ORIGINAL ARTICLE

Novel cationic trypsinogen (PRSS1) N29T and R122C mutations cause autosomal dominant hereditary pancreatitis

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Background and aims: Hereditary pancreatitis (HP) is caused by mutations R122H or N29I in the cationic trypsinogen (PRSS1) gene in 60–75% of HP families but the cause of autosomal dominantly inherited pancreatitis in other families is unknown. Our aim was to identify additional disease associated mutations in HP families.

Methods: Over 150 unique families were recruited through the Midwest Multicenter Pancreatic Study Group (MMPSG) and 101 families were recruited through the European Registry of Hereditary Pancreatitis and Familial Pancreatic Cancer (EUROPAC). The proband from each family was initially screened for the common cationic trypsinogen mutations (PRSSI gene): R122H, N29I, K23R, and A16V. If one of the four common mutations was not identified, the entire PRSSI gene was sequenced.

Results: Two novel mutations were detected in two independent families resulting in R122C and N29T amino acid substitutions. The mutations appear to segregate with the disease in an autosomal dominant fashion. The R122C mutation exhibited incomplete penetrance while penetrance in the N29T family could not be determined. The restriction fragment length polymorphism screening test for the R122H mutation using *AflIII* failed to detect the novel R122C mutation.

Conclusions: Mutations in codons 29 and 122 of the PRSS1 gene are central to the pathogenesis of HP. The R122C mutation eliminates the arginine autolysis site, as does the R122H mutation. The N29T mutation may also enhance intrapancreatic trypsin activity as has been demonstrated in vitro. Identification of these new mutations requires special attention as commonly used detection methods may fail.

Hereditary pancreatitis (HP) is a genetic disorder of the pancreas characterised by multiple episodes of acute pancreatitis, development of chronic pancreatitis, and high incidence of pancreatic cancer.[1 2] The classic HP disorder follows an autosomal dominant mendelian inheritance pattern with a penetrance rate of approximately 80%.[1 3] Genetic linkage studies[4 5] and candidate gene analysis[6] revealed an R122H substitution mutation in the cationic trypsinogen gene (PRSS1) which was originally reported as R117H using the chymotrypsinogen numbering system (chy No 117).[7] Mechanistic and molecular modelling suggest that this mutation confers gain of function characteristics to the mutated cationic trypsinogen by eliminating a "fail safe" inhibitory mechanism that destroys trypsin when the trypsinogen molecule is prematurely activated.[3 6] A second PRSS1 mutation, N29I (also reported as N21I[7 8]), was also identified with nearly identical clinical features to the R122H mutation.[8–10] To date, molecular screening of over 300 families with a characteristic autosomal dominant pattern of HP reveals the presence of the cationic trypsinogen R122H or N29I in the majority. However, significant heterogeneity exists in that neither the R122H nor the N29I mutation in the cationic trypsinogen gene can be found in about 30–40% of families.

Additional PRSS1 mutations appear in patients with hereditary or idiopathic chronic pancreatitis. These include the A16V,[11–13] D22G,[14] K23R and –28delTCC[15] mutations clustered in the activation peptide region and 5′ untranslated region of the gene. These mutations may also cause gain of function changes with early activation of trypsin,[14] but they do not necessarily confer the clinical phenotype of autosomal dominant inheritance with high penetrance rate.[11] Recently, Chen and colleagues[16] reported mutational screening of the PRSS1 gene in a large cohort with idiopathic chronic pancreatitis. Single subjects were identified with P36R, E79K, 683E, K92N, and V123M missense mutations. However, it is yet to be determined if these are disease causing or disease enhancing mutations.

In 1995, the Midwest Multicenter Pancreatic Study Group (MMPSG) began collecting families with inherited diseases of the pancreas as part of the University of Pittsburgh's Hereditary Pancreatitis study.[5] An analogous study, the European Registry of Hereditary Pancreatitis and Familial Pancreatic Cancer (EUROPAC) has been ascertaining similar families in Europe. Both groups have been investigating trypsinogen related genes through DNA sequencing efforts. Herein we report the findings of two novel mutations associated with an autosomal dominant inheritance pattern characteristic of HP.

