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Complete Analysis of the Human Mesotrypsinogen Gene *(PRSS3)* in Patients with Chronic Pancreatitis

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Key Words

Chronic pancreatitis · Mesotrypsinogen · *PRSS3* · Variant · Haplotype · PHASE v2.1

Abstract

Background/Aims: A sustained imbalance of pancreatic proteases and their inhibitors seems to be important for the development of chronic pancreatitis (CP). Mesotrypsin (PRSS3) can degrade intrapancreatic trypsin inhibitors that protect against CP. Genetic variants that cause higher mesotrypsin activity might increase the risk for CP. ***Methods:*** We analyzed all 5 exons and the adjacent non-coding sequences of *PRSS3* by direct sequencing of 313 CP patients and 327 controls. Additionally, exon 4 was investigated in 855 patients and 1,294 controls and a c.454+191G>A variant in 855 patients and 1,467 controls. The c.499A>G (p.T167A) variant was analyzed functionally using transiently transfected HEK 293T cells. ***Results:*** In the exonic regions, the previously described common c.94_96delGAG (p.E32del) variant and a novel p.T167A non-synonymous alteration were identified. Extended analysis of the p.T167A variant revealed no association to CP and in functional assays p.T167A showed normal secretion and activity. Variants of the intronic regions, including the extensively analyzed c.454+191G>A alteration, were not associated with the disease. Haplotype reconstruction using variants with a minor allele frequency of >1% revealed no CP-associated haplotype. ***Conclusions:***

Jonas Rosendahl, Niels Teich and Heiko Witt contributed equally to this work.

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Although the trypsin inhibitor-degrading activity qualified *PRSS3* as a candidate for a novel CP susceptibility gene, we found no association between a specific variant or haplotype and CP in our cohort with a high suspicion of genetically determined disease.

Introduction

A sustained imbalance of pancreatic proteases and their specific inhibitors is thought to be a central pathogenic step in the development of chronic pancreatitis (CP). In the human pancreatic juice, three isoforms of trypsinogen are secreted that are encoded by the *PRSS* (protease, serine) genes. According to their electrophoretic mobility, these enzymes are referred to as cationic trypsinogen, anionic trypsinogen, and mesotrypsinogen (PRSS 1, 2 and 3), respectively.

Gain-of-function variants of the cationic trypsinogen (*PRSS1*, OMIM 276000) have been found in several families with hereditary chronic pancreatitis [1] and in CP patients without a family history (idiopathic CP (ICP)) [2, 3 and references within]. Triplication and duplication of the trypsinogen locus was also found in a subset of patients with hereditary CP and ICP [4]. On the other hand, loss of function variants of the natural intrapancreatic trypsin inhibitor *SPINK1* (OMIM 167790) are associated with a broad range of CP entities [5–7]. The importance of altered protease-antiprotease equilibrium in CP is also supported by the observation that the degradation-sensitive anionic trypsinogen (*PRSS2*, OMIM 601564) variant p.G191R protects against CP [8]. Additionally, we recently demonstrated that loss of function variants of the trypsin-degrading enzyme chymotrypsinogen C (*CTRC*, OMIM 601405) are also associated with CP [9].

Despite the growing number of genetic alterations identified in CP patients, in the majority of affected individuals no disease-associated variant can be detected, indicating that further genetic alterations might be involved.

PRSS3 accounts for 3–10% of trypsin activity in human pancreatic juice and biochemical experiments demonstrated a large degree of resistance to naturally occurring trypsin inhibitors as the most intriguing characteristic of this enzyme [10–12]. Furthermore, PRSS3 was shown to hydrolyze the reactive-site peptide bond of trypsin inhibitors such as SPINK1, suggesting that PRSS3 plays a role in degradation of these inhibitors. The ability of PRSS3 to degrade SPINK1 also raises the possibility that premature PRSS3 activation in the pancreas may contribute to the development of CP.

We hypothesized that activating mutations in the human mesotrypsinogen gene might represent novel risk factors for CP (fig. 1). In our previous study we showed that the common deletion polymorphism p.E32del is not associated with alcoholic CP [13]. Here we present the comprehensive analysis of the entire *PRSS3* gene in a large cohort of patients with CP and controls.

