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A degradation-sensitive anionic trypsinogen (*PRSS2*) variant protects against chronic pancreatitis

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Abstract

Chronic pancreatitis (CP) is a common inflammatory disease of the pancreas. Mutations in the genes encoding cationic trypsinogen (*PRSS1*)¹ and the pancreatic secretory trypsin inhibitor (*SPINK1*)² are associated with CP. Since increased proteolytic activity due to mutated *PRSS1* enhances the risk for CP, mutations in the gene encoding anionic trypsinogen (*PRSS2*) may also act disease predisposing. Here we analyzed *PRSS2* in CP patients and controls and found, to our surprise, that a variant of codon 191 (G191R) is overrepresented in control subjects: G191R was present in 220/6,459 (3.4 %) controls but only in 32/2,466 (1.3 %) patients (odds ratio 0.37; $P = 1.1 \times 10^{-8}$). Upon activation by enterokinase or trypsin, purified recombinant G191R protein showed a complete loss of trypsin activity due to the introduction of a novel tryptic cleavage site that renders the enzyme hypersensitive to autocatalytic proteolysis. In conclusion, the G191R variant of *PRSS2* mitigates intrapancreatic trypsin activity and thereby plays a protective role against chronic pancreatitis.

Three different isoforms of trypsinogen have been described in human pancreatic juice. According to their electrophoretic mobility on isoelectric focusing, they have been designated as cationic trypsinogen (*PRSS1*), anionic trypsinogen (*PRSS2*) and mesotrypsinogen (*PRSS3*)³. Anionic trypsinogen, also referred to as serine protease 2, is one of the most abundant secretory proteins synthesized by the pancreas^{3,4}. The ratio of the anionic to the cationic forms

is approximately 1:2, whereas mesotrypsinogen is only secreted in low quantities³. Compared to the cationic isoenzyme, anionic trypsinogen autoactivates less easily, particularly at acidic pH, and is more sensitive to autolysis^{5,6}. The gene encoding human *PRSS2* (OMIM 601564) maps to 7q35, is approximately 3.6 kb long, and contains 5 exons⁷. The preproprotein comprises 247 amino acids, including a 15-amino acid signal peptide and an 8-amino acid activation peptide.

PRSS1 (OMIM 276000) and *SPINK1* (OMIM 167790) mutations are strongly associated with hereditary and idiopathic CP^{1,2}. However, the majority of patients do not carry mutations in either of these two genes, suggesting that defects in further genes might be involved in the pathogenesis of the disease². Since mutations in *PRSS1* can cause pancreatitis, we hypothesized that, in analogy, gene variants of the anionic isoenzyme *PRSS2* could also modify the susceptibility to CP. We therefore investigated the coding regions and flanking intronic sequences of *PRSS2* in 200 German patients and 240 control subjects by single-strand conformation polymorphism (SSCP) analysis and direct DNA sequencing.

In these subjects we identified three missense *PRSS2* variants. Two of the variants, T8I and V118I, were equally common among patients with CP (12.5% and 5%, respectively) and controls (15% and 4.6%, respectively). In contrast, the third variant, a G→A transition resulting in a substitution of glycine by arginine at codon 191 in exon 4 (G191R), was present in 9/240 (3.8 %) control subjects, but in none of the 200 patients investigated.

We subsequently expanded the study to include additional German patients and controls. By using melting curve analysis we confirmed the higher prevalence of the heterozygous G191R variant in the control group (71/2,019 [3.5 %]) vs. CP patients (19/1,548 [1.2 %]) (OR 0.34; $P = 0.000012$) (Table 1).

In order to investigate whether our findings were applicable to other European populations, we investigated further 918 CP patients and 4,440 control subjects from Austria, the Czech Republic, Denmark, France, Italy, the Netherlands, Romania, Spain, and Switzerland. Because of the low case numbers in some of these populations we pooled them together as European sample without Germans. The frequency (3.4%) and the 95% confidence interval (CI, 2.8-3.9) for the controls in this European sample lay well in the range of the German sample (frequency 3.5%; 95% CI 2.7-4.3). The frequency in controls (149/4,440 [3.4 %]) was significantly higher than in CP patients (13/918 [1.4 %]) (OR 0.41; $P = 0.00097$) (Table 1). Taking all sampled European populations together, the frequency of G191R was 1.3 % (32/2,466) in the patient group compared to 3.4 % (220/6,459) among control subjects (OR=0.37; $P = 1.1 \times 10^{-8}$) (Table 1). Stratification of the subgroups (alcoholic and idiopathic/hereditary pancreatitis) showed no significant differences between the different patient groups. For detailed data see Supplementary File 1.