MATERIALS AND METHODS

Patient identification and ascertainment

Patients and family members were recruited for genetic analysis through the HP study initiated by the MMPSG and EUROPAC. Recruitment, consent, and counselling were as previously described.[5 17] Over 150 unique families have been recruited by the MMPSG and over 100 independent families have been recruited by EUROPAC. A broad delineation of HP

Abbreviations: HP, hereditary pancreatitis; MMPSG, Midwest Multicenter Pancreatic Study Group; EUROPAC, European Registry of Hereditary Pancreatitis and Familial Pancreatic Cancer; RFLP, restriction fragment length polymorphism; PCR, polymerase chain reaction.

Table 1 Polymerase chain reaction (PCR) primers, PCR reaction conditions, and sequencing primers used for amplification and sequencing of the PRSS1 gene

Exon	PCR primers	Product size (bp)	Reaction conditions	Sequencing primers
1	5′-GAA CCT ATG ACA GGA TGC AC-3′ 5′-TGA AGT CAA GGA GAA GGT GA-3′	1018	94°C 15 s 54°C 30 s 72°C 60 s	5′-CCT CAC AGT CAC CTC CTC TCT G-3′ 5′-TAT GCC AGA TGG AGG AAA CG-3′ 5′-CAT CAG CCT GGC TAT GTT TG-3′
2	5′-TGT GAG GAC ATT CCT TGC GA -3′ 5′- TCT TCC TGA AAA TTT TGA CT -3′	805	94°C 10 s 48°C 30 s 72°C 60 s	5′- CGC CAC CCC TAA CAT GCT ATT G -3′ 5′- CCA TCT TAC CCA ACC TCA GTA G -3′
3	5′- GGT CCT GGG TCT CAT ACC TT -3′ 5′- GGG TAG GAG GCT TCA CAC TT -3′	911	94°C 30 s 61°C 30 s 72°C 60 s	5′- TCT CCA TTT GTC CTG TCT CT -3′ 5′- TGA CCC ACA TCC CTC TGC TG -3′
4	5′-GCA CCA GAG AGA TGC AAA CTA-3′ —*	1133	94°C 15 s 52°C 30 s	5′-AGC CCC ACC ACC TTT TGA GTT-3′ 5′-GGG CTG TGT TCC TCT TCA GTT-3′
5	—* 5′-CCT TTC TGA AAC AGG TAT CT-3′		72°C 60 s	5′-TCC AGT GTG AAG GAG TGA GAG-3′ 5′-GAA CAG AGA ATG GGC CAC CAT-3′

*Exon 4 and 5 were amplified in one PCR product.

was used by the MMPSG to include proband individuals with any family history of pancreatitis or patients with a strong clinical suspicion since family history alone is a poor predictor of the R122H mutation.[18] Among the 159 MMPSG families with complete genetic analysis at the time of this publication, 115 kindreds had a strong family history of pancreatitis and 44 families contained a sporadic case of pancreatitis. In the familial/hereditary group, 54 of 115 families tested negative for all known disease causing PRSSI mutations. In the sporadic group, 42 of 44 families tested negative for all known disease causing PRSS1mutations. A total of 101 of the family units from EUROPAC had multiple members affected with pancreatitis and 28 of these families had no detectable mutation in the PRSS1 gene.

DNA extraction

DNA was isolated from whole blood using the PureGene DNA Isolation Kit (Gentra Systems, Minneapolis, Minnesota, USA), diluted to 50 µg/ml, given a unique identifier number, and stored using an honest broker system to ensure patient confidentiality.

