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Gene Conversion between Cationic Trypsinogen (*PRSS1*) and the Pseudogene Trypsinogen 6 (*PRSS3P2*) in Patients with Chronic Pancreatitis

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Abstract

Mutations of the human cationic trypsinogen gene (*PRSS1*) are frequently found in association with hereditary pancreatitis. The most frequent variants p.N29I and p.R122H are recognized as disease-causing mutations. Three pseudogene paralogs in the human trypsinogen family, including trypsinogen 6 (*PRSS3P2*), carry sequence variations in exon 3 which mimic the p.R122H mutation. In routine genetic testing of patients with chronic pancreatitis we identified in two unrelated individuals similar gene conversion events of 24–71 nucleotides length between exon 3 of the *PRSS1* (acceptor) and *PRSS3P2* (donor) genes. The converted allele resulted in three non-synonymous alterations c.343T>A (p.S115T), c.347G>C (p.R116P) and c.365_366delinsAT (p.R122H). Functional analysis of the conversion triple mutant revealed markedly increased autoactivation resulting in high and sustained trypsin activity in the presence of chymotrypsin C. This activation phenotype was identical to that of the p.R122H mutant. In addition, cellular secretion of the triple mutant from transfected HEK 293T cells was increased about two-fold and this effect was attributable to mutation p.R116P. Our observations confirm and extend the notion that recombination events between members of the trypsinogen family can generate high risk

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PRSS1 alleles. The pathogenic phenotype of the novel conversion is explained by a unique combination of increased trypsinogen activation and secretion.

Keywords

hereditary pancreatitis; gene conversion; Trypsinogen; Autoactivation; Genetic risk

Introduction

Recurrent acute pancreatitis and chronic pancreatitis form an inflammatory continuum resulting in progressive destruction of the pancreas parenchyma and function (Gress, et al., 1994). The pathology underlying these clinical entities is driven by genetic risk factors which comprise mostly missense mutations in a variety of susceptibility genes, which include *PRSS1*, *SPINK1*, *CTRC*, *CFTR* and *CPA1*. reviewed in (Keim, et al., 2001; Weiss, et al., 2003; Whitcomb, 2013) Strong risk factors typically cause early-onset disease, often with a family history which can follow an autosomal dominant inheritance pattern, as observed in hereditary pancreatitis (Whitcomb, et al., 1996). Mutations of the protease, serine, 1 gene (*PRSS1*; MIM# 276000) encoding human cationic trypsinogen are the most frequently found genetic alterations in hereditary pancreatitis. Mutations p.R122H (~65%) and p.N29I (~25%) are causative in the majority of cases (Németh and Sahin-Tóth, 2014).

The human trypsinogen gene family consists of nine highly homologous genes, eight of which are located within the T-cell-receptor beta (TCR-beta) locus on chromosome 7, in two groups of tandem repeats (Rowen, et al., 1996). Group I trypsinogens comprise five paralogs; besides *PRSS1* and the other functional gene *PRSS2*, three pseudogenes are also located here, *PRSS3P1* (*TRY5*), *PRSS3P2* (*TRY6*, encoding trypsinogen 6) and *TRY7* (Figure 1A). The TCR-beta locus is a hot spot for gene conversion events to generate a broad variety of TCR-beta genes (Flajnik, 2002). Gene conversion is a non-reciprocal exchange of genetic information between homologous DNA sequences. It occurs frequently in tandem repeat DNA sequences and is most probably the result of mismatch repair following a heteroduplex formation with a DNA strand from the donor gene (Chen, et al., 2007). While an acceptor gene acquires the DNA sequence from the donor gene within the recombined segment, the donor gene remains unchanged. Gene conversion can occur between different paralogs including functional genes or pseudogenes. DNA exchange by gene conversion is essential in the evolution of gene families, but it also is the cause of a number of human diseases [reviewed in (Chen, et al., 2007)].

The high degree of homology among members of the human trypsinogen family and their tandem arrangement in the TCR-beta locus would be expected to promote the generation of novel trypsinogen conversion mutants which can act as pathogenic alleles in chronic pancreatitis. Surprisingly, very few have been discovered so far. First, Chen et al. (2000) hypothesized that gene conversion was a likely cause of pancreatitis-associated mutations within the *PRSS1* gene (Chen and Ferec, 2000a; Chen and Ferec, 2000b; Chen, et al., 2000). Subsequently, Teich et al. (2005) demonstrated a gene conversion in a patient with chronic pancreatitis, which occurred between *PRSS1* and *PRSS2* (Teich, et al., 2005). The

conversion affected exon 2 and the following intron and introduced the pathogenic mutation p.N29I and the harmless variant p.N54S in cationic trypsinogen. More recently, Masson et al. (2008) described a similar recombination event which also resulted in gene duplication and the new *PRSS1-PRSS2* hybrid allele carried both p.N29I and p.N54S mutations (Masson, et al., 2008).

