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REVIEW

Molecular basis of hereditary pancreatitis

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Hereditary pancreatitis (HP) is an autosomal dominant disease. Two heterozygous missense mutations, R122H (R117H) and N29I (N21I), in the cationic trypsinogen gene have been clearly associated with HP. The 'self-destruct' model proposed for the R122H mutation is discussed in connection with the existing theory of pancreatitis, and the basic biochemistry and physiology of trypsinogen, with particular reference to R122 as the primary autolysis site of the cationic trypsinogen. Two different genetic mechanisms are identified which cause the R122H mutation, and gene conversion is the likely cause of the N29I mutation. A unifying model, which highlights an indirect impairment on the R122 autolysis site is hypothesised for the N29I mutation. Possible predisposition to pancreatitis by additional DNA variants in the gene, such as the A16V signal peptide cleavage site mutation and the K23R activation peptide cleavage site mutation is suspected, but not proven. Evidence of genetic heterogeneity of HP is reviewed and cystic fibrosis transmembrane conductance regulator (*CFTR*) gene mutations detected in HP families are re-evaluated. Finally, large scale association studies are expected to clarify the additional variants' role in pancreatitis and to identify new HP genes. *European Journal of Human Genetics* (2000) **8**, 473–479.

Keywords: pancreatitis; hereditary pancreatitis (HP); cationic trypsinogen gene; missense mutation; genetic heterogeneity; cystic fibrosis transmembrane conductance regulator (*CFTR*); gene conversion; genetic predisposition; linkage analysis; association analysis

Introduction

Hereditary pancreatitis (HP; MIM 167800) is an autosomal dominant disease with approximately 80% penetrance and variable expression.[1,2] Apart from an early age of onset and a positive family history, HP is indistinguishable from the sporadic disease.[3,4] Although pancreatitis was hypothesised to result from inappropriate activation of pancreatic zymogens in 1896[5] and the genetic nature of HP was identified in 1952,[6] the molecular basis of HP remained elusive until 1996, when a mutation in the cationic trypsinogen gene was demonstrated as being associated with HP.[7] While first touching on the major historical events in the pursuit of the etiology of HP briefly, this article focuses on the mutational spectrum of the cationic trypsinogen gene and the current understanding of the mechanisms underlying these mutations. It then goes on to discuss the genetic heterogeneity of HP and finally re-evaluates the cystic fibrosis transmembrane conductance regulator (*CFTR*) gene mutations detected in HP families. It is suggested that the reader also refer to recent review papers[8,9] to complement this article.

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Mapping of one HP gene to chromosome 7q35 by genome-wide linkage analysis

The simple Mendelian model of inheritance of HP suggested that a single genetic defect might be responsible for this disorder. Historically, associations between pancreatitis and certain clinical variants have been reported but none of them substantiated (reviewed by Whitcomb *et al*[10]). Thus positional cloning, in which a disease gene is identified by virtue of its location in the genome rather than by knowledge of its biochemical defects, was carried out. Using a genome-wide genetic linkage analysis, we mapped one HP gene to chromosome 7q35 in a large French HP family in 1996.[11] This linkage was concurrently identified by Whitcomb *et al*[10] and confirmed by Pandya *et al*.[12]

Identification of the disease-causing gene expedited by the chance discovery of eight trypsinogen genes to be intercalated within the human T cell receptor (*TCR*) β locus on chromosome 7q35

The next step was to identify the HP-causing gene that must be located somewhere within 7q35. Coincidentally, eight pancreatic trypsinogen genes were discovered intercalated within the human β *TCR* locus in 7q35 as a result of a large scale genomic sequencing effort.[13] The complete genomic sequencing data of these trypsinogen genes enabled Whitcomb *et al*[7] to easily exclude the pseudogenes and to design isoform-specific primers for accurately amplifying the two clearly functional cationic and anionic genes (see Chen and Ferec[9] for details on the human trypsinogen family). Within months, an HP-associated mutation in the cationic trypsinogen gene was identified.[7]

HP-associated mutations in the cationic trypsinogen gene

A few words on the nomenclature for cationic trypsinogen mutations, which we discussed on a previous paper[9] are appropriate at this point. Throughout this review the 'Recommendations for a Nomenclature System for Human Gene Mutations'[14] will be used, ie for amino-acid based systems, the codon for the initiator methionine is codon 1, with the 'comparative chymotrypsin numbering system' in parentheses the first time the mutations are mentioned in the text. To date, two missense mutations R122H (R117H)[7] and N29I (N21I)[15] in the cationic trypsinogen have been unambiguously associated with HP.