DNA sequencing

DNA fragments of the five exons of PRSS1 were amplified and sequenced using the polymerase chain reaction (PCR) primers, PCR reaction conditions, and sequencing primers shown in table 1. PCR was performed using 50 ng of genomic DNA template, 1.25 U Taq DNA polymerase (Life Technologies, Inc., Rockville, Maryland, USA), 1.5 mM $MgCl_2$, 200 µM each dNTP, and 250 nM of each primer in a 50 µl reaction volume. Thirty cycles of PCR were performed. The PCR products were purified with QiaQuick 96 PCR purification kits (Qiagen Inc., Valencia, California, USA). Cycle sequencing was performed using the ABI Prism Big Dye Terminator Cycle Sequencing kit (PE Biosystems, Foster City, California, USA). Sequencing reactions were 100 ng template, 3.2 pM of primer, and 4 µl of Cycle Sequencing premix in a 10 µl reaction. Reaction products were purified using a standard ethanol precipitation protocol following the manufacturer's recommendations, resuspended in deionised H_2O and run on an ABI Prism 3700 DNA Analyzer (PE Biosystems, Foster City, California, USA). Sequence analysis was performed using Sequencher 3.1 (GeneCodes Corp., Ann Arbor, Minnesota, USA). Possible mutations were verified by resequencing the opposite DNA strand.

Restriction fragment length polymorphism (RFLP) analysis

DNA from affected individuals with new mutations were screened with specific restriction enzymes previously demonstrated to be useful in identifying the R122H[6] and N29I mutations. For codon 29, RFLP analysis was performed using a previously unpublished method using *Bst4CI* (SibEnzyme Ltd, Novosibirsk-117, 630117, Russia). The wild-type PCR product has three recognition sites for *Bst4CI*, with four digestion products of 415, 160, 151, and 79 bp. A mutation at position 131945 causes loss of one ACNGT recognition site so that a mutant allele has three digestion products of 415, 230, and 151 bp. Restriction endonuclease digestion was performed using 5 µl of PCR product, 3 units of *Bst4CI*, 0.2 µl of bovine serum albumin (New England BioLabs, Beverly, Massachusetts, USA), and 2 µl of *Bst4CI* buffer (SibEnzyme Ltd) in a 20 µl reaction. Digestion was performed at 65°C for two hours. Fragments were separated on a 2% agarose gel and imaged after ethidium bromide staining.

For codon 122, RFLP analysis was performed using *AflIII* restriction endonuclease digestion as described previously.[6]

Genotype and phenotype correlation

Families with newly identified mutations in the proband were re-contacted to invite additional relatives to participate in the study. All affected participants without evidence of one of the previously reported mutations on initial screening were studied by direct sequencing of the cationic trypsinogen gene. Genotypes and phenotypes were compared with ascertain penetrance and inheritance patterns.

RESULTS

Two novel PRSS1 mutations were identified in two independent families, each resulting in an amino acid change in codons 29 and 122, respectively.

Pedigree 1 (fig 1)

Family characteristics

The proband was a 25 year old Caucasian woman from the USA diagnosed with pancreatitis at age 18 years but reported similar symptoms of recurrent abdominal pain and nausea

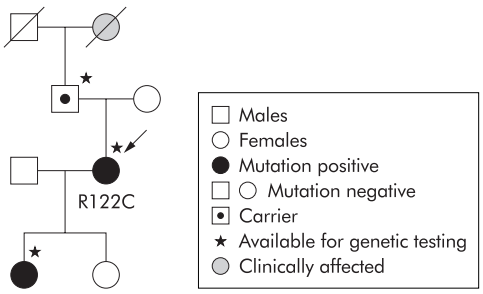

Figure 1 Pedigree of the family with a R122C mutation. The arrow points to the index case.