Materials and Methods

Patients

This study was approved by the Medical Ethical Review Committee of the University of Leipzig and of the Charité University Hospital. All individuals gave informed consent. The diagnosis of CP was based on two or more of the following findings: presence of a typical history of recurrent pancreatitis, pancreatic calcifications and/or pancreatic ductal irregularities revealed by endoscopic retrograde pancreaticography or by magnetic resonance imaging of the pancreas and/or pathological sonographic findings. Hereditary CP was diagnosed when 1 first-degree relative or 2 or more second-degree relatives suffered from recurrent acute CP or CP without any apparent precipitating factor. Affected individuals were classified as having ICP when precipitating factors, such as alcohol abuse, trauma, medication, infection, metabolic disorders or a positive family history were absent. Alcohol-induced CP was diagnosed in patients who consumed >60 g (females) or >80 g (males) of ethanol per day for more than 2 years.

In the two centers, Berlin and Leipzig, we investigated 313 unrelated patients with CP by DNA sequencing of the coding region of *PRSS3* (180 female and 133 male patients; Berlin n = 208, Leipzig n = 105; 263 ICP, 31 hereditary CP, 19 alcoholic CP; median age 16 years, mean age 22.7 years, range 0–83 years). In addition, 327 controls were enrolled (187 female, 140 male; Berlin n = 219, Leipzig n = 108; median age 72 years, mean age 62.8 years, range 20–100 years). To evaluate the role of the exon 4 variant c.499A>G (p.T167A), we screened exon 4 in 1,168 CP patients (487 female, 681 male; Berlin n = 882, Leipzig n = 286; 811 ICP, 82 hereditary CP, 275 alcoholic CP; median age 36 years, mean age 34.6 years, range 0–87 years) and in 1,621 controls (960 female, 661 male; Berlin n = 1,513, Leipzig n = 108; median age 32 years, mean age 39.8 years, range 17–100 years). In total, variant c.454+191G>A was investigated in 1,168 patients (details see above) and in 1,794 controls (1,093 female, 701 male; Berlin n = 1,513, Leipzig n = 281; median age 33 years, mean age 40.5 years, range 17–100 years).

Sequence Analysis of PRSS3

We extracted DNA from peripheral blood leukocytes. We analyzed all 5 exons and the intron/exon junctions of *PRSS3* by unidirectional DNA sequencing. PCR reactions were performed using slightly different conditions at the two centers. In Leipzig, 0.75 U AmpliTaq Gold polymerase (PerkinElmer), 450 μmol/l deoxynucleoside triphosphates, and 0.3 μmol/l of each primer were used in a total volume of 25 μl. In Berlin, 0.5 U AmpliTaq Gold

