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A 93 year old man with the *PRSS1* R122H mutation, low *SPINK1* expression and without pancreatitis: Insights into phenotypic non-penetrance.

Short Title— *PRSS1* R122H and *SPINK1* expression in HP

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Abstract

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), 1 with chronic pancreatitis and 1 with 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 nonpenetrant subjects with R122H genotypes using allele-specific quantitative RT-PCR and intron-spanning nested RT-PCR followed by cDNA sequencing. Compared to *PRSS1* mRNA levels, *SPINK1* mRNA levels were low in normal-appearing tissue but marked increased in samples with chronic inflammation independent of *PRSS1* genotype.

Conclusion: Attacks of acute pancreatitis in hereditary pancreatitis 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.

Introduction

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 ~80% of individuals before age 20 years with pancreatitis-associated mutations, a 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 about 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 nonpenetrant) (4, 6, 7).

The mechanism of nonpenetrance 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 centers 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. Upon 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 nonpenetrance in HP. These include: (a) Does nonpenetrance reflect an inability to detect sub-clinical pancreatitis? (b) Is non-penetrance due to epigenetic factors that alter the expression of the gain-of-function mutation (e.g. expression of R122 but not H122)? (c) Is non-penetrance due to relative over-expression of the pancreatic secretory trypsin inhibitor (PSTI, serine protease inhibiter, Kazal-type 1; UniGene Symbol *SPINK1*, MIM 167790) compared to *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 nonpenetrance.

Alternatively, the relative expression of the trypsin inhibitor gene, *SPINK1*, may be enhanced compared to trypsinogen in nonpenetrant patients. This hypothesis is based on the assumption that SPINK1 is the first line of defense 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). Since the stochiometry of the trypsin-SPINK1 inhibition is one to one, the relative expression of the two genes is likely relevant. Thus, relative expression of these two critical genes was also investigated.

Methods

Patients:

Studies were conducted with the approval of the University of Pittsburgh

Institutional Review Board (IRB) 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 93 year old phenotypically nonpenetrant hereditary pancreatitis (R122H) study subject were obtained during a rapid autopsy (within 2 hours of death), snap-frozen and stored at -80° C until analysis. Pancreatic tissue for comparison was obtained as surgical waste from an affected patient with hereditary pancreatitis, a patient with chronic pancreatitis and normal pancreatic tissue.

Histology:

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

Differential R122 and H122 Expression:

Frozen tissue samples were homogenized in TRIzol (Life Technologies, Grand Island, NY) on dry ice, extracted in chloroform, and precipitated in isopropyl alcohol. The pellet was washed in 75% ethanol, re-suspended 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° C. The 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.

a). Allele specific quantitative PCR of cDNA: The cDNA prepared was used to determine relative allelic expression of R122 and H122 trypsinogen (qPCR). Fluorescent probes and primers used are illustrated in Figure 1 with the probes aligning genomic DNA sequence of H122 and R122 trypsinogen. Probe cross hybridization was evaluated using PCR amplified R122 and H122 bacteriophage clone. The samples were run on an ABI 7700 Sequence Detection system (Applied Biosystems). The reaction master mix contained 12 µl DEPC-treated H_2O, 25 µl TaqMan Universal PCR master mix (Applied Biosystems), 2.5 µl of forward and reverse primer (250 nM final concentration), and 1.0 µl of each probe (100nM final concentration). All reactions were performed in quadruplicate. Negative controls included samples from the PCR amplification reaction without cDNA. Cycling conditions were 50° C for 2 minutes, 95° C for 12 minutes, then 95° for 15 seconds and 64° C for 1 minute X 40 cycles. The results were analyzed using sequence detector (Applied Biosystems). The experiment was repeated 3 times to assure reproducibility.