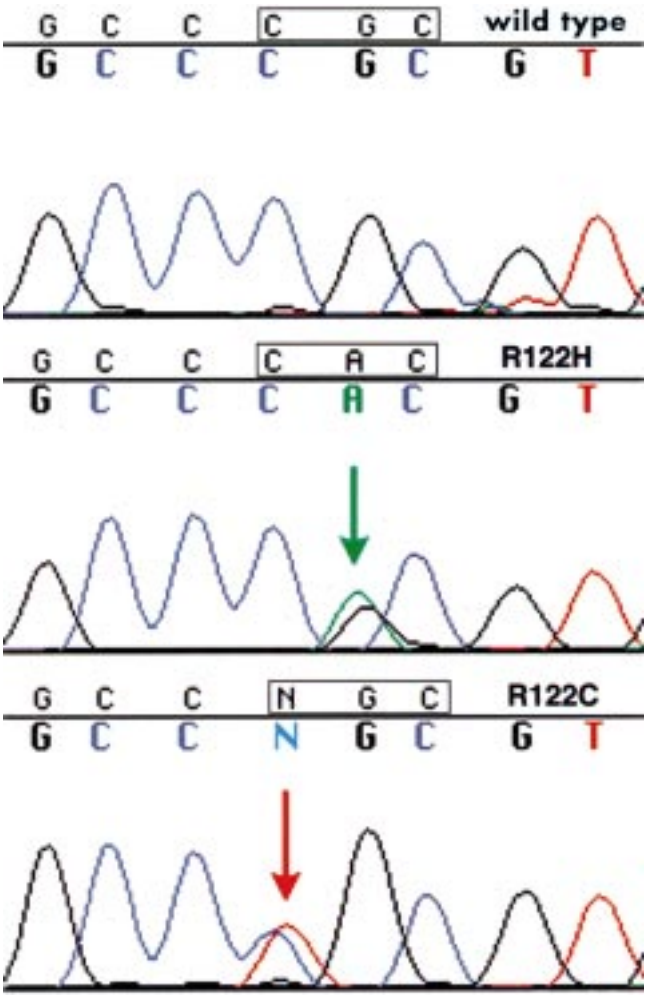

Figure 2 C>T transition mutation at position 133282 (Genbank accession U66061), resulting in a R122C amino acid substitution. A mutation negative sample (top), the R122C mutation (middle), and the R122H mutation (bottom) are shown.

beginning at age five years. Her parents had no history of pancreatitis or unexplained abdominal pain. The deceased paternal grandmother was diagnosed with chronic pancreatitis at the age of 34 years. One of the two daughters of the index patient had a single episode of pancreatitis at the age of five years.

Sequencing data

Direct sequencing of exon 3 of PRSS1 revealed a C to T transition mutation at position 133282 of the sequence (Genbank accession U66061), resulting in a R122C amino acid substitution (fig 2). The presence of the R122C mutation was investigated in all remaining MMPSG and EUROPAC families without a previously detected PRSS1 mutation. This mutation was not found in any of the remaining 54 MMPSG families or 28 EUROPAC families with HP, nor was this mutation found in any of the remaining 42 MMPSG families with a sporadic case of pancreatitis or in 130 healthy controls.

The mutation was also found in the affected daughter of the patient. A sample of the unaffected sibling was not available for testing. The phenotypically unaffected father of the patient also carried the mutation.

RFLP analysis

Figure 3 shows the results of restriction endonuclease digestion with *AflIII*. While *AflIII* digestion of a positive control for the known R122H mutation with a G to A transition mutation at position 133283 results in three bands, a wild-type control and the sample with the R122C mutation show no cleavage. Thus the *AflIII* digestion fails to detect the novel R122C mutation.

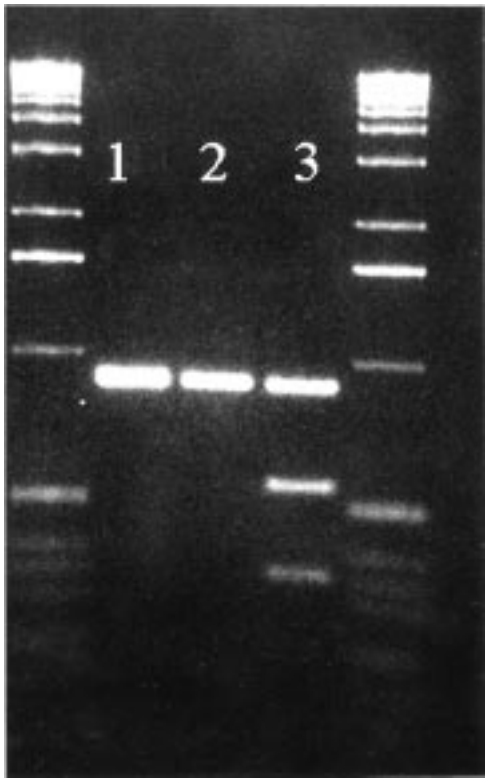

Figure 3 *AflIII* digestion of the 911 bp product resulting in two products of 565 and 346 bp in a R122H positive control (lane 3). No cleavage is seen in a healthy control (lane 1) or the index patient with the R122C mutation (lane 2). Lanes 0 and 4 are 1 kb ladder bands.