Here, we extend the repertoire of trypsinogen conversion mutants associated with chronic pancreatitis and report a new *PRSS1* allele, which was generated through gene conversion with the expressed pseudogene *PRSS3P2*.

Materials and Methods

Patients

A 20-year-old male with two episodes of acute pancreatitis at age of 4 and 19 was referred to genetic testing at the Department of Medicine, University Medicine Greifswald, Germany. A 7-year-old girl was referred to the Children's Memorial Health Institute (ChMHI), Warsaw, Poland, following three episodes of acute pancreatitis. Both centers perform routine testing for genetic risk factors of hereditary pancreatitis. Local ethics committee approval and informed consent of patients and participants of the study were obtained before genetic analysis.

Gene symbols, reference sequences and numbering

Gene symbols: *CFTR*, cystic fibrosis transmembrane conductance regulator; *CPA1*, carboxypeptidase A1; *CTRC*, chymotrypsin C; *PRSS1*, serine protease 1 (human cationic trypsinogen), *PRSS2*, serine protease 2 (human anionic trypsinogen), *PRSS3P2* (*TRY6*), serine protease 3 pseudogene 2 (trypsinogen 6), *SPINK1*, serine protease inhibitor Kazal type 1 (pancreatic secretory trypsin inhibitor). Reference sequence accession numbers: NG_001333.2 (*PRSS1* and *PRSS3P2* genomic; this was derived from the original sequencing data of the Leroy Hood group [7], see accession L36092.2); NM_002769.4 (*PRSS1* mRNA); NR_001296.3 (*PRSS3P2* mRNA). Note that nucleotide c.375 in exon 3 of *PRSS3P2* is different in the two reference sequences (G versus C), suggesting this position may be polymorphic. We used c.375C in our alignment with *PRSS1* (see Figure 1B). Nucleotide numbering uses +1 as the A of the ATG translation initiation codon in the reference sequence, with the initiation codon as codon 1. Amino acid residues in human cationic trypsinogen were numbered starting with the initiator methionine of the primary translation product; according to the recommendations of the Human Genome Variation Society. The conversion mutations have been submitted to www.pancreasgenetics.org.

Molecular analyses

At the Greifswald center, genomic DNA was isolated from peripheral blood mononuclear cells (PBMCs) using Quick-gDNA blood mini kit (ZymoResearch, Irvine, CA, USA) according to the manufacturer's protocol. Coding regions of *PRSS1* and *SPINK1* (all exons including exon-intron junctions), as well as *CTRC* (exons 2, 3 and 7) were amplified by PCR using specific oligonucleotide primers, followed by Ampure (Beckmann Coulter GmbH, Krefeld, Germany), clean-up procedure and Sanger sequencing using the BigDye

v3.1 terminator sequencing kit and a 3130xl genetic analyzer (Life technologies GmbH, Darmstadt, Germany).

At the Warsaw center, genomic DNA was isolated from PBMCs using Genomic Maxi AX (A&A Biotechnology). DNA was also isolated from saliva and buccal cells (GenTech TD). DNA was amplified by PCR and sequenced using the Sanger method. Sequence analysis of exon 3 of *SPINK1* and of all exons (including exon-intron junctions) of *PRSS1*, *CTRC* and *CFTR* was performed. Large unbalanced rearrangements in *PRSS1* were excluded by MLPA (SALSA MLPA P242 Pancreatitis, MRC Holland). Additionally, the whole *PRSS1* gene was amplified in one amplicon to verify location of the detected variants within *PRSS1* (primers: PRS1i5_F: GTG TTT GTG CTG GGA GGA GT; PRS1i5_R: GGG GAC AGT CGT GTC TAA CC). To exclude genetic mosaicism, exon 3 of *PRSS1* was analyzed from DNA isolated from the patient's saliva and buccal cells. Furthermore, in the patient's parents exon 3 of *PRSS1* was sequenced and microsatellite segregation analysis was performed using PowerPlex16 (Promega) and Sefiler Plus (Applied Biosystems; Forensic Medicine Department, Medical University of Warsaw). Exon 3 of *PRSS3P2* of the patient was sequenced. (primer: PRS3P2_F: 5'-ATG AGC AGG AAG CTT GAG GA-3'; PRS3P2_R: 5'-CCA GTG CAG AAC CTG TGT GT-3').

