R) that have occurred within the signal peptide of PSTI in the CHO line, yielded similar results to those obtained independently in the human embryonic kidney 293T cell line[22] and (iii) that the N34S polymorphism did not cause any significant reduction of PSTI expression in the CHO cells concurred with the analysis using *Saccharomyces cerevisiae* BJ1991 as a model system.[20]

What is the mechanism underlying the significantly reduced or abolished expression of PSTI in the p.G48E, p.D50E, p.Y54H, p.R65Q, and p.R67C mutants?

This could be explained by two different mechanisms. Since PSTI is a secretory protein, these missense mutations may have resulted in the intracellular retention of their respective mutant proteins. This is an attractive mechanism because it points to a potential unifying pathological basis underlying both the signal peptide and mature

peptide mutations. There are precedents for such a mechanism, best exemplified by the multiple *RS1* missense mutations causing X-linked retinoshisis;[33,34] and the intracellular retention of the functional mutants was attributable to large conformational changes in the protein.[33] In this regard, it is important to reiterate that all five missense mutations that caused a significantly reduced or complete loss of PSTI expression (Figure 3a and b) always occurred in well or strictly conserved residues and often involved radical amino acid changes (Figure 2); this is highly suggestive of significant conformational changes in the PSTI mutants. Unfortunately, this postulate cannot be experimentally tested since the anti-PSTI antibody used in this study is not suitable for confocal fluorescent immunocytochemistry. Alternatively, the 'reduced expression' may result from reduced or lack of binding activity of the mutant protein to the anti-hPSTI antibody. However, since the antibody we used for Western blotting is monoclonal, this alternative mechanism seems unlikely to serve as a common basis underlying all five functional missense mutations. Nevertheless, irrespective of the exact underlying mechanism, the significantly reduced expression of the five mutants as compared with the wild-type PSTI as well as the two polymorphic mutants (Figure 3a and b), can be reasonably attributed to dramatic conformational changes in the mutant proteins. Additional analysis such as trypsin inhibitor activity determination[22] would help to clarify this issue.

How to interpret the obtained functional analytic data in terms of the mutants' likely clinical consequences?

On the basis of a combined consideration of all the current available genetic, evolutionary and functional analytical data, it is not unreasonable to conclude that the p.N34S and p.P55S polymorphisms appear to make a very minor (if any) contribution to the etiology of pancreatitis. Here, one may argue that p.N34S is nevertheless by far the most frequent pancreatitis-associated *SPINK1* variant. In this regard, it is important to note that the p.N34S polymorphism is in complete linkage disequilibrium with two intronic variants;[13,35] and the IVS3-66_-65insT4 variant, 'which changes a T5 sequence to a T9 sequence near the end of intron 3 of the *SPINK1* gene, is interesting, even it is not within the immediate splicing site as the IVS8-Tn variant of the cystic fibrosis transmembrane conductance regulator gene'.[35] In short, it is possible that the p.N34S polymorphism simply represents a marker of a truly functional variant. (Note that while the association between 'N34S' and chronic pancreatitis has been firmly established, the presence of 'N34S' in patients carrying a known *PRSS1* mutation appeared to affect neither the disease phenotype nor its penetrance.[36] More studies are warranted to clarify this issue.) With respect to the five missense mutations shown to be functional in the current study, again on the basis of a combined consideration of all the current available data, we believe that most, if not all, of them can be safely interpreted as pancreatitis-predisposing genetic factors. Note that this conclusion does not, by any means, exclude the participation of any other genetic and environment factors in causing the disease.

In summary, in addition to the identification of a novel pancreatitis-associated missense mutation in the *SPINK1* gene, we have performed the first functional characterization of missense mutations that have occurred within the mature peptide of PSTI in a mammalian expression system. This work, as well as our previous work, not only serves to determine the functionality of the naturally occurring missense mutations in the *SPINK1* gene, but also suggests a potential unifying pathological mechanism – a disturbance of the intracellular transportation of PSTI – underlying both the signal peptide and mature peptide mutations.

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