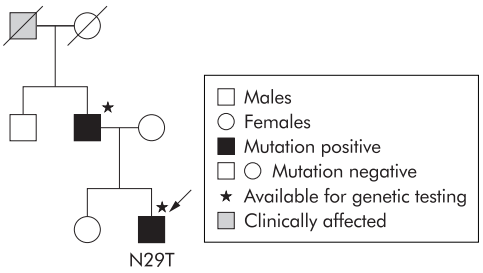

Figure 4 Pedigree of the family with a N29T mutation. The arrow points to the index case.

Pedigree 2 (fig 4)

Family characteristics

The proband was a 23 year old Caucasian male from Ireland who reported a history of recurrent abdominal pain since infancy, with more than 20 episodes of pancreatitis. His 70 year old father also had recurrent abdominal pain starting in infancy; he had developed insulin dependent diabetes mellitus and signs of malabsorption in his late twenties. The deceased grandfather of the patient reportedly also had symptoms of pancreatitis. In 1984 the diagnosis of autosomal dominant pattern HP was made based on family history. The other family members showed no signs of pancreatitis.

Sequencing data

Direct sequencing of exon 2 of PRSS1 revealed an A to C transition mutation at position 131945 of the sequence (Genbank accession U66061), resulting in a N29T amino acid substitution of cationic trypsinogen (fig 5). The N29T mutation was found in the proband and his affected father. No further relatives were available for mutational analysis. As seen with the

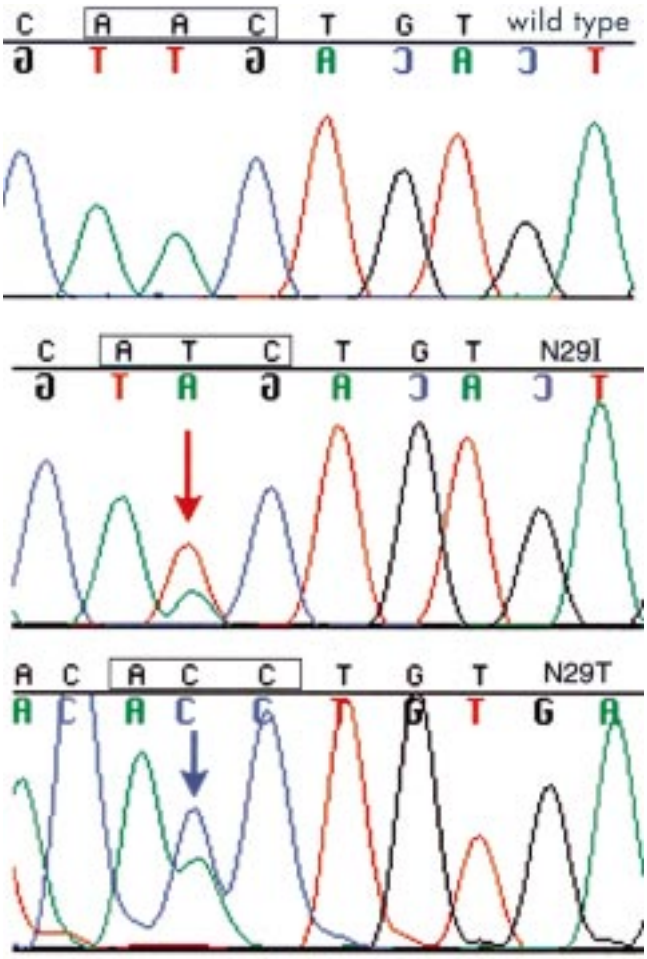

Figure 5 A to C transition mutation at position 131945, resulting in a N29T amino acid substitution of cationic trypsinogen. The novel N29T mutation (top), the N29I mutation (middle), and a mutation negative sample (bottom) are shown.