The PCR product of *PRSS1* exon 3 from the Polish patient was cloned into the pJet1.2/blunt plasmid (Clone JetTM PCR Cloning kit, Fermentas) to determine whether detected variants were on the same allele (primer: TRY3e5_F: 5'-TCC ATG AGC AGA GAG CTT GAG GAA-3'; TRY3ie_R: 5'-TGT GAG GAT GGA GGG AAG TAG AAG GAC T-3'). Eleven clones were sequenced.

Expression plasmids and mutagenesis

Construction of the pTrapT7-intein-PRSS1 (GenBank DQ371396) and pcDNA3.1(-)-PRSS1 expression plasmids harboring the coding sequence for human cationic trypsinogen was reported previously (Király, et al., 2006; Nemoda and Sahin-Tóth, 2006). In the pTrapT7-intein-PRSS1 plasmid the native N terminus of human cationic trypsinogen is fused to the C terminus of a mini-intein (Király, et al., 2006). The p.R122H mutant in the pTrapT7-intein-PRSS1 plasmid was constructed previously (Szabó and Sahin-Tóth, 2012b). This mutant was used as template to generate the conversion triple mutant (p.S115T, p.R116P, p.R122H) in the pTrapT7-intein-PRSS1 plasmid by overlap-extension PCR mutagenesis. The PCR product was subcloned with the NcoI and SacI restriction sites. In the pcDNA3.1(-)-PRSS1 plasmid the conversion mutant was constructed by gene synthesis. A 427-nucleotide fragment containing all eight nucleotide variants identified in the German index patient was custom synthesized (GenScript, Inc.) and subcloned using XhoI and PflMI restriction sites. Single mutants p.S115T and p.R116P in the pcDNA3.1(-)-PRSS1 plasmid were constructed by overlap-extension PCR and subcloned using XhoI and BamHI sites. Single mutant p.R122H in the pcDNA3.1(-)-PRSS1 plasmid was constructed previously (Kereszturi, et al., 2009). All plasmids were verified by sequencing the entire construct between the flanking restriction sites.

Expression and purification of human cationic trypsinogen

Trypsinogens were expressed as intein fusion proteins in aminopeptidase P deficient *E. coli* strain LG-3, as described previously (Király, et al., 2011; Király, et al., 2006). During expression the fusion protein undergoes spontaneous self-cleavage and cationic trypsinogen with an authentic N-terminal sequence is liberated. Trypsinogen was solubilized from inclusion bodies, refolded *in vitro* and purified by ecotin affinity chromatography according to protocols we published (Király, et al., 2011; Király, et al., 2006; Lengyel, et al., 1998). Trypsinogens eluted from the ecotin column were dialyzed overnight against 3 liters of 50 mM HCl. Concentrations of trypsinogen preparations were calculated from their UV absorbance at 280 nm using the extinction coefficient $37,525 \text{ M}^{-1} \text{ cm}^{-1}$.

Trypsin activity assay

Trypsin activity was measured in 0.1 M Tris-HCl (pH 8.0) containing 1 mM CaCl_2 and 0.05% Tween 20 with 150 μM N-CBZ-Gly-Pro-Arg-p-nitroanilide substrate (final concentration) in 200 μL volume at 22°C. The release of the yellow p-nitroaniline was followed for 1 min at 405 nm in a SpectraMax Plus384 microplate reader (Molecular Devices) and rates of substrate cleavage were determined from fits to the initial, linear portion of the curves.

Cell culture and transfection

We used HEK 293T cells for cellular secretion studies because efficient transfection of pancreatic acinar cells is not feasible. HEK 293T cells were cultured at a density of 1.5×10^6 cells per well in six-well tissue culture plates in DMEM medium supplemented with 10% fetal bovine serum, 4 mM glutamine and 1% penicillin/streptomycin at 37°C. Transfections were carried out using 5 μL Lipofectamine 2000 (Invitrogen) and 2 μg expression plasmid in 2 mL DMEM final volume. After overnight incubation, cells were washed and the transfection media was replaced with 2 mL OptiMEM containing 1 mM benzamidine (final concentration) to prevent autoactivation. Conditioned media were collected after 24 h incubation.

Gel electrophoresis and densitometry

Conditioned media samples (200 μL) were precipitated with 10% trichloroacetic acid (final concentration), the precipitate was collected by centrifugation, dissolved in 20 μL Laemmli sample buffer containing 100 mM dithiothreitol (final concentration), and heat-denatured at 95°C for 5 min. Electrophoretic separation was achieved on 15% SDS-PAGE mini gels in standard Tris-glycine buffer. Gels were stained with Brilliant Blue R (Coomassie Blue) and photographed on Gel Doc XR+ gel documentation system (Bio-Rad). Quantitation of trypsinogen bands was carried out with the Image Lab (Bio-Rad) software.