b) cDNA sequencing: A complimentary approach was used to verify of R122 and H122 RNA. In order to eliminate the amplification of genomic DNA external PCR primers were designed to span the junction between exon 1 and 2 in the forward direction (CT**C**-TTGCTGCCCCCTTT; dash indicating the location of the exon 1-2 junction, bold indicating the *PRSS1*-specific base) and exon 5 and 4 in the reverse direction (CCAGAATCACCCTGACATGA). A *PRSS1* gene specific RT reaction included: 1 ug RNA, 1x PCR buffer II (ABI), 7.5 mM MgCl2, 1 mM dNTPs, 40 nM external reverse primer, 0.4u/ul RNase inhibitor (Promega), and 0.1 u/ul MMLV (Epicentre). Thermal cycler conditions were as follows: 48˚C x 40 minutes, 95˚C x 5 minutes. PCR was performed as follows on the cDNA as well as 200 ng gDNA to verify the primers specifically amplify cDNA: 1x PCR buffer II (ABI), 1.5 mM MgCl2, 200 uM dNTPs, 200 nM forward and reverse primer, 0.05u/ul AmpliTaq Gold (ABI) and 25ng cDNA (2x dilution of stock cDNA). PCR temperature parameters were: 1 cycle 95 ˚C x 12 minutes,

35 cycles 95˚C x 15 seconds, 64˚C x 1 minute, 72˚C x 1 min. Internal sequencing primers for PRSS1 were designed to span the junction between exon 2 and 3 in the forward direction (CACTGCTACAAGTC-CCGCAT) with a reverse primer within exon 4 (TTCACACT**T**AGCCTGGCTCA). The external PCR product was treated with exonuclease prior to sequencing. Sequencing was performed on an ABI 3730 Sequencer. Results were analyzed 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 10X SybrGreen PCR Buffer, 2.5 µl of forward and reverse primer (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 are as those for differential allelic discrimination.

Results:

The pancreas of the 93 year old nonpenetrant 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 nonpenetrant 0.26 and 0.13 cycles, normal pancreas 0.17 and 0.77 cycles, hereditary pancreatitis 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 2 alleles was used to calculate difference in mRNA quantity based on the assumption that 1 PCR cycle equals a 2 fold difference in mRNA. A one cycle difference suggests twice the starting RNA amount for the allele reaching cycle threshold 1 cycle earlier. For example, if CT for allele A=20 and B=22 than allele A has 4 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 (0.19 +/- 0.25 cycles) and the HP nonpenetrant samples (-0.2 +/- 0.44 cycles) was similar (Figure 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 nonpenetrant 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.

Direct sequencing of the cDNA exon 3 spanning rtPCR product in both the forward and reverse direction for the HP nonpenetrant subject verified that both the R122 and H122 alleles were expressed and the signal amplitude was equal (Figure 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 amongst the samples. The SPINK1:PRSS1 ratio for the normal and HP non-penetrant

samples was <1:1000. The 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 was >6:1 (Figure 4).

Discussion

The current study provides rare insights into the biology and genetics of the pancreas from a phenotypically nonpenetrant *PRSS1* R122H subject well beyond the typical age of hereditary pancreatitis onset. The histological evaluation excluded significant sub-clinical 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-hybridization, since control experiment with phage template suggested highly specific probes. These data suggest that promoter methylation and gene suppression was not the mechanism of nonpenetrance in this subject.

We anticipated that the 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, i.e. the phenotypically nonpenetrant *PRSS1* R122H carrier had a low, rather than 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 represent a novel and important finding, it does not explain nonpenetrance in hereditary pancreatitis.

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 subjects' 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 nonpenetrance in subjects with cationic trypsinogen R122H mutations provides clues to genetic mechanisms of protection from unregulated trypsinogen activation. The current study suggests that the determinants of penetrance and nonpenetrance 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.

COMPETING INTEREST STATEMENT Dr Whitcomb has patented clinical testing of the trypsinogen genes and patients with hereditary pancreatitis. The genetic testing has been licensed to Ambry Genetics, Irvine CA. None of the other authors have any competing interests.

ETHICS APPROVAL The study was reviewed and approved by the University of Pittsburgh Institutional Review Board.