Further analyses showed that patients with G191R were of higher age than those without the protective variant. In the idiopathic/hereditary pancreatitis group we found G191R in 24/1,256 (1.9 %) patients older than 20 years compared to 3/601 (0.5 %) patients of younger age ($P = 0.021$), however, due to limited sample size these data should be interpreted with caution. A similar tendency was observed in the alcoholic chronic pancreatitis group: None of the 162 patients with an age of 40 years or younger showed G191R compared to 5/447 (1.1 %) of patients older than 40 years ($P = 0.332$) (see Supplementary File 1). No age-related differences were observed in the control group (3.4 % vs. 3.3 % in subjects at or below 20 years vs. subjects above 20 years and 3.2 % vs. 3.5 % in subjects at or below 40 years vs. subjects above 40 years, respectively).

In contrast to unaffected Europeans, who showed a G191R frequency of 3.4 %, the variant was rare in individuals of African descent (2/948) with an estimated overall frequency was 0.2 %

(95% CI 0-0.5), which was significantly different from the frequency we found in the European population ($P = 10^{-11}$).

One of the 6,459 control subjects (originating from the Netherlands) but none of the patients was homozygous for G191R. When analyzing the G191R variation among 204 parents of Austrian or German CP patients, we detected 6 G191R carriers. However, in none of the cases was the variant transmitted from the unaffected parent to the affected child.

To exclude that G191R is in linkage disequilibrium with other variants in *PRSS2* or *PRSS1*, which is located only 40 kb centromeric to *PRSS2*, we bi-directionally sequenced the complete *PRSS1* and *PRSS2* coding sequence in 60 *PRSS2* G191R heterozygotes (10 patients and 50 control individuals). With the exception of the common *PRSS2* polymorphisms T8I and V118I, which were present in a similar frequency as found in the above mentioned German patients and control subjects (15 % and 1.7 %, respectively), no other *PRSS2* variant within the coding region was found. Moreover, none of these 60 G191R heterozygotes carried a *PRSS1* missense mutation.

In order to determine the haplotype structure in the genomic context of G191R, we analyzed 17 SNPs (going from *rs2156965* to *rs985180*) around *PRSS2* (Fig. 1). From a cohort of 95 subjects corresponding to 36 families with at least a carrier of G191R we found a small common haplotype always associated with G191R (233112 or CGGAAC in Table 2a; Block 1 in Fig. 1). This minimum haplotype starts at *rs10273639* (located within *PRSS1* 40 kb centromeric from *PRSS2*) and ends with *rs2367487* (located 15 kb telomeric from *PRSS2*) corresponding to an interval of ~60 kb ($D' = 0.90$). In contrast, we found 8 different haplotypes on 72 chromosomes bearing the wild-type (Table 2a). Taking into account a SNP 51 kb centromeric from *PRSS2* (*rs1964986*) and 22 kb telomeric from *PRSS2* (*rs2071361*) we found 3 haplotypes: The first haplotype was found on 10/22 (46 %), the second on 8/22 (36 %), and the third on 4/22 chromosomes (18 %) (Table 2b).

To exclude the genetic effect of potential causative variant(s) in any non-233112 haplotypes, we further analyzed in all German patients and control subjects *rs10273639* and *rs997222*, which tag the two most common haplotypes (233312 and 411314) and the only other one with a significant frequency (233342; 10 %), respectively (Table 2a). Allele frequencies of both SNPs did not significantly differ between both groups: *rs10273639* T 1,277/3,096 (41.2 %) vs. 1,715/4,038 (42.5 %); $P = 0.31$ and *rs997222* T 242/3,096 (7.8 %) vs. 355/4,038 (8.8 %); $P = 0.14$. In addition, haplotype analysis as well as the very rare frequency of G191R in individuals of African descent indicate that G191R is a founder allele and not a recurrent mutation.

The glycine at position 191 is strictly conserved in vertebrate trypsinogens. Although the crystal structure of human anionic trypsin remains to be solved, comparison with rat trypsin or the homologous human cationic trypsin (*PRSS1*) structure (Fig. 2) suggest that Gly191 is located on the surface of the enzyme near the primary substrate binding pocket. Therefore, the G191R alteration is expected to generate a surface-exposed, positively charged, bulky arginine side chain. The novel Arg191-Gly192 peptide bond would be predicted to be highly amenable to cleavage by trypsin. *PRSS2* lacks the conserved Cys139-Cys206 disulfide bond which would prevent the dissociation of the C-terminal proteolytic fragment⁸. This fragment contains the catalytic Ser200 residue, which is essential for enzymatic activity (Fig. 2). The cleavage should, therefore, inactivate trypsin(ogen) rapidly and irreversibly.