Results

Case report from Greifswald, Germany

The German index patient, a 20-year-old male, had suffered two episodes of acute pancreatitis at age of 4 and 19 years. Regular consumption of tobacco and alcohol were

denied. The patient had no family history of pancreatitis and autoimmune markers (ANA, IgG4) were not elevated. On magnetic resonance imaging (MRI) no morphological changes of the pancreas, gallstones or irregularities of bile and pancreatic ducts could be detected. Genetic testing for risk factors of hereditary pancreatitis revealed the presence of eight heterozygous nucleotide substitutions within exon 3 of the *PRSSI* gene: c.343T>A, c.347G>C, c.351A>C, c.354A>C, c.360C>T, c.365_366delinsAT and c.390C>T. Sequence alignments indicated that these variations resulted from a gene conversion event between *PRSS3P2* and *PRSSI* in one allele of the *PRSSI* gene (Figure 1B). Because *PRSSI* and *PRSS3P2* sequences flanking the variants are identical between c.331 and c.342 and between c.391 and c.401, the 5' and 3' conversion breakpoints could not be localized to exact nucleotides. The minimal converted sequence was 48 nucleotides (from c.343 to c.390) and the maximal was 71 nucleotides (from c.331 to c.401). No additional mutations were identified in the pancreatitis risk genes *SPINK1* and *CTRC*. Genetic testing of the parents confirmed inheritance of the gene conversion from the patient's father, who had been diagnosed with type 1 diabetes at the age of 38. However, the father, aged 54 today, had never experienced any attacks of acute pancreatitis and he did not recall that his parents had any pancreatic disease. The index patient's paternal grandfather was presumed to have suffered also from diabetes. The healthy mother of the index patient carried a *SPINK1* p.N34S mutation which, however, was not inherited by the son.

Case report from Warsaw, Poland

A similar gene conversion was identified in Poland in a 7-year-old girl who was referred to the Children's Memorial Health Institute (ChMHI), Warsaw, following three episodes of acute pancreatitis within the previous eight months. Autoimmune pancreatitis, biliary disease and anatomic anomalies of the pancreatic duct were excluded. Serum lipids, hepatic panel and calcium were within the reference range. Sweat test was normal. Magnetic resonance cholangiopancreatography (MRCP) showed a slightly dilated pancreatic duct without dilated branches and ultrasound scan revealed an irregular, heterogeneous pancreas with calcifications. At age 8, the patient was hospitalized twice for recurrent pancreatic attacks. Endoscopic retrograde cholangiopancreatography (ERCP) showed a pre-ampullar stricture with upstream dilation of the pancreatic duct (5–6mm) and side branches; a picture diagnostic for chronic pancreatitis. Genetic testing revealed the presence of seven heterozygous nucleotide substitutions within exon 3 of the *PRSSI* gene that were also observed in the German index patient. The c.390C>T variant detected in the German subject was not present in the Polish patient (Figure 1C) and no additional mutations were found by sequence analysis of *SPINK1*, *CTRC* and *CFTR* genes. Analysis of the cloned *PRSSI* exon 3 of the patient showed that all nucleotide substitutions were present *in cis*, on the same allele. Since *PRSSI* and *PRSS3P2* sequences are identical between c.331 and c.342 and between c.367 and c.389, the minimal converted sequence was 24 nucleotides (from c.343 to c.366) and the maximal converted length was 59 nucleotides (from c.331 to c.389). As genetic testing for *PRSSI* exon 3 mutations in both parents was negative and parental relationship with the patient was confirmed by microsatellite testing, the conversion in the Polish index patient – in contrast to the German patient – was the result of a *de novo* event. Sequence analysis of exon 3 of *PRSS3P2* showed no difference from the reference sequence. The

novel conversion variant was absent in 383 other patients with acute or chronic pancreatitis referred to this center for genetic testing.

Comparison of the clinical course of the Polish index patient with that of 14 other children hospitalized in the ChMHI with hereditary pancreatitis harboring the p.R122H mutation alone revealed no appreciable differences.