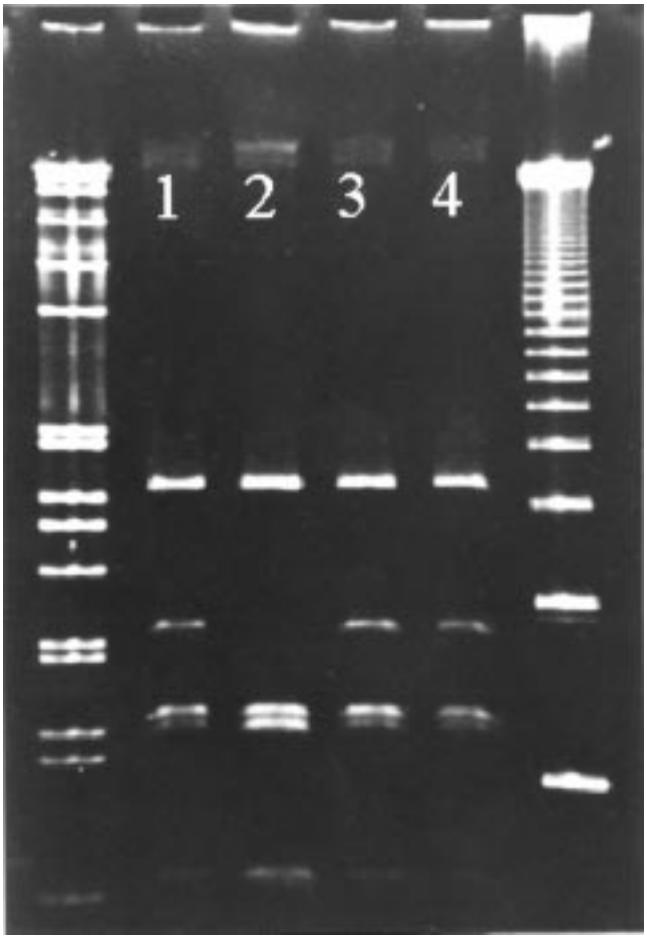

Figure 6 Restriction endonuclease digestion with *Bst4CI*, resulting in cleavage in four fragments of 79, 151, 160, and 415 bp in the wild-type (lane 2), and an additional band of 230 bp in the N29I (lane 1) and N29T (lanes 3 and 4) heterozygous samples, representing the combined products of 79 and 151 bp due to loss of one restriction site.

R122C mutation, the N29T mutation was not found in any of the remaining 54 MMPSG families or 28 EUROPAC families with HP, nor was this mutation found in any of the remaining 42 MMPSG families with a sporadic case of pancreatitis or in 130 healthy controls.

RFLP analysis

Restriction endonuclease digestion with *Bst4CI* resulted in cleavage in four fragments of 79, 151, 160, and 415 bp in the wild-type. In the N29T and N29I mutants, an additional band of 230 bp was observed, representing the combined products of 79 and 151 bp due to loss of one restriction site (fig 6). Thus *Bst4CI* detects both of the known codon 29 mutations.

DISCUSSION

Our results demonstrate two novel mutations in the PRSS1 gene that were associated with an autosomal dominant pattern of HP. Remarkably, both novel mutations alter codons where previously gain of function mutations associated with an autosomal dominant inheritance pattern were found. While other mutations associated with pancreatitis may also cause a gain of function or early activation efforts through different mechanisms, it appears that codons 29 and 122 are hot spots for activation and/or inactivation of the PRSS1 gene. These findings are also important in that previously described screening methods for the PRSS1 codon 29 and codon 122 mutations may miss the new mutations.