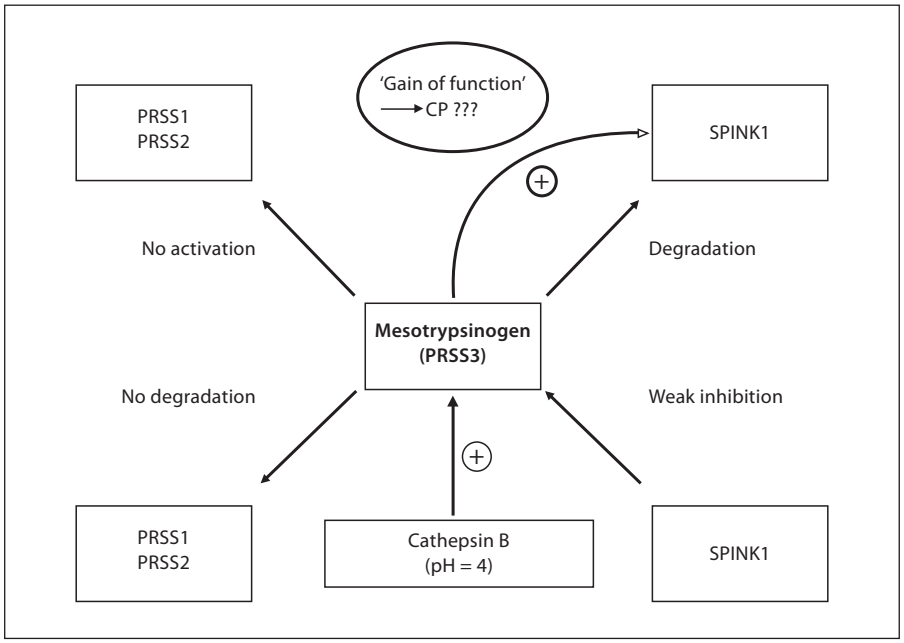

Fig. 1. Functional properties of mesotrypsinogen [11]. PRSS3 is weakly inhibited by SPINK1. PRSS3 does not activate or degrade PRSS1 or PRSS2. Cathepsin B activates PRSS3 at a higher rate than PRSS1 and PRSS2 at pH = 4. Gain-of-function mutations of *PRSS3* might lead to CP by disturbing the intrapancreatic protease-antiprotease equilibrium, due to enhanced degradation of trypsin inhibitors. SPINK1 = Serine protease inhibitor, Kazal type 1; CP = chronic pancreatitis; PRSS1 = cationic trypsinogen; PRSS2 = anionic trypsinogen; PRSS3 = mesotrypsinogen.

polymerase, 400 μmol/l deoxynucleoside triphosphates and 0.1 μmol/l primers were used. Cycle conditions were as follows: initial denaturation for 6 min at 95°C; 40 cycles of 20 s denaturation at 95°C, 40 s annealing (table 1) and 90 s primer extension at 72°C, and a final extension step for 6 min at 72°C. In Berlin, 12 min initial denaturation and 2 min final extension were used. PCR products were digested with shrimp alkaline phosphatase (USB) and exonuclease I (USB). Cycle sequencing was performed using BigDye terminator mix (Applied Biosystems). The reaction products were purified with ethanol precipitation or on a Sephadex G-50 column (Amersham) and loaded onto an ABI 3100-Avant or an ABI 3730 fluorescence sequencer (Applied Biosystems). DNA mutation numbering is based on a cDNA sequence (GenBank: NM_002771.2) that uses the A of the ATG start codon as nucleotide +1. The mutations are described according to the nomenclature recommended by the Human Genome Variation Society [http://www.hgvs.org/mutnomen]. Haplotype reconstruction was performed using PHASE software (v2.1) [14, 15]. For the reconstruction of haplotypes, only SNPs with a minor allele frequency of >1% were considered.

The significance of the differences between variant frequencies in affected individuals and controls was tested by two-tailed Fisher's exact test and was calculated using GraphPad Prism (v 4.03). p values <0.05 were considered to be of statistical significance. When appropriate, correction according to Bonferroni was carried out.

Functional Analysis of the p.T167A Variant

Mutation p.T167A was introduced into the pcDNA3.1(–)_PRSS3 expression plasmid by PCR mutagenesis. Human embryonic kidney (HEK) 293T cells were transfected with wild-type and p.T167A PRSS3 plasmids and secretion of mesotrypsinogen to the growth medium was measured at 24 and 48 h by activity assays after enterokinase activation. Mesotrypsinogen expression was also confirmed by SDS-PAGE and Coomassie Blue staining. Protocols for culturing and transfection of HEK 293T cells were described previously [9].

Results

In our initially screened cohort of 313 patients and 327 control subjects, we identified a novel c.499A>G transition resulting in a predicted threonine to alanine change at codon 167 (p.T167A), which was present in 2/327 controls (0.6%), but was not found in patients. Aside the novel p.T167A variant, the only other exonic alteration detected was the common c.94_96delGAG (p.E32del) variant, which was present with similar minor allele frequencies in patients (123/626, 19.6%) and controls (144/654, 22%) (table 2).