R122H (R117H)

R122H, which results from a G > A (CGC > CAC) single nucleotide change in exon 3 of the cationic trypsinogen gene, is the first and most frequent mutation identified as being associated with HP.[7,16–26] Whilst no one doubts its disease-causing role in HP, some[27] do argue that 'self-destruct' mechanism proposed for R122H[7] has not yet been proven. In our opinion, however, this may be due to inadequate evaluation rather than a lack of good supporting data and, accordingly, we wish to highlight several issues.

On the one hand, trypsinogens are specifically activated in the intestine by enterokinase, and the newly formed trypsins are responsible for the activation of all other pancreatic digestive zymogens.[28] Conceivably, premature activation of trypsinogens within the pancreas would be disastrous. Indeed, pancreatitis was proposed to be an autodigestive process more than 100 years ago,[5] and intracellular premature activation of trypsinogen has been demonstrated to be an early event in both *in vitro* and three different *in vivo* models of pancreatitis.[29] Although the 'co-localisation of trypsinogen with lysosomal hydrolases' in these models may not be relevant in HP, it is important to note that the human cationic trypsinogen possesses the ability to autoactivate, and that it autoactivates more easily than other trypsinogen isoforms.[30] Furthermore, activated proteolytic enzymes such as trypsin and elastase can be detected within the pancreas in both clinical[31] and experimental[32] forms of pancreatitis.

On the other hand, the body employs a series of protective mechanisms against premature zymogen activation.[28] Of great importance is that trypsinogen/trypsin is subject to hydrolysis by trypsin itself. Most importantly, as in the case of the bovine[33] and porcine[34] trypsinogens, residue R122 of the human cationic trypsinogen was identified to be the primary autolysis site by co-sequencing of the first 14 residues of the partially proteolysed protein.[35] Protection of this autolysis site by a monoclonal antibody against human cationic trypsinogen resulted in increased enzyme activity,[35,36] and substitution of this site with certain amino acids,[37,38] especially histidine[39] in the rat trypsinogen showed increased enzyme stability.

Thus, the pathogenesis of the R122H mutation, considered in connection with the existing theory of pancreatitis, the basic biochemistry and physiology of trypsinogen, and combined with the molecular modelling and X-ray crystallography data provided in the original paper by Whitcomb *et al*,[7] is clear and evident. The R122H mutation would eliminate the R122 primary autolysis site, which clearly underlies the unique 'fail-safe' or 'self-destruct' mechanism against pancreatic autodigestion[7] simply because histidine (H) is not a trypsin cleavage site. Consequently, during excessive trypsinogen activation the stabilised mutant enzyme would trigger the pancreatic autodigestive process resulting in pancreatitis[7,8] (Figure 1). What has yet to be proven is under what condition and in which subcellular compartment the premature activation of cationic trypsinogen is initiated.

Since R122 has been confirmed to be the primary autolysis site[35] and trypsin specifically catalyses the hydrolysis of peptide bonds on the carboxyl side of arginine (R) and lysine (K) residues, we surmise that *in vitro* experiments on the human R122H mutant would not yield any more useful information beyond these fundamental observations. Transgenic models are attractive but certainly will be confounded by the existence of multiple functional trypsinogen genes and different protective mechanisms against trypsinogen activation in mice.

