PANCREAS

A 93 year old man with the *PRSS1* R122H mutation, low *SPINK1* expression, and no pancreatitis: insights into phenotypic non-penetrance

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Background: The cationic trypsinogen (*PRSS1*) R122H mutation causes autosomal dominant hereditary pancreatitis (HP) with multiple attacks of acute pancreatitis, but the penetrance, frequency, and severity of attacks are highly variable. HP twins study suggests that modifier genes influence severity but not penetrance.

Aim: To investigate potential trypsin associated factors in subjects with the PRSS1 R122H mutation and phenotypic non-penetrance.

Methods: Two subjects from HP families (including a 93 year old subject with *PRSS1* R122H without pancreatitis), one with chronic pancreatitis and one with a normal pancreas, were studied. Relative expression of: (a) the *PRSS1* R122 and H122 alleles; and (b) the *PRSS1* and *SPINK1* genes in pancreatitis were determined using complementary methods.

Results: *PRSS1* wild-type (R122) and mutant (H122) allele expression was equivalent in multiple (>3) samples from the phenotypically affected and non-penetrant subjects with R122H genotypes using allele specific quantitative reverse transcription-polymerase chain reaction (RT-PCR) and intron spanning nested RT-PCR followed by cDNA sequencing. Compared with *PRSS1* mRNA levels, *SPINK1* mRNA levels were low in normal appearing tissue but markedly increased in samples with chronic inflammation, independent of *PRSS1* genotype.

Conclusion: Attacks of acute pancreatitis in HP subjects appear to be independent of the relative expression of the mutant *PRSS1* H122 allele or *SPINK1* gene expression. The marked increase in *SPINK1* gene expression with inflammation is consistent with its regulation as an acute phase protein.

Hereditary pancreatitis (HP; MIM 167800) is a syndrome in which two or more individuals within a family have unexplained recurrent acute or chronic pancreatitis, appearing in an autosomal dominant pattern.[1 2] The phenotype includes attacks of acute pancreatitis in approximately 80% of individuals before the age of 20 years with pancreatitis associated mutations, median age of onset of about 10 years, progression to chronic pancreatitis occurs in about half of the patients with acute pancreatitis, and of these approximately 40% may develop pancreatic cancer, usually after the fifth decade of life.[1–4] Mutations in the cationic trypsinogen gene (protease, serine, 1; UniGene symbol *PRSS1*; MIM 276000), especially *PRSS1* R122H[6] or N29I,[3] are the most common causes.[4–6] Approximately 20% of *PRSS1* R122H and N29I carriers never develop pancreatitis (phenotypically non-penetrant).[4 6 7]

The mechanism of non-penetrance remains elusive. Our previous study involving seven sets of identical twins from HP kindreds[7] suggested that genetic and environmental factors play an important role in determining susceptibility and disease progression but genetic factors alone could not explain penetrance.[7]

The present report centres on a 93 year old Caucasian male from a large HP kindred with the *PRSS1* R122H mutation. Genetic testing proved that the subject had the R122H mutation yet never suffered an attack of pancreatitis. On his death from unrelated causes, rapid autopsy and study of snap frozen and fixed sections of his pancreas allowed us to address several unanswered questions about non-penetrance in HP. (A) Does non-penetrance reflect an inability to detect subclinical pancreatitis? (B) Is non-penetrance due to epigenetic factors that alter expression of the gain of function mutation (for example, expression of R122 but not H122)? (C) Is non-penetrance due to relative overexpression of the pancreatic secretory trypsin inhibitor (PSTI, serine protease inhibiter, Kazal-type 1; UniGene Symbol *SPINK1*, MIM 167790) compared with *PRSS1*? The first of these questions was addressed with histological examination of the entire pancreas. The second and third questions required complex investigations.