The intronic variants c.200+212G>A, c.200+256T>A, c.592-66C>G and c.592-188T>C showed no statistically significant difference in the distribution of genotypes (table 2) or allele frequencies (not shown). Additionally, several rare intronic variants were detected in each group (Berlin: c.200+123G>A, 0/208 patients, 1/219 (0.5%) controls; c.200+272G>A, 0/208 patients, 1/219 (0.5%) controls; c.454+9_454+10insTCCTCACAgCTgACTA, 1/208 (0.5%) patients, 2/219 (0.9%) controls; c.591+180C>T, 1/208 (0.5%) patients, 0/219 controls; c.592-220C>T, 1/208 (0.5%) patients, 0/219 controls; c.*8G>A, 0/208 patients, 1/219 (0.5%) controls); (Leipzig: c.200+67G>A,

Table 1. Oligonucleotide sequences used for PCR amplification and sequencing of *PRSS3* (Berlin upper section; Leipzig lower section)

Exon	Primer	Sequence (5′→3′)	Annealing temperature, °C
1–2	E1-2F-P	GCTGTGTGATCCGGGGATTGGT	60
	E1-2R-P	CACACTGTCTTCCTGAGAAG	
3–5	E3-5F-P	GCTGGGAAAACTACGAGGAGCTCT	64
	E3-5R-P	GGCGGCTCTGCTTACCCTTC	
1	E1F-S	AGCTGATGCAAGACCCTGGC	56
2	E2F-S	CCCTTGCCTGGCCTCACTGG	56
	E2R-S	CACACTGTCTTCCTGAGAAG	56
3	E3F-S	CCCCACCCCACTACCACCAG	56
4	E4F-S	TATTTGAATCCTCTTGCCTCCC	56
5	E5F-S	CGATACCCAGGCCCCACCG	56
1–2	E1-2F-P	CGTAGCCGAGCTGATGCAAGA	58
	E1-2R-P	CTGCAGGTGGTTTTGACGCATACT	
3–5	E3-5F-P	GCGCAGTTAGCAAAGACCT	60
	E3-5R-P	GCGGCTCTGCTTACCCTTC	
1	E1R-S	CCACAGCTCGGATATGGATG	60
2	E2F-S	GGCTTGTTAAGGATTTCTAATTG	60
	E2R-S	GGCAGCGGTTCTCAGCTCTGT	60
3	E3F-S	AGCAGTGAGCTTGAGGAC	60
4	E4F-S	CAGATTATTGTCTCCTTCTC	58
5	E5R-S	CCAGTGTGAAGGAGTGTGAG	62

Table 2. Variants investigated in 313 CP patients (Berlin/Leipzig) and in 327 controls (Berlin/Leipzig)

Variant	Genotype	Patients	Controls	p value
p.E32del c.94_96delGAG	WT/WT WT/del del/del	201/313 (64.2%) 101/313 (32.3%) 11/313 (3.5%)	198/327 (60.5%) 114/327 (34.9%) 15/327 (4.6%)	not significant
c.200+212G>A	G/G G/A A/A	309/313 (98.7%) 4/313 (1.3%) 0/313	327/327 0/327 0/327	not significant
c.200+256T>A rs83921	T/T T/A A/A	115/313 (36.7%) 151/313 (48.2%) 47/313 (15%)	111/325 (34.2%) 164/325 (50.5%) 50/325 (15.4%)	not significant
c.592-188T>C	T/T T/C C/C	263/313 (84%) 48/313 (15.3%) 2/313 (0.6%)	264/327 (80.7%) 59/327 (18%) 4/327 (1.2%)	not significant
c.592-66C>G	C/C C/G G/G	263/313 (84%) 48/313 (15.3%) 2/313 (0.6%)	264/327 (80.7%) 59/327 (18%) 4/327 (1.2%)	not significant

p values were calculated using two-tailed Fisher's exact test in a dominant (e.g. c.94_96delGAG (p.E32del): WT/WT+WT/del vs. del/del) and in a recessive (e.g. c.94_96delGAG (p.E32del): WT/WT vs. WT/del+del/del) model. None of the p values reached statistical significance. In this table only the variants are listed that were analyzed in both groups (Berlin and Leipzig). All variants were in Hardy-Weinberg equilibrium ($p > 0.05$).