To test this hypothesis, we recombinantly expressed wild-type anionic trypsinogen and the G191R variant. Both the physiological activator enteropeptidase (enterokinase) and trypsin strongly activated wild-type *PRSS2*, and the trypsin activity generated remained stable over the entire time period studied. In contrast, the G191R variant showed only minimal or no tryptic

activity after activation (Fig. 3a-c). Analysis of the activation process by reducing SDS-PAGE showed a time-dependent conversion of trypsinogen to trypsin for wild-type PRSS2. In contrast, activation of the G191R variant led to a strong novel band corresponding to the N-terminal proteolytic fragment of trypsinogen cleaved after Arg191 (Fig. 3a-c). Removal of the activation peptide from this proteolytic fragment by enteropeptidase yielded a smaller fragment corresponding to the N-terminal fragment of trypsin cleaved after Arg191 (Fig. 3a). The proteolytic processing of the G191R mutant was further characterized by mass spectrometry (Fig. 4). After incubation with enteropeptidase, wild-type trypsinogen (25,069.2 Da) was converted to trypsin (24,034 Da), whereas the G191R mutant proenzyme (25,168.3 Da) was rapidly converted to cleavage products of molecular masses corresponding to the N-terminal peptide 16-191 (19,233.7 Da) and the C-terminal peptide 192-247 (data not shown). Intact G191R trypsin was nearly undetectable. Enteropeptidase also converted the N-terminal G191R trypsinogen fragment to its corresponding trypsin fragment (18,196.5 Da) (Fig. 4). Incubation with trypsin as activating enzyme similarly demonstrated rapid cleavage of the G191R mutant at the Arg191-Gly192 peptide bond (data not shown). Taken together, this functional analysis confirms that the G191R substitution generates a supersensitive tryptic cleavage site and destines anionic trypsin(ogen) for autocatalytic degradation.

It is currently thought that pancreatitis results from an imbalance of proteases and their inhibitors within the pancreatic parenchyma. Whitcomb *et al.* first showed that a gain-of-function mutation in the cationic trypsinogen gene is a cause of hereditary pancreatitis¹. A more recent study provided evidence that loss-of-function mutations in the specific trypsin inhibitor, *SPINK1*, can also predispose to chronic pancreatitis². Thus, it is now generally believed that inappropriate intrapancreatic activation of trypsinogen is a precondition for acinar cell injury and pancreatitis. Along the same line of thought, it is conceivable that loss-of-function trypsinogen mutations should afford protection against pancreatitis. The present study is the first to experimentally support this notion not only by identifying a novel *PRSS2* variant which is significantly underrepresented in pancreatitis patients of various ethnic origins but also by providing experimental evidence that this variant conveys a loss of function. Albeit the overall contribution of G191R to disease pathogenesis is low, the functional characterization of the G191R mutation reported here provides the first example in pancreatitis - and a rare example as far as inheritable diseases in general are concerned - for a disease-protective genetic variant.

Methods

Patients

The study was approved by the medical ethical review committee of the Charité University Hospital. All patients gave informed consent for genetic analysis. The study population included 2,466 patients suffering from chronic pancreatitis (609 with alcohol-related disease and 1,857 with either idiopathic or hereditary etiology) originating from Austria (n = 24), the Czech Republic (n = 92), France (n = 359), Germany (n = 1,548), Italy (n = 141), the Netherlands (n = 139), Romania (n = 3), Spain (n = 64), and Switzerland (n = 96). The clinical diagnosis of CP was based on two or more of the following criteria: presence of a typical history of recurrent pancreatitis, radiological findings such as pancreatic calcifications and/or pancreatic ductal irregularities revealed by endoscopic retrograde pancreatography or magnetic resonance imaging of the pancreas and/or pathological sonographic findings. Hereditary CP was diagnosed when two first-degree relatives or three or more second-degree relatives, suffered from recurrent acute or chronic pancreatitis without any apparent precipitating factor. Patients were classified as having idiopathic CP when precipitating factors such as alcohol abuse, trauma, medication, infection, metabolic disorders and/or a positive family history, were

absent. Alcohol-induced CP was diagnosed in patients who consumed more than 60 g (females) or 80 g (males) of ethanol per day for more than two years.