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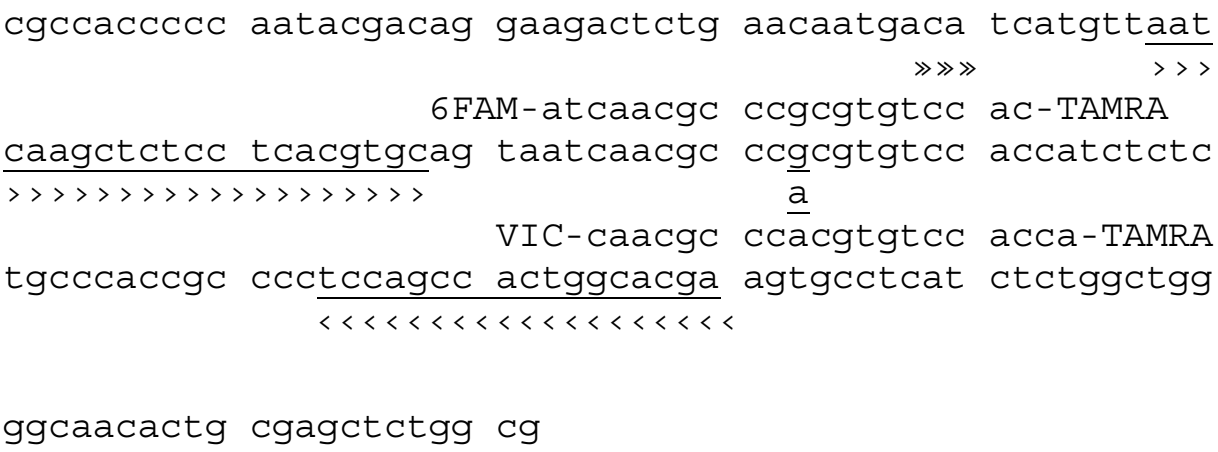
Figure 1: represents a portion of exon 3 of the human trypsin cDNA sequence. The specific probes are shown in bold overlapping a portion of the sequence including the mutation. The location of the mutation is underlined, the ***g*** is the wild type sequence and in the mutant allele the ***g*** is substituted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

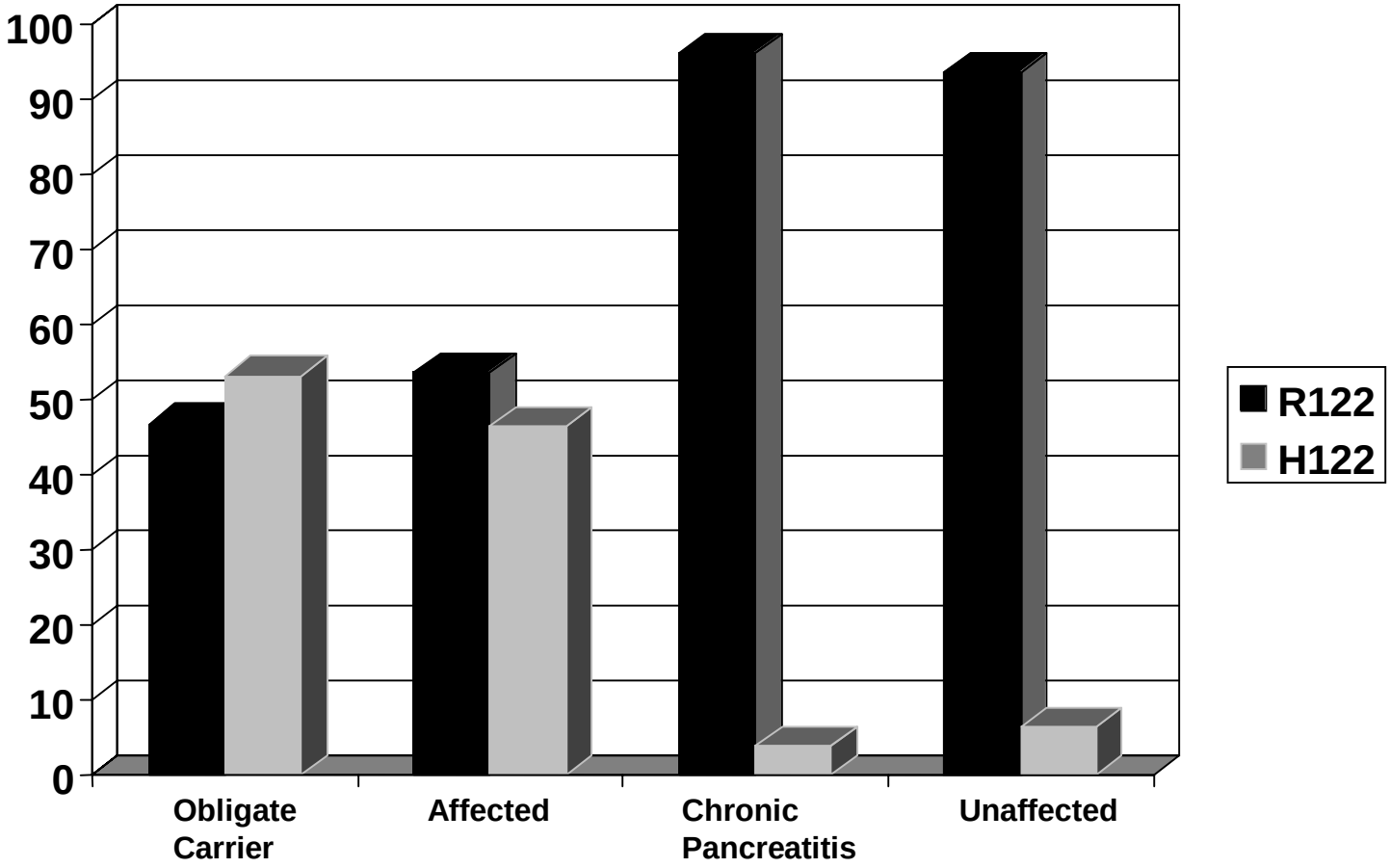
Figure 2: Quantitative relative allelic expression of HP and non-HP samples. R122 and H122 ratios are similar for the HP (obligate carrier/nonpenetrant and affected) samples with negligible H122 amounts expressed in the non-HP (chronic pancreatitis and normal) samples.