Based on the discovery of the R122H mutation in the PRSS1 gene, we proposed a mechanistic model for the initiation of acute pancreatitis.[6] A modified version of this model is illustrated in fig 7. In this model, three events must occur in order for acute pancreatitis to be initiated by activated digestive enzyme mediated pancreatic autodigestion and subsequent inflammation. Firstly, trypsinogen must be activated to trypsin. This event could be enhanced by certain mutations in trypsinogen[14] or by enzymatic activation of trypsinogen by trypsin or other enzymes.[19–22] The second event is for active trypsin to overcome the first line of defence, which is trypsin inhibition by pancreatic secretory trypsin inhibitor (PSTI or SPINK1). This event occurs when more trypsin becomes activated than SPINK1 can inactivate and is enhanced by excessive trypsinogen activation or by diminished SPINK1 inhibitory capacity (for example, through specific SPINK1 mutations).[23 24] The third event is to overcome the second "fail safe" line of defence, which is trypsin autolysis beginning at R122. This event occurs when trypsin is resistant to autolysis because of an amino acid substitution at R122 (for example, R122H or R122C), in the presence of elevated calcium levels[25–28] or possibly other mechanisms. This model is gaining support from a variety of recent biochemical studies.[29–34] Taken together, a number of mutations appear to increase the risk of developing pancreatitis.

Mutations in cationic trypsinogen at codon 122 remain of major importance because of the large number of HP families with mutations at this codon, and therefore have prompted an extensive amount of biochemical work investigating the significance of this site. The arginine associated with codon 122 (chy No 117) has long been recognised by biochemists as the initial hydrolysis site of trypsin and trypsinogen hydrolysis by trypsin.[35] In some non-human species, hydrolysis also

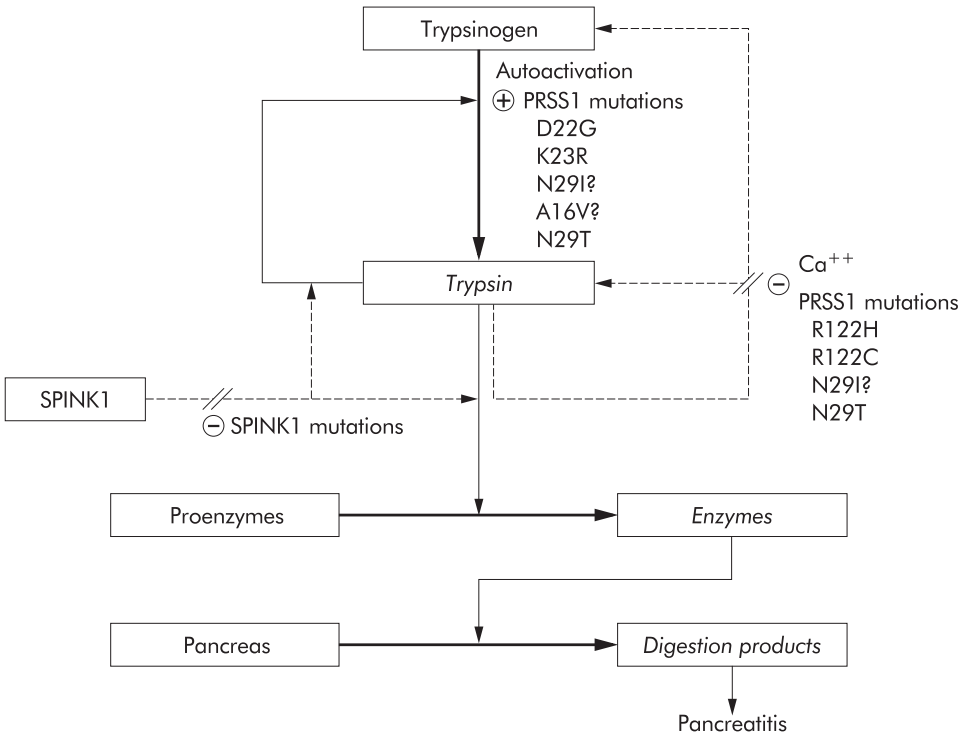

Figure 7 Enhanced trypsin activity model of acute pancreatitis. Three general mechanisms may lead to excessive amounts of active trypsin within pancreatic acinar cells: (1) enhanced activation of trypsinogen through autoactivation (especially with D22G, K23R, N29T, and possibly N29I and A16V mutations), or by trypsin; (2) limited inhibitory capacity of PSTI/SPINK1 (further reduced by N34S and other SPINK1 mutations); or (3) reduced autolysis by elevated calcium concentrations, R122H, R122C, N29T, and possibly N29I mutations of PRSS1. Thick arrows indicate pathways, thin arrows catalytic activation, and broken lines inhibition. Plus and minus signs indicate factors influencing the individual components.