The R122H mutation, in most cases,[7,16–26] resulted from a single G > A (CGC > CAC) transition, which most probably occurred as a spontaneous deamination of 5-methylcytosine to give thymine in the CpG dinucleotides on the opposite strand. Interestingly, we identified a GC > AT (CGC > CAT) 2 bp nucleotide substitution, which also resulted in a R122H mutation but clearly arose via a different genetic mechanism – gene conversion.[40] This theory was strongly supported by the presence of AT in the corresponding position of cationic trypsinogen's two homologous genes and a *Chi*-like sequence in the 3' vicinity of the mutation. This genetic finding also

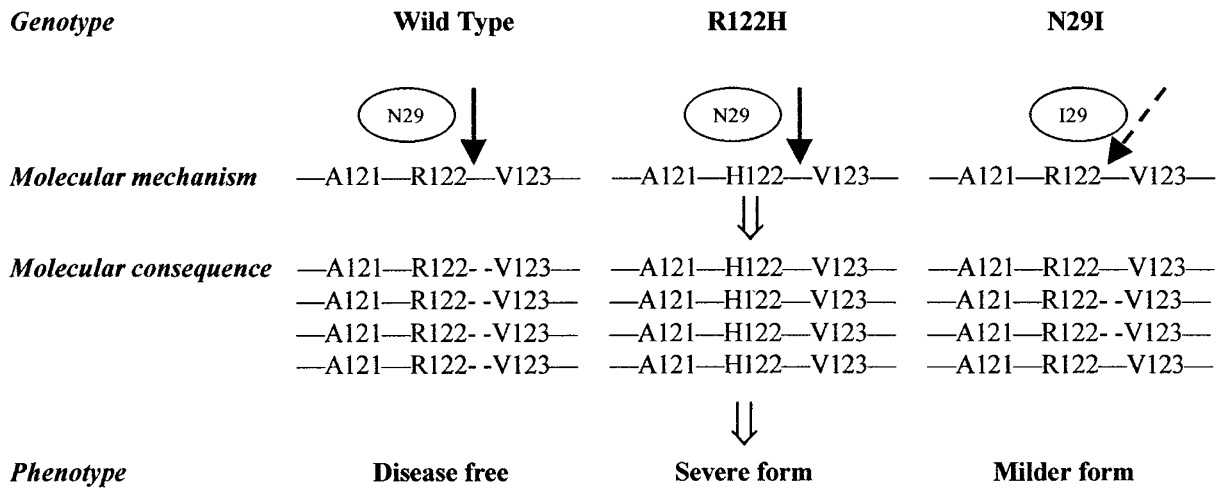

Figure 1 A unifying model of the R122H and N29I mutations in the pathogenesis of HP. This model is based mainly on the observations that R122 is the primary autolysis site of the cationic trypsinogen,[35] thereby constituting the unique 'self-destruct' defence system of the body against pancreatic autodigestion,[7] and that N29 is near R122 on the trypsin surface.[35] Arrows indicate the accessibility of trypsin-like enzymes to residue 122. R122H is presumed to impair the 'self-destruct' defence system directly by abolishing the trypsin-like enzyme sensitive site R122; N29I is hypothesised to induce an as yet unknown conformational change, which in turn would hinder the accessibility of trypsin-like enzymes to R122 (indicated by the dashed arrow). As a result, unchecked mutant trypsin would initiate the activation cascade leading to pancreatic autodigestion and pancreatitis. However, N29I manifests a milder form of disease due to its 'indirect' impairment of the 'self-destruct' defence mechanism compared with R122H.

raises practical concerns. Since a single G > A nucleotide change creates a novel Alf III site (A▼CRYGT), the amplification of exon 3 followed by Alf III digestion[7] has been widely adopted to screen for the R122H mutation.[16–19,21] Obviously, if R122H mutation arose as a result of a gene conversion event, it would not be detected by this simple method.[40]

N29I (N21I)

Whilst the R122H has been demonstrated to be the most frequent mutation in HP, another missense mutation named N29I, which results from a single A > T (AAC > ATC) nucleotide substitution in exon 2 of the cationic trypsinogen gene, also turns out to be present worldwide.[15,20–22,25] However, unlike R122, N29 is clearly not a trypsin cleavage site and thus could not be readily explained by the 'self-destruct' mechanism. Some authors[20] have even questioned whether the N29I mutation could alter the molecular nature of the enzyme dramatically enough to produce pancreatitis, based mainly on the observation that the first 38 N-terminal amino acids of the secreted form of cationic trypsinogen containing this mutation happen to be identical with that of the native anionic trypsinogen isoform (Figure 2). It is this observation that attracted our attention and prompted us to provide an explanation for N29I's origin in the context of the trypsinogen family and based on this, unify the available knowledge to highlight an indirect 'self-destruct' model for the N29I.