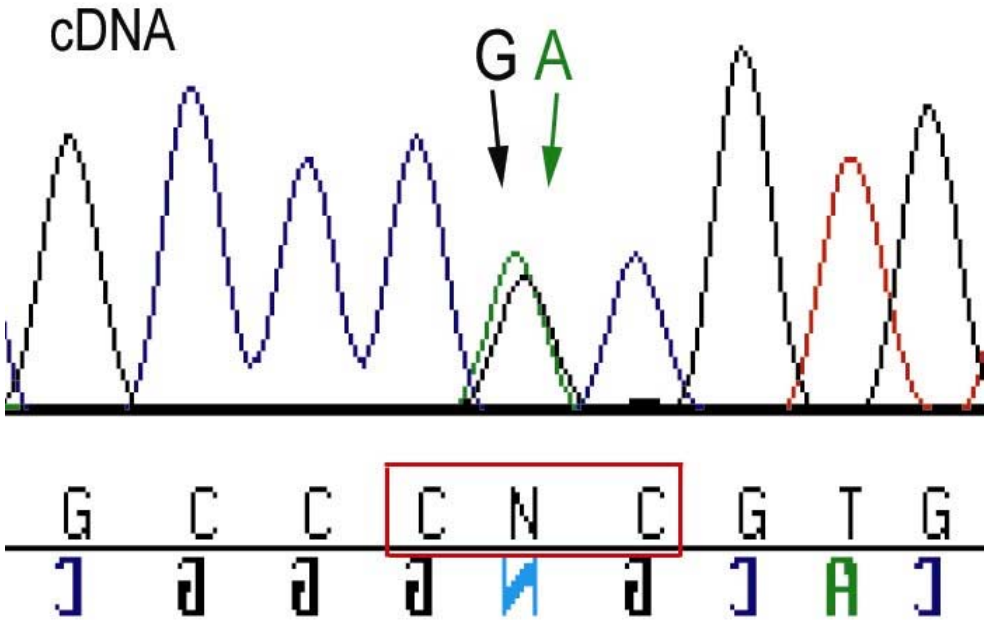
Figure 3: Sequencing of PRSS1-specific cDNA. Note that the signal at the second position of codon 122 is similar in amplitude for the G (normal) and A (mutant) nucleotide.