One important epigenetic event could be the stochastic methylation of critical elements within the promoter region of a gene to interfere with gene expression.[8 9] Reduced expression of the mutant allele (H122) with continued expression of the wild-type allele (R122) might then explain phenotypic non-penetrance.

Alternatively, relative expression of the trypsin inhibitor gene, *SPINK1*, may be enhanced compared with trypsinogen in non-penetrant patients. This hypothesis is based on the assumption that SPINK1 is the firstline of defence that must be overcome before pancreatitis develops[10] and the observation that patients presumed to have reduced SPINK1 function through germline mutations in the *SPINK1* gene are more likely to develop pancreatitis.[11 12] As the stochiometry of the trypsin-SPINK1 inhibition is one to one, relative expression of the two genes is likely relevant. Thus relative expression of these two critical genes was also investigated.

CASE REPORT

Methods

Patients

Studies were conducted with the approval of the University of Pittsburgh Institutional Review Board and with the consent of the patients and/or immediate family. DNA samples for genetic analysis were obtained and analyzed as previously described.[5 12] Multiple pancreatic samples from a

Abbreviations: HP, hereditary pancreatitis; PCR, polymerase chain reaction; RT, reverse transcription

93 year old phenotypically non-penetrant HP (R122H) study subject were obtained during rapid autopsy (within two hours of death), snap frozen, and stored at −80℃ until analysis. Pancreatic tissue for comparison was obtained as surgical waste from an affected patient with HP, from a patient with chronic pancreatitis, and from normal pancreatic tissue.

Histology

Samples of pancreatic tissue were fixed, stained with haematoxylin-eosin, and examined by light microscopy.

Differential R122 and H122 expression

Frozen tissue samples were homogenised in TRIzol (Life Technologies, Grand Island, New York, USA) on dry ice, extracted in chloroform, and precipitated in isopropyl alcohol. The pellet was washed in 75% ethanol and resuspended in RNase free water. A sample was run on a 5% polyacrylamide gel to verify the presence of 28s and 18s bands and the remainder was stored at −20℃. RNA was used as a template for cDNA reverse transcription, as previously described.[13] Two methods were used to detect expression of the R122 and H122 alleles.

(1) Allele specific quantitative polymerase chain reaction (PCR) of cDNA. Prepared cDNA was used to determine relative allelic expression of R122 and H122 trypsinogen (quantitative PCR). Fluorescent probes and primers used are illustrated in fig 1, with the probes aligning genomic DNA sequences of H122 and R122 trypsinogen. Probe cross hybridisation was evaluated using PCR amplified R122 and H122 bacteriophage clone. Samples were run on an ABI 7700 sequence detection system (Applied Biosystems, Foster City, California, USA). The reaction master mix contained 12 µl of DEPC treated H_2O, 25 µl TaqMan Universal PCR master mix (Applied Biosystems), 2.5 µl of forward and reverse primers (250 nM final concentration), and 1.0 µl of each probe (100 nM final concentration). All reactions were performed in quadruplicate. Negative controls included samples from the PCR amplification reaction without cDNA. Cycling conditions were 50℃ for two minutes, 95℃ for 12 minutes, then 95° for 15 seconds, and 64℃ for one minute ×40 cycles. Results were analysed using a sequence detector (Applied Biosystems). The experiment was repeated three times to assure reproducibility.