The 6,459 control subjects were recruited from Austria (n = 637), the Czech Republic (n = 522), Denmark (n = 95), France (n = 1,120), Germany (n = 2,019), Italy (n = 326), the Netherlands (n = 565), Romania (n = 163), Spain (n = 518), and Switzerland (n = 494).

To determine the frequency of G191R in non Europeans, we studied 948 individuals of African descent originating from Benin (n = 161), Cameroon (n = 412), Ethiopia (n = 155), and Ecuador (n = 220). Detailed characteristics of patients are available online as Supplementary File 2.

Mutation screening

Oligonucleotide sequences and PCR conditions are available online as Supplementary File 3. We analyzed DNA by sequencing both strands of the entire coding region.

Melting curve analysis

We performed melting curve analysis to detect the G191R variant using a pair of fluorescence resonance energy transfer (FRET) probes and the LightCycler (Roche Diagnostics). The sensor probe was 5'-CAGGAATCCTTGCCTCCCTC-FL (FL: 5,6-carboxyfluorescein attached to 3'-O-ribose) and the anchor probe was 5'-LC-AGGAAGCCCACACAGAACATGTTGTTG-ph (LC: LightCycler Red 640 attached to 5' terminus; ph: 3' phosphate). The sensor probe was complementary to the wild-type sequence resulting in an allele-specific melting curve (62.5 °C vs. 56 °C). For further details on melting curve analysis and assay validation see the Supplementary File 3 available online.

Haplotype analysis

For haplotype analysis we selected a total of 17 SNPs (going from rs2156965 located 117 kb centromeric from *PRSS2* to rs985180 located 63 kb telomeric from *PRSS2*; Fig. 1) from HapMap (<http://www.hapmap.org>). We determined the genotypes of 95 subjects from 36 families with at least a carrier of G191R by direct DNA sequencing. In five cases we were unable to determine the haplotype. Oligonucleotide sequences and PCR conditions for amplifying the five SNPs defining Block 1 (rs10273639, rs1985888, rs2855983, rs997222, rs2367487) as well as the two nearest SNPs flanking Block 1 (*rs1964986* and *rs2071361*) are available online as Supplementary File 3. Pair-wise LD was calculated and visualized using the Haploview program¹⁰.

Mutation Modeling

We modeled the effect of the G191R variant using the human cationic trypsin crystal structure (Protein Data Bank file 1TRN)¹¹. The image was rendered using DeepView/Swiss-PdbViewer v. 3.7 (www.expasy.org/spdbv/)¹².

Generation of recombinant PRSS2 and activation assays

Site-directed mutagenesis, recombinant expression, in vitro refolding and purification of human trypsinogens were carried out as described previously^{13,14}. Recombinant trypsinogen preparations (2 µM final concentration) were incubated at 37°C, in 0.1 M Tris-HCl (pH 8.0) and 1 mM CaCl₂, in a final volume of 100 µL. Activation was initiated with 20 ng/mL bovine enteropeptidase (Biozyme) or 0.1 µM trypsin (final concentrations). Aliquots of 2.5 µL were withdrawn from reaction mixtures at indicated times and trypsin activity was determined with 0.14 mM N-CBZ-Gly-Pro-Arg-p-nitroanilide (final concentration). Activity was expressed as percentage of the potential total activity, which was determined on a separate wild-type trypsinogen sample after activation with enteropeptidase at 22 °C in 0.1 M Tris-HCl (pH 8.0).

and 10 mM Ca^{2+} . For electrophoretic gel analysis, samples were precipitated with 10 % trichloroacetic acid (final concentration), resolved by 12 % SDS-PAGE under reducing conditions, and stained with Coomassie Blue, as described earlier¹⁵.

Mass spectrometric determination of cleavage sites

Identification of the cleavage sites within Arg191 trypsinogen was performed using Matrix-Assisted-Laser-Desorption/Ionisation-Time-Of-Flight-Mass-Spectrometry (MALDI-TOF-MS). Wild-type and Arg191 trypsinogens were incubated in 10 μL of 50 mM ammonium bicarbonate buffer, pH 8.0, containing 10 mM CaCl_2 and 10 mU enteropeptidase (Sigma). At the indicated time points, 0.5 μL aliquots were taken and subsequently co-crystallized with 0.5 μL sinapinic acid (saturated in 70% acetonitrile) on a SCOUT 384 -MALDI-Target. Mass spectrometry was performed on a MALDI-TOF-MS (Reflex III, Bruker Daltonics, Germany) instrument in linear mode with internal calibration. Annotation of trypsinogen fragments was performed with the BioTools 2.0 software (Bruker Daltonics, Germany).