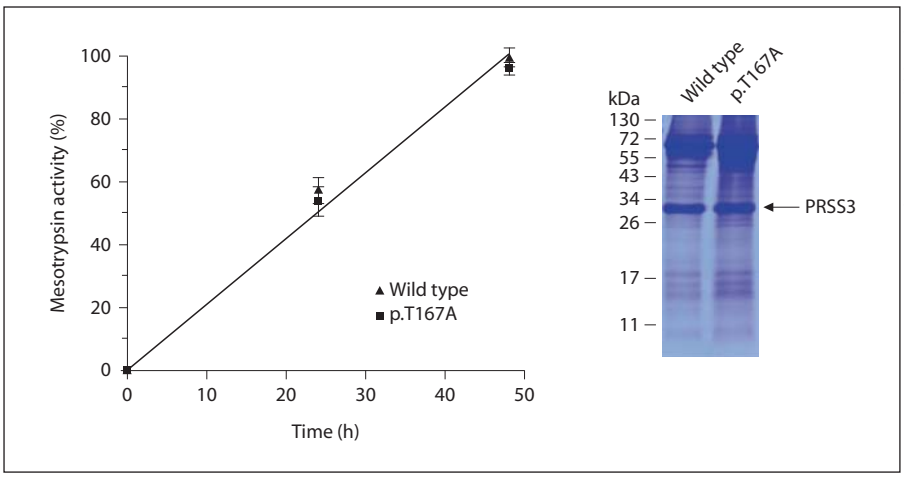

Fig. 2. Secretion of wild-type and p.T167A mutant mesotrypsinogen from transiently transfected HEK 293T cells. At 24 and 48 h after transfection, 2-μl aliquots of conditioned media were collected, activated with enterokinase, and trypsin activity was measured using the *N*-CBZ-Gly-Pro-Arg-*p*-nitroanilide substrate at 0.14 mM final concentration. Trypsin activities were expressed as percentage of the 48 h wild-type trypsin activity. The average of two independent transfection experiments is shown. Error bars indicate the standard error of the mean. For SDS-PAGE analysis, 48 h after transfection, 100-μl aliquots of conditioned media were precipitated with 10% trichloroacetic acid (final concentration), heat denatured at 95°C for 5 min in reducing Laemmli sample buffer and electrophoresed on 15% SDS-polyacrylamide gels. Gels were stained with Coomassie blue.

Table 3. Haplotype reconstruction using PHASE v2.1 considering *PRSS3* variants with a minor allele frequency of >1% in comparison to controls. In summary, 8 haplotypes were defined. The alleles of the different haplotypes are shown in the section alleles

Haplotype	Alleles	Patients	Controls	p value	p_c value
1	WT-A-G-T-C	75/626 (12%)	54/654 (8.3%)	0.03	n.s.
2	WT-A-G-C-G	37/626 (5.9%)	47/654 (7.2%)	n.s.	n.s.
3	WT-A-A-C-G	11/626 (1.8%)	20/654 (3.1%)	n.s.	n.s.
4	WT-T-G-T-C	380/626 (60.7%)	388/654 (59.3%)	n.s.	n.s.
5	Del-A-G-T-C	118/626 (18.8%)	144/654 (22%)	n.s.	n.s.
6	Del-A-G-C-G	4/626 (0.64%)	0/654	n.s.	n.s.
7	Del-T-G-T-C	1/626 (0.16%)	0/654	n.s.	n.s.
8	WT-A-A-C-T	0/626	1/654 (0.16%)	n.s.	n.s.

p values were calculated using two-tailed Fisher's exact test and were corrected according to Bonferroni (p_c values). p_c values <0.05 were regarded as statistically significant. n.s. = Not significant.

0/105 patients, 1/108 (0.9%) controls) (Berlin/Leipzig: c.201-22C>T, 1/313 (0.3%) patients, 0/327 controls). None of these variants showed a significant difference in the distribution between patients and controls and all investigated variants complied with Hardy-Weinberg equilibrium.

To clarify whether a distinct haplotype might be associated with CP, we reconstructed haplotypes including variants with a minor allele frequency of >1% (c.94_96delGAG (p.E32del), c.200+256T>A, c.454+191G>A, c.592-188T>C, c.592-66C>G) and identified 8 different haplotypes (table 3). Haplotype 1 was significantly overrepresented in the patient population (p = 0.03, OR 1.51, 95% CI 1.1–2.2), but this did not withstand Bonferroni correction for multiple testing (p = 0.3).