(2) cDNA sequencing. A complimentary approach was used to verify R122 and H122 RNA. In order to eliminate amplification of genomic DNA, external PCR primers were designed to span the junction between exons 1 and 2 in the forward direction (CT**C**-TTG CTG CCC CCT TT; dash indicating the location of the exon 1–2 junction, bold indicating the *PRSS1* specific base) and exons 5 and 4 in the reverse direction (CCA GAA TCA CCC TGA CAT GA). A *PRSS1* gene specific reverse transcription (RT) reaction included: 1 µg RNA, 1× PCR buffer II (ABI), 7.5 mM $MgCl_2$, 1 mM dNTPs, 40 nM external reverse primer, 0.4 µ/µl RNase inhibitor (Promega, Madison, Wisconsin, USA), and 0.1 µ/µl MMLV (Epicentre, Madison, Wisconsin, USA). Thermal cycler conditions were as follows: 48℃ ×40 minutes, 95℃ ×5 minutes. PCR was performed as follows on the cDNA as well as 200 ng of gDNA to verify the primers specifically amplify cDNA: 1× PCR buffer II (ABI), 1.5 mM $MgCl_2$, 200 µM dNTPs, 200 nM forward and reverse primers, 0.05 µ/µl AmpliTaq Gold (ABI) and 25 ng cDNA (2× dilution of stock cDNA). PCR temperature parameters were: one cycle 95℃ ×12 minutes, 35 cycles 95℃ ×15 seconds, 64℃ ×1 minute, and 72℃ ×1 minute. Internal sequencing primers for PRSS1 were designed to span the junction between exons 2 and 3 in the forward direction (CAC TGC TAC AAG TC-CCG CAT) with a reverse primer within exon 4 (TTC ACA CT**T** AGC CTG GCT CA). The external PCR product was treated with exonuclease prior

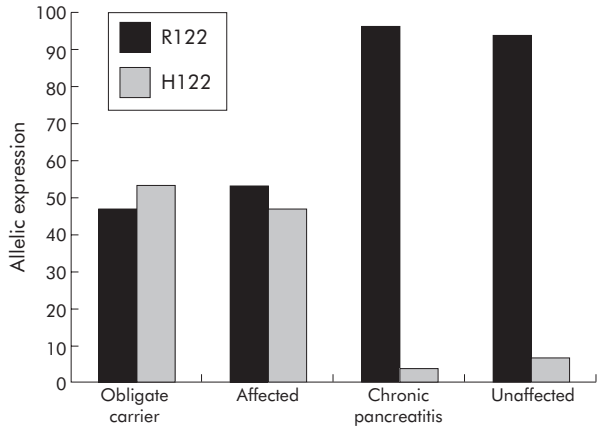

Figure 2 Quantitative relative allelic expression of hereditary pancreatitis (HP) and non-HP samples. R122 and H122 ratios were similar for the HP (obligate carrier/non-penetrant and affected) samples with negligible H122 amounts expressed in the non-HP (chronic pancreatitis and unaffected) samples.

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cgccaccccc aatacgacag gaagactctg aacaatgaca tcatgttaat
                                          »»»       >>>
                    6FAM-atcaacgc ccgcgtgtcc ac-TAMRA
caagctctcc tcacgtgcag taatcaacgc ccgcgtgtcc accatctctc
>>>>>>>>>>>>>>>>>>>                   a
                       VIC-caacgc ccacgtgtcc acca-TAMRA
tgcccaccgc ccctccagcc actggcacga agtgcctcat ctctggctgg
               <<<<<<<<<<<<<<<<<<<