Functional analysis of the converted *PRSS1* allele

The gene conversion events described in the German and Polish index patients resulted in three amino acid substitutions in the primary structure of human cationic trypsinogen, p.S115T, p.R116P and p.R122H. Mutation p.R122H is the most frequently identified genetic change in hereditary pancreatitis (Németh and Sahin-Tóth, 2014; Whitcomb, et al., 1996) and previous biochemical studies demonstrated that p.R122H leads to increased autoactivation of human cationic trypsinogen by rendering trypsinogen resistant to chymotrypsin C (CTRC)-mediated degradation (Szabó and Sahin-Tóth, 2012b). Mutations p.S115T and p.R116P are novel variants which were not analyzed functionally before. To compare biochemical properties of the conversion mutant with that of p.R122H, we expressed and purified wild-type, p.R122H and triple-mutant (p.S115T, p.R116P, p.R122H) human cationic trypsinogens. Autoactivation was measured at pH 8.0 in 1 mM calcium (Figure 2). In the absence of CTRC, there was no significant difference in the activation kinetics between mutant and wild-type trypsinogens (Figure 2A). In sharp contrast, when autoactivation was tested in the presence of 20 nM human CTRC, dramatic differences emerged. Thus, trypsin levels generated from wild-type trypsinogen were markedly reduced due to CTRC-mediated degradation, whereas mutant p.R122H and the triple mutant both reached comparably high trypsin activity which was sustained over the time-course studied (Figure 2B). The lack of a discernible difference in the activation pattern indicated that mutation p.R122H determined the activation properties of the conversion mutant and variants p.S115T and p.R116P were unimportant in this regard.

Enzyme kinetic parameters of the active trypsins were also determined using a small peptide substrate. Michaelis-Menten parameters were similar for wild-type, p.R122H mutant and the triple mutant trypsins (Table 1).

Previously, we found that mutation p.R122H had no effect on cellular secretion of cationic trypsinogen (see Figure 2 in (Kereszturi, et al., 2009)). To test whether the conversion mutant behaved similarly, we measured trypsinogen secretion from transiently transfected HEK 293T cells. Unexpectedly, secreted levels of the triple mutant were increased about two-fold relative to wild-type and p.R122H trypsinogen. When single mutants p.S115T and p.R116P were tested, a similar two-fold increase was detected with p.R116P, indicating that this mutation was responsible for the observed stimulatory effect on secretion (Figure 3).

Discussion

In this study we report the first pathogenic *PRSS1* allele generated through a gene conversion event between a functional trypsinogen gene (*PRSS1*) and an expressed pseudogene (*PRSS3P2*). Remarkably, this event was simultaneously identified in two

patients suffering from recurrent acute pancreatitis and chronic pancreatitis, respectively, in two European centers, in Germany and Poland. In the German index patient the converted allele was inherited from the father whereas in the Polish index patient it was generated *de novo*. Spontaneous mutations affecting the trypsinogen locus have been identified previously (Simon, et al., 2002) and are suggestive of a mutational hotspot. The length of the converted region was comparable in the two cases and it affected part of exon 3 of *PRSSI* resulting in seven (Polish patient) or eight (German patient) heterozygous nucleotide substitutions. Only three of these variations were non-synonymous, leading to amino-acid changes p.S115T, p.R116P, and p.R122H in human cationic trypsinogen.

PRSSI and *PRSS3P2* are more than 91% identical at the nucleotide level and 89% identical at the amino acid level. Interestingly, *PRSS3P2* contains several variants that correspond to pancreatitis-associated mutations in *PRSSI*, e.g. p.A16V, p.N29T and p.R122H and thus may serve as a template for gene conversions to create pathogenic *PRSSI* alleles. *PRSS3P2* has been annotated as an expressed pseudogene on the basis of an apparently intact exon-intron structure which should generate a normal transcript (Chen, et al., 2001; Rowen, et al., 1996). However, evidence for actual mRNA expression is scarce. A recent search of the human expressed sequence tag (EST) database with the predicted coding DNA for trypsinogen 6 did not result in any convincing hits. Due to the high degree of homology among the trypsinogen genes, interpretation of a published account claiming to quantitate *TRY6* (*PRSS3P2*) expression is problematic (Diederichs, et al., 2004). In any event, even if the gene is transcribed and then translated to a protein product, levels are expected to be very low and biologically insignificant. This notion is supported by the fact that a common deletion polymorphism results in the absence of *PRSS3P2* in a large segment of the population (Rowen, et al., 1996); in a recent study 17% of the cohort was homozygous and 48% heterozygous for this deletion (Wagner, et al., 2007).