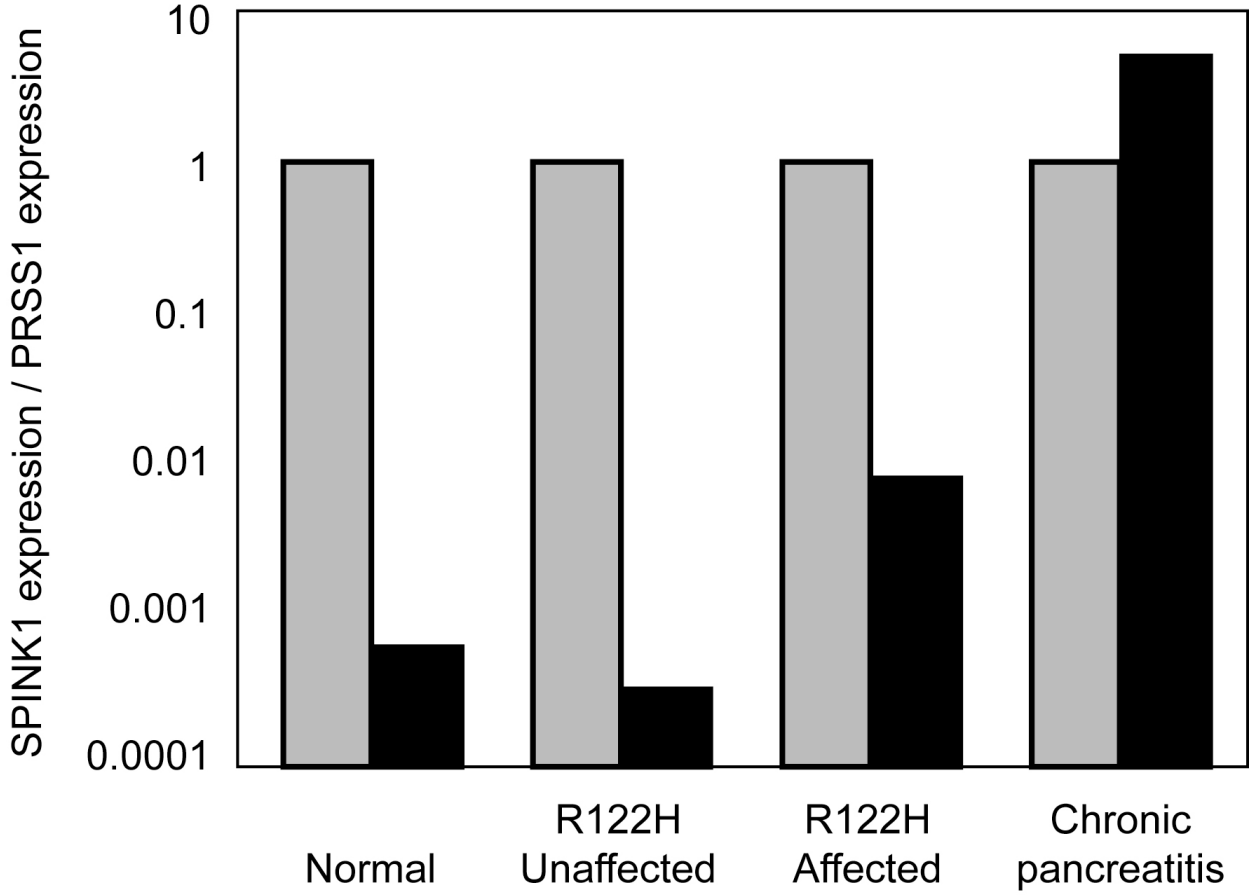
Figure 4: Relative abundance of PSTI/ SPINK1 mRNA to Trypsin/ PRSS1 mRNA. All data is normalized to PRSS1 mRNA (grey bars). PSTI/ SPINK1 mRNA levels (black bars) are relatively low compared to PRSS1 (<1:1000) in normal and R122H unaffected subjects, but SPINK1 mRNA levels are markedly increased relative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

Figure 1:









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