Strong evidence suggested that N29I arose from a gene conversion event in which the wild-type functional anionic trypsinogen gene acted as the donor sequence and, more importantly, N29 and I29 might represent rare mutations of the two functional human trypsinogen genes, positively selected by evolution to somehow endow an as yet unknown advantageous effect on their respective protein's structure and function.[41] Thus, a rare, non-reciprocal genetic information exchange between the anionic and the cationic trypsinogen genes at this position could be disastrous. In support of this, the physico-chemical characteristics of Ile are quite different from those of Asn, and a structural difference for

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          1          11   16       23    29 31          41          51
CT  MNPLLILTFV AAALAAPFDD DDKIVGGYNC EENSVPYQVS LNSGYHFCGG SLINEQWVVS
AT  --L------- ---V------ --------I- ---------- ---------- ---S------
MT  ---F---A-- G--V-V---- --------T- ----L----- ----S----- ---S------
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Figure 2 Partial alignment of the deduced amino acid sequences of cationic (CT), anionic (AT), and meso (MT) pretrypsinogens. Numbering of amino acids begins at the ATG initiator codon. Dashes indicate identity with the CT sequence. Signal peptides are in italics, activation peptides are underlined, and residues with mutations are in bold. Note the presence of Val at residue 16 of the premesotrypsinogen and the presence of Ile at residue 29 of the anionic pretrypsinogen, and the interesting locations of A16 and K23 in the CT sequence. Also compare the CT sequence containing N29I with AT.

this substitution has been suggested by the different affinity constants of a monoclonal antibody towards the human anionic and cationic trypsinogen isoforms.[35,36] A computer analysis of the N29I substitution in the cationic trypsinogen protein also predicted a secondary structural change from a native 'turn' into a 'sheet' in the flanking region.[20]

The next pertinent question is how this gene conversion-like event could produce pancreatitis. Considering the facts that N29I is a disease-causing mutation in HP, that HP is a 'gain-of-function' disorder, that the N29I does not seem to affect either the intracellular transportation or the autoactivation of the protein, and that N29 is near R122 on the surface of trypsin,[35] we believe that N29I must somehow exert an indirect impairment on the R122 autolysis site.[9,42] This is given strong support by the recent molecular modelling data. An electrostatic bond at E32–R122, presumably induced by the N29I substitution, would hinder the accessibility of trypsin-like enzymes to the R122 autolysis site.[8] In addition, recent *in vitro* mutagenesis experiments have shown that N29I mutants in the rat[43] and human[44] trypsinogens demonstrate increased enzyme stability. This indirect model could further reconcile the clinical observations that N29I causes a milder form of HP in terms of a relatively later age of disease onset and fewer hospitalisations compared with R122H[15,20] (Figure 1).

Novel DNA variants identified in cationic trypsinogen gene in sporadic pancreatitis

The nature of HP as an autosomal dominant disorder implies that the variety or number of causal mutations in the cationic trypsinogen gene will be limited, since 'loss-of-function' mutations would not cause the disease. This holds true in the context of HP in which only the R122H and N29I are associated. However, large scale mutational screening efforts in sporadic disease revealed new DNA variants, some of which may predispose someone to pancreatitis. Due to the scarcity of data about these DNA variants, this section is not meant to give any definitive conclusions but rather intends to initiate discussions.