Mutation p.R122H is the most frequently identified genetic variant in subjects with hereditary chronic pancreatitis (Németh and Sahin-Tóth, 2014; Whitcomb, et al., 1996). The mutation destroys a trypsin-sensitive cleavage site and thereby blocks CTIRC-dependent trypsinogen degradation (Szabó and Sahin-Tóth, 2012b; Szmola and Sahin-Tóth, 2007). CTIRC is a digestive serine protease which controls trypsinogen activation through proteolytic cleavages of regulatory nick sites (reviewed in (Zhou and Sahin-Tóth, 2011)). The dominant effect of CTIRC on trypsinogen is degradation through cleavage of the Leu81-Glu82 peptide bond in the calcium binding loop (Szabó and Sahin-Tóth, 2012a; Szabó and Sahin-Tóth, 2012b). Importantly, for trypsinogen degradation to occur, simultaneous cleavage of the Arg122-Val123 peptide bond by trypsin is necessary (Szabó and Sahin-Tóth, 2012b; Szmola and Sahin-Tóth, 2007). Mutation of Arg122, therefore, blocks CTIRC-mediated trypsinogen degradation resulting in elevated trypsin levels during autoactivation (Szabó and Sahin-Tóth, 2012b). Functional analysis demonstrated that the conversion triple mutant exhibited the same activation kinetics as mutant p.R122H, achieving high and sustained trypsin activity in the presence of CTIRC. Based on this observation, we conclude that activation properties of the conversion mutant are determined by the p.R122H mutation solely and variants p.S115T and p.R116P are inconsequential. On the other hand, we found that mutation p.R116P increased cellular secretion of cationic trypsinogen, which may

represent an additional mechanism of risk independent of the p.R122H mutation. The mechanism of increased trypsinogen secretion is unclear but we speculate that mutation p.R116P facilitates folding of trypsinogen and thereby accelerates its transit in the secretory pathway. Overall, functional studies suggest that the conversion triple mutant should confer a stronger disease risk than the p.R122H mutation alone. Interestingly, however, the father of the German index patient was an asymptomatic carrier, indicating incomplete penetrance.

Gene conversion events explain pathogenic alleles for a variety of human diseases, as reviewed by Chen et al. (Chen, et al., 2007). With respect to the pancreas, gene conversion mutations account for the majority of pathogenic alleles found in Shwachman-Bodian-Diamond syndrome, an autosomal recessive disorder causing pancreatic exocrine insufficiency, hematologic dysfunction, and skeletal abnormalities (Boocock, et al., 2003). The conversion occurs between the *SBDS* gene and its paralogous duplicated pseudogene *SBDSP*. Even more relevant to the present study is the finding that a recombination allele of the carboxyl ester lipase gene (*CEL*) and its pseudogene *CELP* is a novel genetic risk factor for chronic pancreatitis (Fjeld, et al., 2014). Finally, taken together with previous findings of conversions between *PRSS1* and *PRSS2* (Masson, et al., 2008; Teich, et al., 2005), the present study confirms and extends the importance of gene conversions between trypsinogen genes in the generation of new pathogenic alleles in chronic pancreatitis.

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References

- Boocock GR, Morrison JA, Popovic M, Richards N, Ellis L, Durie PR, Rommens JM. Mutations in *SBDS* are associated with Shwachman-Diamond syndrome. *Nat Genet.* 2003; 33(1):97–101. [PubMed: 12496757]
- Chen JM, Cooper DN, Chuzhanova N, Férec C, Patrinos GP. Gene conversion: mechanisms, evolution and human disease. *Nat Rev Genet.* 2007; 8(10):762–775. [PubMed: 17846636]
- Chen JM, Férec C. Gene conversion-like missense mutations in the human cationic trypsinogen gene and insights into the molecular evolution of the human trypsinogen family. *Mol Genet Metab.* 2000a; 71(3):463–469. [PubMed: 11073713]
- Chen JM, Férec C. Origin and implication of the hereditary pancreatitis-associated N21I mutation in the cationic trypsinogen gene. *Hum Genet.* 2000b; 106(1):125–126. [PubMed: 10982192]
- Chen JM, Montier T, Férec C. Molecular pathology and evolutionary and physiological implications of pancreatitis-associated cationic trypsinogen mutations. *Hum Genet.* 2001; 109(3):245–252. [PubMed: 11702203]
- Chen JM, Raguene O, Férec C, Deprez PH, Verellen-Dumoulin C. A CGC>CAT gene conversion-like event resulting in the R122H mutation in the cationic trypsinogen gene and its implication in the genotyping of pancreatitis. *J Med Genet.* 2000; 37(11):E36. [PubMed: 11073545]
- Diederichs S, Bulk E, Steffen B, Ji P, Tickenbrock L, Lang K, Zänker KS, Metzger R, Schneider PM, Gerke V, et al. S100 family members and trypsinogens are predictors of distant metastasis and