ggcaacactg cgagctctgg cg
```

Figure 1 A portion of exon 3 of the human trypsin cDNA sequence. The specific probes are shown overlapping a portion of the sequence, including the mutation. The location of the mutation is underlined; ***g*** is the wild-type sequence and in the mutant allele ***g*** is substituted for ***a***. Sequences up and downstream from the mutation that correspond to the primers are underlined, with arrows to indicate direction (that is, 5′>3′ or 3′>5′).

to sequencing. Sequencing was performed on an ABI 3730 sequencer. Results were analysed using ABI's Sequencher software.

SPINK1 and *PRSS1* real time amplification

Real time PCR was performed to quantitate *SPINK1* and *PRSS1* mRNA levels and thereby calculate their relative expression. cDNA (5 µl) from each of the samples was amplified in 25 µl DEPC treated H_2O, 5 µl 10× SybrGreen PCR Buffer, 2.5 µl of forward and reverse primers[12] (250 nM final concentration), 6 µl of 25 mM $MgCl_2$, 4 µl of dNTP blend (200 µM dA/C/GTP, 400 µM dUTP final concentration), 0.5 U AmpErase-UNG, and 1.25 U AmpliTaq Gold (Applied Biosystems). The reaction was performed on samples in quadruplicate and a mean value calculated. Negative controls, cycling conditions, and analysis were the same as those for differential allelic discrimination.

Results

The pancreas of the 93 year old non-penetrant mutation carrier appeared histologically normal (not shown).

Relative expression of R122 and H122 in a non-penetrant subject

Cycle threshold values for the quadruplicate samples from each specimen were similar (SD for wild-type and mutant probes: R122H non-penetrant 0.26 and 0.13 cycles, normal pancreas 0.17 and 0.77 cycles, HP 0.25 and 0.45 cycles, chronic pancreatitis 0.03 and 0.12 cycles, respectively). The mean difference in the number of PCR cycles at which fluorescence was detected for the two alleles was used to calculate the difference in mRNA quantity, based on the assumption that one PCR cycle equals a twofold difference in mRNA. A one cycle difference suggests twice the starting RNA amount for the allele reaching cycle threshold one cycle earlier. For example, if CT for allele A = 20 and B = 22, then allele A has four times higher RNA at the beginning of PCR *or* 4:1 *or* 80% of the total RNA amount. Expression of the wild-type and mutant alleles in the HP affected (mean 0.19 (SD 0.25) cycles) and HP non-penetrant samples (mean −0.2 (SD 0.44) cycles) was similar (fig 2). Expression of the wild-type and mutant alleles in the normal and chronic pancreatitis samples was 4.54 and 3.8 cycles, respectively. In repeated experiments, the cycle difference for the HP penetrant and non-penetrant never exceeded 0.7 cycles. The probes were highly specific (1.18% H122 phage amplification with the R122 probe and 0.01% R122 phage amplification with the H122 probe). The relative ratio of the R122 and H122 alleles contributing to total allele expression for HP non-penetrant was 46.75% and 53.25%, HP penetrant 53.0% and 47.0%, chronic pancreatitis 97% and 3%, and normal pancreas 93% and 7%, respectively.

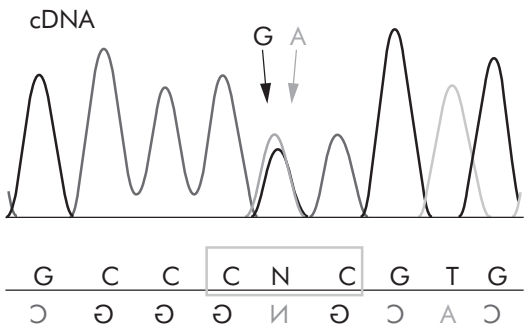

Figure 3 Sequencing of PRSS1 specific cDNA. Note that the signal at the second position of codon 122 is similar in amplitude for the G (normal) and A (mutant) nucleotides.

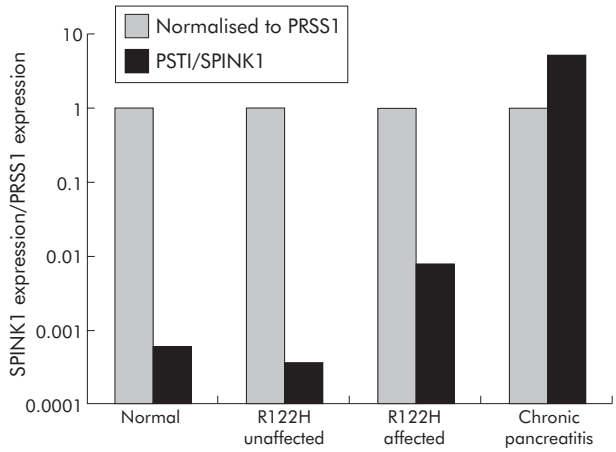

Figure 4 Relative abundance of PSTI/SPINK1 mRNA to trypsin/PRSS1 mRNA. All data are normalised to PRSS1 mRNA. PSTI/ SPINK1 mRNA levels were relatively low compared with PRSS1 (<1:1000) in normal and R122H unaffected subjects but SPINK1 mRNA levels were markedly increased relative to PRSS1 mRNA levels in patients with pancreatitis from either R122H or alcoholic chronic pancreatitis.