Statistics

The significance of the differences between mutation frequencies in patients and controls as well as between different control populations were tested by two-tailed Fisher's Exact tests and additional odds ratios have been calculated using SAS/STAT software, Version 9.1 of the SAS System for Windows.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig. 1.

Haploview graph of the pair-wise LD among 17 SNPs around the *PRSS2* locus. Block 1 indicates the minimal haplotype always associated with *PRSS2* G191R. $D'=1.00$ in the blank red squares; numbers inside the squares are $D' \times 100$. Genomic locations of the different SNPs are illustrated above the LD plot.

Fig. 2. Ribbon diagram of human cationic trypsin (Protein Data Bank file 1TRN) showing Gly191 (chymotrypsin numbering [chymo#] Gly187) mutated to Arg (in red). Also shown is the primary specificity determinant Asp194 (in green; chymo#Asp189) and the catalytic triad His63, Asp107 and Ser200 (in orange, chymo# His57, Asp102 and Ser195). The yellow peptide segment is removed after cleavage of the Arg191-Gly192 peptide bond.

Fig. 3. Activation of wild-type and Arg191 mutant anionic trypsinogens with enteropeptidase (a), human cationic trypsin (b) or human anionic trypsin (c). Activation assays and gel electrophoresis were carried out as described in Methods. Trypsin activity was expressed as percentage of the potential total activity. In experiments b and c, the activity of the added trypsin was subtracted from the total activity measured. The single asterisk indicates the N-terminal trypsinogen fragment generated by cleavage of the Arg191-Gly192 peptide bond. This trypsinogen fragment is further processed by enteropeptidase to the corresponding trypsin fragment (double asterisk). The C-terminal peptide is not detected under these electrophoretic conditions. Control experiments using human pancreatic trypsin inhibitor demonstrate that

enteropeptidase cannot cleave the Arg191-Gly192 peptide bond and that the degradation observed during enteropeptidase-activation is entirely attributable to trypsin (data not shown).

Fig. 4.

Mass spectrometric analysis of trypsinogen activation. Wild-type and Arg191 variant anionic trypsinogen were incubated at pH 8.0 in the presence of 10 mM CaCl_2 , as described in Methods. At the indicated time points, samples were withdrawn and directly analyzed by MALDI-TOF mass spectrometry. The left shoulders observed on the trypsinogen peaks are due to partial N-terminal processing of recombinant trypsinogen by *E. coli* aminopeptidases; the right shoulders are sinapinic acid adducts. The molecular masses indicated correspond to average peptide masses with cysteines oxidized as disulfides. Note that recombinant trypsinogens contain an extra methionine at the N terminus.

Table 1
Frequency of G191R *PRSS2* variant in patients and controls

Population	Patients		Controls		OR	P value
	Mutation/Total	%	Mutation/Total	%		
German	19/1548	1.2	71/2019	3.5	0.34	0.000012
European (w/o German)	13/918	1.4	149/4440	3.4	0.41	0.00097
European (all)	32/2466	1.3	220/6459	3.4	0.37	1.1×10^{-8}

Number of mutations and sample sizes, frequencies of mutations, 95 % confidence intervals (95% CI) of the frequencies for patients and for controls, odds ratio (OR), and the P-value of the Fisher's Exact Test.

Table 2a
Haplotype analysis of the G191R region

rs10273639 -40 kb	rs1985888 -36 kb	rs2855983 -1 kb	G191R PRSS2	rs907222 +12 kb	rs2367487 +15 kb	n	%
2	3	3	1	1	2	27	100%
2	1	1	3	1	4	2	3%
2	3	1	3	1	4	2	3%
2	3	3	3	1	2	29	40%
2	3	3	3	1	4	1	1%
2	3	3	3	4	2	7	10%
2	3	3	3	4	4	1	1%
4	1	1	3	1	2	1	1%
4	1	1	3	1	4	29	40%

Table 2b

Haplotype analysis of the G191R region

rs1964986 -51 kb	rs10273639 -40 kb	rs1985888 -36 kb	rs2855983 -1 kb	G191R PRSS2	rs997222 +12 kb	rs2367487 +15 kb	rs2071361 +22 kb	n	%
1	2	3	3	1	1	2	3	10	46%
2	2	3	3	1	1	2	2	8	36%
2	2	3	3	1	1	2	3	4	18%