To confirm the enrichment of p.T167A in controls relative to patients, we extended the analysis of exon 4. However, after screening an additional 855 subjects with CP

Table 4. Extended screening of exon 4, variant c.454+191G>A and the c.591+10C>T variant in patients (Berlin/Leipzig) and in controls (Berlin/Leipzig)

Variant	Genotype	Patients	Controls	p value
c.454+191G>A	G/G	1,125/1,168 (96.3%)	1,709/1,794 (95.3%)	not significant
	G/A	43/1,168 (3.7%)	85/1,794 (4.7%)	
	A/A	0/1,168	0/1,794	
p.T167A (c.499A>G)	A/A	1,163/1,168 (99.6%)	1,611/1,621 (99.4 %)	not significant
	A/G	3/1,168 (0.3%)	10/1,621 (0.6 %)	
	G/G	0/1,168	0/1621	
c.591+10C>T	C/C	1,163/1,168 (99.6%)	1,603/1,621 (98.9%)	not significant
	C/T	5/1,168 (0.4%)	18/1,621 (1.1%)	
	T/T	0/1,168	0/1621	

p values were calculated using Fisher's exact test in a dominant (e.g. c.454+191G>A: G/G+G/A vs. A/A) and in a recessive (e.g. c.454+191G>A: G/G vs. G/A+A/A) model.

and 1,294 controls, the distribution of p.T167A was not significantly different between the two groups (patients 0.3% (3/1,168) vs. controls 0.6% (10/1,621); $p > 0.05$) (table 4). In line with the genetic results, functional studies on the p.T167A PRSS3 protein revealed normal activity and secretion (fig. 2). Intronic variant c.591+10C>T was also found in similar frequencies in patients and controls (patients 0.4% (5/1,168) vs. 1.1% (controls 18/1,621); $p > 0.05$) (table 4).

A c.454+191G>A variant was significantly overrepresented in controls when provisional results were analyzed after extension of screening for the p.T167A variant (patients 2.7% (16/660), controls 5.1% (64/1,265), $p = 0.0174$, OR 1.9, 95% CI 1.117–3.235). However, after the screening for the c.454+191G>A variant was extended there was no significant difference between the frequencies in both groups (patients 3.7% (43/1,168), controls 4.7% (85/1,794); $p > 0.05$) (table 4).

Discussion

We did not find disease-associated *PRSS3* variants in 313 patients with a high suspicion of genetically determined CP. In the control group, the p.T167A variant was detected twice in our initially screened group of 327 controls, but showed no significant enrichment after extension of the investigated patient and control groups. Furthermore, functional analysis of recombinant p.T167A showed normal activity and secretion, in agreement with the negative genetic results.

Several intronic variants were found that were not significantly enriched in patients or controls. The c.454+ 191G>A variant was significantly overrepresented in controls when we analyzed provisional results after extension of screening for the p.T167A variant. Therefore, we extended the screening for the c.454+191G>A variant and ruled out an association to CP.

To further elucidate whether a distinct haplotype might be associated with CP, haplotypes were reconstructed using PHASE v2.1. Even though one haplotype was enriched in the patient group, this did not reach statistical significance after correction for multiple testing.

The common p.E32del variant has been ruled out previously as a potential disease causing mutation in patients with hereditary and alcoholic CP [13, 16]. The p.E32del variant has no effect on the catalytic properties, inhibitor resistance and inhibitor digesting ability of PRSS3. Activation of p.E32del PRSS3 by human enteropeptidase, trypsin or cathepsin B was also unaffected and degradation of wild-type and p.E32del PRSS3 by human trypsin was almost identical [13]. Again, functional results support genetic data that showed no different distribution of p.E32del in patients and controls.

Our negative result also concurs with an earlier preliminary investigation of six French families with hereditary pancreatitis where only the p.E32del variant was found in 1 patient [16].

Noteworthy, a recent study did not find copy number variations of *PRSS3* in patients with CP further supporting our genetic data [17]. In conclusion, our investigation

did not reveal an association between *PRSS3* variants and CP, which was further supported by functional data of PRSS3 variants.

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