Direct sequencing of the cDNA exon 3 spanning RT-PCR product in both the forward and reverse directions for the HP non-penetrant subject verified that both the R122 and H122 alleles were expressed and signal amplitude was equal (fig 3). We also identified a D162D polymorphism in exon 4 for this individual.

Relative expression of *PRSS1* and *SPINK1* in subjects with and without pancreatitis

Relative amounts of SPINK1 RNA to PRSS1 RNA varied dramatically among the samples. The SPINK1:PRSS1 ratio for the normal and HP non-penetrant samples was <1:1000. Relative expression of SPINK1 increased in the context of pancreatic inflammation. In the HP affected sample, the SPINK1:PRSS1 ratio was ~1:100 and for the chronic pancreatitis sample >6:1 (fig 4).

DISCUSSION

The current study provides rare insights into the biology and genetics of the pancreas from a phenotypically non-penetrant *PRSS1* R122H subject well beyond the typical age of HP onset. Histological evaluation excluded significant subclinical pancreatic injury and fibrosis.

Our findings demonstrate physiologically similar levels of R122 and H122 expression, regardless of the phenotype for the R122H samples. The reason for minimal H122 (mutant allele) expression that appeared in the non-HP samples is unknown but likely reflects minimal probe cross hybridisation as control experiments with phage template suggested highly specific probes. These data suggest that promoter methylation and gene suppression were not the mechanisms of non-penetrance in this subject.

We anticipated that relative expression of *SPINK1* to *PRSS1* would be on the order of 1:5.[14] Our findings demonstrate that the ratio of SPINK1:PRSS1 mRNA in normal human pancreas is closer to 1:1000. Also, the ratio of SPINK1 to PRSS1 correlates with inflammation rather than pancreatitis risk—that is, the phenotypically non-penetrant *PRSS1* R122H carrier had a low, rather than a high, SPINK1:PRSS1 ratio. This finding is consistent with the observation that SPINK1 may be regulated as an acute phase reactant.[15] While the

marked difference in SPINK1 and PRSS1 mRNA levels in normal pancreas and inflamed pancreas represents a novel and important finding, it does not explain non-penetrance in HP.

There are several limitations to the present study. Although the unaffected cationic trypsinogen R122H carrier was 93 years old and had no clinical or histological evidence of pancreatitis, this only represents a single case. Furthermore, delay between the subject's death and recovery of the pancreas may have unpredictable consequences on pancreatic mRNA survival. We assumed that any degradation of SPINK1 and PRSS1 mRNA in the pancreas occurred in parallel, but this is unproven. Finally, the question as to the degree that PRSS1 and SPINK1 mRNA levels reflect protein levels is unanswered, but is believed to be fairly direct.

Understanding the mechanism of disease penetrance and non-penetrance in subjects with cationic trypsinogen R122H mutations provides clues to the genetic mechanisms of protection from unregulated trypsinogen activation. The current study suggests that the determinants of penetrance and non-penetrance are not at the level of mutant trypsinogen expression or SPINK1 expression. Likely a triggering event is needed to initiate the process leading to pancreatitis. A better understanding of the trigger mechanism leading to trypsinogen activation is needed to determine how individuals who appear to be at high risk of pancreatitis remain symptom free for a lifetime.

Conflict of interest: declared (the declaration can be viewed on the *Gut* website at http://www.gutjnl.com/supplemental).

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