occurs at other sides, such as lysine 66 (chy No 61).[29 35] The single trypsin autolysis susceptibility site at R122 in humans would appear to confer an increased risk to autolysis resistant trypsin in the case of a specific mutation at this site, as illustrated in HP. Indeed, the impact of the R122H mutation on autolysis in rat and human trypsin has been demonstrated in biochemical studies.[29 30] It is highly likely that the same mechanism of autolysis resistance occurs with the R122C mutation reported here. Indeed, the clinical characteristics of a multigenerational family with an apparent autosomal dominant inheritance pattern matches the characteristics of the R122H mutation kindreds.[9] Recently, human R122H mutations[34] but not rat R122H mutations[32] were reported to be associated with enhanced trypsinogen autoactivation under high and low calcium conditions in vitro. Finally, we suggest that the relative infrequency of the R122C variant compared with R122H most likely reflects the history of the disease founders rather than being a functionally less important mutation.

Mutations at cationic trypsinogen codon 29 are clearly associated with HP[8] but the mechanism conferring a gain of function remains controversial.[3 33 36] Theories on how the cationic trypsinogen N29I mutation leads to pancreatitis include enhanced activation[33] or limitation of trypsin autolysis.[3 8 37] Sahin-Toth has recently expressed mutants in human cationic trypsin at codon 29 (that is, N29I and N29T) and completed in vitro studies comparing them with wild-type human cationic trypsinogen.[33] These biochemical studies failed to identify a functional change with the N29I mutation that would explain increased susceptibility to pancreatitis. However, the effects of the N29T mutation were striking. In vitro, the N29T mutation markedly enhanced autoactivation and also decreased autolysis.[33] Our finding of a family with the N29T mutation provides clinical evidence that the early trypsinogen activation and resistance to degradation of the N29T variants seen in vitro predicts pancreatitis in humans.

Most of the pancreatitis associated mutations in cationic trypsinogen merely substitute the regular amino acid with one from another trypsinogen family member. For example, the sequence of cationic trypsinogen at the mutation site becomes that of trypsin 6 in R122H, anionic trypsinogen (PRSS2) in N29I, and mesotrypsinogen in A16V.[7] Likewise, the amino acid sequence of cationic trypsinogen becomes that of trypsin 6 or mesotrypsinogen in the N29T mutation. One possible explanation is gene conversion[38]—that is, a process where one allele directs another allele to take on its form during DNA mismatch repair.[39] This has yet to be proved and it remains unclear why these changes would lead to pancreatitis.

Chen *et al* reported fine novel missense mutations in the PRSS1 gene.[16] We did not identify any of these mutations in our cohorts. In addition, the D22G and K23R mutations were not observed. The present study raises important considerations for genetic screening. RFLP analysis of exon 3 (and in some laboratories exon 2) has proved to be a useful and cost effective way to screen for PRSS1 mutations. Our present findings and the report of Howes and colleagues[17] clearly challenge this paradigm as the R122C mutation and some R122H mutations cannot be detected with the *AflIII* digest. Thus only studies that have used direct sequencing or other mutation independent mutation detection methods[40] are expected to give accurate information on trypsinogen mutations in previously uncharacterised families. Likewise, the N29T mutation would be missed by some mutation specific screening methods.[41] Therefore, the prevalence of the R122C and N29T mutations may be underestimated.

In conclusion, we report two novel PRSS1 mutations, R122C and N29T, in families with autosomal dominant appearing pancreatitis. Our findings confirm the important role of these amino acids for regulating trypsinogen function and predisposing individuals to pancreatitis. The mechanisms predisposing patients with codon 29 mutations to pancreatitis remain obscure and underscore the need for additional structural and functional studies. Finally, RFLP analysis and similar mutation specific screening strategies may miss important mutations that clearly predispose some individuals to pancreatitis.

Authors' note

Two other groups have now confirmed the R122C mutations[42 43] that was first presented by Pfutzer at the Digestive Disease Week, May 2001 and included in table 1 of Applebaum-Shapiro *et al*.[44]

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