- survival in early-stage non-small cell lung cancer. *Cancer Res.* 2004; 64(16):5564–5569. [PubMed: 15313892]
- Fjeld K, Johansson S, Rosendahl J, Chen J-M, Lasher D, Cnop M, Berg Johansson B, Masson E, Mayerle J, Mössner J, et al. A recombination allele of the lipase gene CEL and its pseudogene CELP encodes a hybrid protein and is a genetic risk factor for chronic pancreatitis. *Pancreatology.* 2014:S16.
- Flajnik MF. Comparative analyses of immunoglobulin genes: surprises and portents. *Nat Rev Immunol.* 2002; 2(9):688–698. [PubMed: 12209137]
- Gress TM, Müller-Pillasch F, Lerch MM, Friess H, Büchler M, Beger HG, Adler G. Balance of expression of genes coding for extracellular matrix proteins and extracellular matrix degrading proteases in chronic pancreatitis. *Z Gastroenterol.* 1994; 32(4):221–225. [PubMed: 8017097]
- Keim V, Bauer N, Teich N, Simon P, Lerch MM, Mössner J. Clinical characterization of patients with hereditary pancreatitis and mutations in the cationic trypsinogen gene. *Am J Med.* 2001; 111(8): 622–626. [PubMed: 11755505]
- Kereszturi E, Szmola R, Kukor Z, Simon P, Weiss FU, Lerch MM, Sahin-Tóth M. Hereditary pancreatitis caused by mutation-induced misfolding of human cationic trypsinogen: a novel disease mechanism. *Hum Mutat.* 2009; 30(4):575–582. [PubMed: 19191323]
- Király O, Guan L, Sahin-Tóth M. Expression of recombinant proteins with uniform N-termini. *Methods Mol Biol.* 2011; 705:175–194. [PubMed: 21125386]
- Király O, Guan L, Szepessy E, Tóth M, Kukor Z, Sahin-Tóth M. Expression of human cationic trypsinogen with an authentic N terminus using intein-mediated splicing in aminopeptidase P deficient *Escherichia coli*. *Protein Expr Purif.* 2006; 48(1):104–111. [PubMed: 16542853]
- Lengyel Z, Pál G, Sahin-Tóth M. Affinity purification of recombinant trypsinogen using immobilized ecotin. *Protein Expr Purif.* 1998; 12(2):291–294. [PubMed: 9518472]
- Masson E, Le Maréchal C, Delcenserie R, Chen JM, Férec C. Hereditary pancreatitis caused by a double gain-of-function trypsinogen mutation. *Hum Genet.* 2008; 123(5):521–529. [PubMed: 18461367]
- Nemoda Z, Sahin-Tóth M. Chymotrypsin C (caldecrin) stimulates autoactivation of human cationic trypsinogen. *J Biol Chem.* 2006; 281(17):11879–11886. [PubMed: 16505482]
- Németh BC, Sahin-Tóth M. Human cationic trypsinogen (PRSS1) variants and chronic pancreatitis. *Am J Physiol Gastrointest Liver Physiol.* 2014; 306(6):G466–G473. [PubMed: 24458023]
- Rowen L, Koop BF, Hood L. The complete 685-kilobase DNA sequence of the human beta T cell receptor locus. *Science.* 1996; 272(5269):1755–1762. [PubMed: 8650574]
- Simon P, Weiss FU, Zimmer KP, Rand S, Brinkmann B, Domschke W, Lerch MM. Spontaneous and sporadic trypsinogen mutations in idiopathic pancreatitis. *JAMA.* 2002; 288(17):2122. [PubMed: 12413370]
- Szabó A, Sahin-Tóth M. Determinants of chymotrypsin C cleavage specificity in the calcium-binding loop of human cationic trypsinogen. *FEBS J.* 2012a; 279(23):4283–4292. [PubMed: 23035638]
- Szabó A, Sahin-Tóth M. Increased activation of hereditary pancreatitis-associated human cationic trypsinogen mutants in presence of chymotrypsin C. *J Biol Chem.* 2012b; 287(24):20701–20710. [PubMed: 22539344]
- Szmola R, Sahin-Tóth M. Chymotrypsin C (caldecrin) promotes degradation of human cationic trypsin: identity with Rinderknecht's enzyme Y. *Proc Natl Acad Sci U S A.* 2007; 104(27):11227–11232. [PubMed: 17592142]
- Teich N, Nemoda Z, Köhler H, Heinritz W, Mössner J, Keim V, Sahin-Tóth M. Gene conversion between functional trypsinogen genes PRSS1 and PRSS2 associated with chronic pancreatitis in a six-year-old girl. *Hum Mutat.* 2005; 25(4):343–347. [PubMed: 15776435]
- Wagner K, Grzybowska E, Butkiewicz D, Pamula-Pilat J, Pekala W, Tecza K, Hemminki K, Försti A. High-throughput genotyping of a common deletion polymorphism disrupting the TRY6 gene and its association with breast cancer risk. *BMC Genet.* 2007; 8:41. [PubMed: 17598925]
- Weiss FU, Simon P, Witt H, Mayerle J, Hlouschek V, Zimmer KP, Schnakenburger J, Domschke W, Neoptolemos JP, Lerch MM. SPINK1 mutations and phenotypic expression in patients with pancreatitis associated with trypsinogen mutations. *J Med Genet.* 2003; 40(4):e40. [PubMed: 12676913]