A16V

Witt *et al*[26] first described a strong association between a novel DNA variant named A16V with chronic pancreatitis. In their study, four out of 44 children and adolescents with chronic pancreatitis were found to carry the A16V variant. We independently identified this variant in two out of 312 patients with the sporadic disease.[45]

In contrast to the 80% penetrance of the R122H and N29I mutations, the penetrance of A16V is clearly low – only one of the seven first-degree relatives carrying the A16V mutation in Witt *et al*'s report was affected, and neither of the two A16V carriers in our study had a family history of pancreatitis. Even more interestingly, as an Ile is present at residue 29 of the functional anionic pretrypsinogen, a Val is present at residue 16 of the functional premesotrypsinogen (Figure 2). However, whilst a N29I mutation results in a non-conservative amino acid substitution, which is considered to be important in causing the disease,[42] an A16V mutation results in a conservative substitution, which may underlie its lower penetrance.[46]

Since A16 happens to be the signal peptide cleavage site (Figure 2), the A16V substitution was suggested to cause a 'loss' rather than a 'gain' of trypsin function in a recent discussion.[47] We disagree with this assumption due to two interrelated considerations. First, A16V seems to be associated with pancreatitis. Second, a 'loss of function' of the cationic trypsinogen could not cause the disease. Alternatively, we argue that an A16V substitution would most likely disrupt the intracellular transportation of the pretrypsinogen and, in this vein, it may even be tempting to speculate that this substitution could lead to the co-localisation of pretrypsinogen with lysosomal hydrolases, the very well documented trypsinogen activation mechanism in animal models of pancreatitis.[29,46]

K23R

The K23R variant was only detected in two affected members of one family and was not present in 400 control chromosomes.[22] Unfortunately, no other family members were available for segregation analysis. Since K23 is the last residue of the highly conserved eight amino acid activation peptide of trypsinogens (Figure 2), a substitution could be expected to have an effect on phenotype, ie to enhance autoactivation. However, the fact that the physico-chemical properties between K and R are quite similar, that an R is used in the corresponding position of rat trypsinogen V[48] and that R is also cleaved by trypsin suggests a neutral effect.

V123M

This variant was detected in a single case with sporadic disease and was not present in 400 control chromosomes (JM Chen *et al* in preparation). Could it affect the R122 autolysis site (Figure 1)?

Other variants

In addition to the above three interesting DNA variants, further missense mutations have been identified separately in single cases.[45] Among them, the P36R and G83E resulted in non-conservative amino acid substitutions at well conserved residues. The fact that frameshift, nonsense and splicing mutations were never detected is consistent with a 'gain-of-function' disease.

Genetic heterogeneity of HP

Mutations in the cationic trypsinogen gene do not appear to be the whole story and, in hindsight, Sarles *et al*'s report that HP is a group of at least two diseases having a similar clinical picture and pathological features but with different chemical

compositions of calculi (protein lithiasis *vs* calcic lithiasis) presented in pancreatic duct[49] may have suggested genetic heterogeneity. Nevertheless, definite proof was first provided by Dasouki *et al*[17] in 1998. Two of eight American HP families were found not to carry the R122H mutation. Although they did not analyse the N29I mutation in their patients, the involvement of this mutation and other possible mutations, if there are any, in the cationic trypsinogen gene was ruled out by the negative linkage between the disease and the loci D7S661 and D7S676 in at least one family. We provided further evidence in French HP families and, especially, a segregation analysis using 7q35 markers in family N indicated that the affected II2 and II3 inherited different haplotypes from I1, and III2 did not receive I1's haplotype,[22] excluding not only the cationic trypsinogen gene, but also all the other genes on chromosome 7. There are additional studies supporting genetic heterogeneity[20,25,26] and a preliminary report suggests that a new HP gene may be located on chromosome 12.[50] However, the observed linkage with a lod score < 3.0 needs further confirmation. Additionally, possible involvement of anionic trypsinogen gene on chromosome 7, mesotrypsinogen gene on chromosome 9,[51] pancreatic secretory trypsin inhibitor gene on chromosome 5[52] and pancreatitis-associated protein gene on chromosome 2[53] in HP has been excluded by candidate gene approach.

Is *CFTR* a new HP gene?

It is worth pointing out that in 1996, the same year the identification of the cationic trypsinogen gene was associated with HP, mutations in the *CFTR* gene were reported to be detected in HP families.[54] Of the two new mutations identified, a heterozygous L327R was found to segregate with the disease in one family. The possibility that this family might be, in fact, a CF family was excluded by the normal sweat chloride levels, normal sputum cultures, forced vital capacity (FVC) and forced expiratory volume (FEV) values over 100% and no family history of CF.[54]

To date, there have been no further reports describing *CFTR* gene mutations in HP families. Our haplotype analysis in family N[22] definitively excluded the involvement of the *CFTR* gene due to its location on chromosome 7, and the potential linkage of a new HP gene was to chromosome 12 in another American family.[50] However, two simultaneous articles reported an association between sporadic chronic pancreatitis and mutations in the *CFTR* gene.[55,56] In these two studies, none of the patients carrying *CFTR* mutations had the combination of sinopulmonary disease, high sweat electrolyte concentrations, and low nasal potential-difference values that are diagnostic of CF, and the vast majority of these patients had mutations in only one copy of the *CFTR* gene. Analogous with other atypical diseases of CF, including congenital absence of the vas deferens, chronic bronchitis and sinusitis with nasal polyposis, sporadic chronic pancreatitis with abnormal *CFTR* alleles was referred to as a monosymptomatic form of CF.[57]

These observations prompted us to re-evaluate the role of the L327R missense mutation in the *CFTR* gene identified in a single HP family.[54] There could be three explanations: first, this substitution is indeed a disease-causing mutation. If this were the case, this family may be more adequately diagnosed as having monosymptomatic pancreatitis of CF. Second, the L327R substitution represents a mere coincidence, provided that it does not have any phenotype-modifying effect on the disease. Thus, the real disease-causing mutation, probably a cationic trypsinogen mutation in this family, remains to be identified. Third, it is tempting to speculate that *CFTR* may act as a modifier gene in the pathogenesis of HP, and more work is warranted to explore this possibility.

Conclusions

The identification of mutations in the cationic trypsinogen gene as the molecular basis for most cases of HP confirmed for the first time the existing theory of pancreatitis as an autodigestive disease and marked the beginning of a new era in pancreatic research. This finding also demonstrates how genetic investigation can provide insights into protein structure and function. Furthermore, if N29I indeed arose as a gene conversion event, it would represent the first example of such an event occurring between two functional wild-type genes which resulted in a disease. However, as we have discussed, several issues remain to be resolved.

First, more extensive association studies are needed to correlate the clinical features of pancreatitis (phenotype) with the different cationic trypsinogen missense mutations (genotype), particularly those rare DNA variants detected in sporadic disease. At present, the possibility that slight variations in the primary structure of the cationic trypsinogen induced by these variants may render a degree of predisposition to pancreatic autodigestion cannot be excluded. Second, the identification of another HP gene promises to not only provide further insights into the molecular pathogenesis of HP, but also better means for diagnosis, family testing and treatment. Based on the patchy, focal nature of pancreatitis and the examples of the cationic trypsinogen and *CFTR* gene mutations, the new HP gene is expected to result in a defect in either the acinar or ductal cells of the exocrine pancreas. Since the remaining HP families are generally small, we envision that association studies, fuelled by the approaching availability of the entire human genome sequence and the increasing number of single nucleotide polymorphisms (SNPs), will play an important role in this enduring effort. Finally, the co-examination of *CFTR* and cationic trypsinogen gene mutations in pancreatitis subjects would shed light on gene–gene interaction in the disease process. Hopefully, answers to these questions may come sooner through the co-operative effort of the two major study groups for HP – the Midwest Multicenter

Pancreatic Study Group in North America (website: http://www.pitt.edu/~whitcomb/HPRN/) and the EUROPAC Registry of Hereditary Pancreatic Disease in the UK and Europe (website: http://www.liv.ac.uk/surgery/europac/html).

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