- Whitcomb DC. Genetic risk factors for pancreatic disorders. *Gastroenterology*. 2013; 144(6):1292–1302. [PubMed: 23622139]
- Whitcomb DC, Gorry MC, Preston RA, Furey W, Sossenheimer MJ, Ulrich CD, Martin SP, Gates LK, Amann ST, Toskes PP, et al. Hereditary pancreatitis is caused by a mutation in the cationic trypsinogen gene. *Nat Genet*. 1996; 14(2):141–145. [PubMed: 8841182]
- Zhou J, Sahin-Tóth M. Chymotrypsin C mutations in chronic pancreatitis. *J Gastroenterol Hepatol*. 2011; 26(8):1238–1246. [PubMed: 21631589]

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Figure 1. Gene conversion between *PRSS1* and *PRSS3P2*

Group I trypsinogen genes are located at the TCR-beta locus on chromosome 7. The conversion of exon 3 between the donor pseudogene *PRSS3P2* and the acceptor functional gene *PRSS1* is indicated (A). Electropherogram of *PRSS1* exon 3 in the German index patient (B). Heterozygous variations are indicated by arrows. Alignment of the relevant sequence tracts of *PRSS1* and *PRSS3P2* are also shown. Electropherogram of *PRSS1* exon 3 in the Polish index patient (C). Note the absence of the c.390C>T variant. Nucleotide

numbering is based on cDNA and uses +1 as the A of the ATG translation initiation codon in the reference sequence, with the initiation codon as codon 1.

Figure 2. Autoactivation of wild-type and mutant cationic trypsinogen

Trypsinogens were incubated at 1 μ M concentration with 10 nM initial trypsin in 0.1 M Tris-HCl (pH 8.0), 1 mM CaCl_2 and 0.05% Tween-20 (final concentrations) at 37 $^{\circ}\text{C}$ in the absence (A) or presence (B) of 20 nM chymotrypsin C (CTRC). At the indicated times, 2 μ L aliquots were removed and trypsin activity was measured as described in *Experimental Procedures*. Trypsin activity was expressed as percent of the maximal activity in the absence of CTRC.

Figure 3. Secretion of wild-type and mutant cationic trypsinogens

HEK 293T cells were transfected as described in *Experimental Procedures* with expression constructs for wild-type human cationic trypsinogen, mutants p.S115T, p.R116P, p.R122H and the conversion triple mutant (p.S115T, p.R116P, p.R122H). Conditioned media were collected after 24 h. Aliquots (200 μ L) were precipitated with 10% trichloroacetic acid (final concentration) and analyzed by SDS-PAGE. A representative gel from three experiments is shown in (A). Secretion of trypsinogen was quantified by densitometric evaluation of

Coomassie Blue stained gels (**B**). Trypsinogen levels were expressed relative to wild type as percent values (average $\pm$ SD, n=3).

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Table 1

Enzyme kinetic parameters

genotype	K_M (μM)	k_{cat} (s^{-1})	k_{cat}/K_M ($\text{M}^{-1}\cdot\text{s}^{-1}$)
wild type	36.2 ± 3.7	110.5 ± 6.3	3.1×10^6
p.R122H	33.1 ± 2.9	112.6 ± 5.4	3.4×10^6
triple mutant	38.3 ± 3.5	109.8 ± 5.7	2.9×10^6

Kinetic measurements of wild-type human cationic trypsinogen, mutant p.R122H and the conversion triple mutant (p.S115T, p.R116P, p.R122H) were performed using the CBZ-Gly-Pro-Arg-p-nitroanilide substrate in 0.1 M Tris-HCl (pH 8.0), 1 mM CaCl_2 and 0.05% Tween 20 (final concentrations) at 22 °C. The trypsin concentration in the assay was 5 nM and the substrate concentration was varied between 5 and 180 μM . Data from three independent experiments were fitted globally to the Michaelis-Menten hyperbolic equation and the error of the fit is indicated.