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Novel chymotrypsin C (*CTRC*) variants from real-world genetic testing of pediatric chronic pancreatitis cases

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ABSTRACT

Background: Chymotrypsin C (CTRC) protects the pancreas against unwanted intrapancreatic trypsin activity through degradation of trypsinogen. Loss-of-function *CTRC* variants increase the risk for chronic pancreatitis (CP). The aim of the present study was to characterize novel *CTRC* variants found during genetic testing of CP cases at a pediatric pancreatitis center.
Methods: We used next-generation sequencing to screen patients. We analyzed the functional effects of *CTRC* variants in HEK 293T cells and using purified enzymes.
Results: In 5 separate cases, we detected 5 novel heterozygous *CTRC* variants: c.407C>T (p.Thr136Ile), c.550G>A (p.Ala184Thr), c.627Cdup (p.Ser210Leufs*?, where the naming indicates a frame shift with no stop codon), c.628T>C (p.Ser210Pro), and c.779A>G (p.Asp260Gly). Functional studies revealed that with the exception of p.Ser210Leufs*?, the *CTRC* variants were secreted normally from transfected cells. Enzyme activity of purified variants p.Thr136Ile, p.Ala184Thr, and p.Asp260Gly was similar to that of wild-type CTRC, whereas variant p.Ser210Pro was inactive. The frame-shift variant p.Ser210Leufs*? was not secreted but accumulated intracellularly, and induced endoplasmic reticulum stress, as judged by elevated mRNA levels of *HSPA5* and *DDIT3*, and increased mRNA splicing of *XBP1*.
Conclusions: *CTRC* variants p.Ser210Pro and p.Ser210Leufs*? abolish CTRC function and should be classified as pathogenic. Mechanistically, variant p.Ser210Pro directly affects the amino acid at the bottom of the substrate-binding pocket while the frame-shift variant promotes misfolding and thereby blocks enzyme secretion. Importantly, 3 of the 5 novel *CTRC* variants proved to be benign, indicating that functional analysis is indispensable for reliable determination of pathogenicity and the correct interpretation of genetic test results.

1. Introduction

Genetic testing has become a commonplace medical procedure in search for etiologies and risk factors of acute recurrent pancreatitis (ARP) or chronic pancreatitis (CP), particularly in the pediatric population where genetic risk factors are more likely to underlie the disease [1–5]. The *CTRC* gene encoding the digestive protease chymotrypsin C is one of the well-established susceptibility genes for CP, which is routinely evaluated during genetic testing [6–9]. CTRC protects the pancreas against the development of harmful intrapancreatic trypsin activity by degrading trypsinogen, the precursor to trypsin [6,8–12]. Loss-of-function variants in *CTRC* weaken this defensive mechanism and result in elevated risk for CP.

The large majority of pathogenic *CTRC* variants are missense mutations typically found in the heterozygous state. There are 4 variants, which occur relatively more frequently; these include the

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missense variants p.Ala73Thr, p.Val235Ile, and p.Arg245Trp, and the microdeletion p.Lys247_Arg254del. According to a meta-analysis [13], average global carrier frequencies in CP cohorts ranged between 1.0 and 2.4 % and the highest carrier frequency reported by a single study was around 5 % for each variant. The variants increased CP risk on average by 6.5-, 4.5-, 2.6-, and 5.4-fold, respectively. Homozygosity or compound heterozygosity for these variants were rare and might be associated with higher risk.

The synonymous *CTRC* variant c.180C>T (p.Gly60=) is commonly found in patients (14.2 % allele frequency) and controls (8.7 % allele frequency), and increases CP risk by 2.2-fold, as shown by a recent meta-analysis [14]. When genotypes were considered, c.180CT heterozygosity was found in 22.9 % of CP patients and in 15.5 % of controls, indicating a 1.9-fold increased risk relative to the c.180CC genotype. Homozygosity with the c.180TT genotype was detected in 3.9 % of CP patients and in 1.2 % of controls, indicating a 5.3-fold increased CP risk. Restricting the analysis to adults of European origin, the risk was 1.7-fold for heterozygosity and 3.3-fold for homozygosity.

The pathogenic *CTRC* variants cause functional impairment by multiple mechanisms, which ultimately diminish the protective role of CTRC in the pancreas. Missense *CTRC* variants may be associated with loss of catalytic function, diminished secretion due to misfolding, increased susceptibility to degradation by trypsin, or resistance to activation by trypsin [6,15,16]. Variants that diminish secretion also elicit endoplasmic reticulum (ER) stress, the magnitude of which seems to be proportional to the secretion defect [15,17]. It remains unclear whether ER stress contributes to CP risk in carriers of misfolding CTRC variants. The synonymous variant c.180C>T (p.Gly60=) is associated with reduced mRNA expression, for reasons that are unexplained [14].

In the present study, we used functional analysis to investigate the clinical significance of novel, rare *CTRC* variants identified in real-world genetic testing at a pediatric pancreatitis center in the USA. The results demonstrate that discovery of novel pathogenic variants in the clinical setting is likely, and correct interpretation of pathogenic potential may require a combination of biochemical and cell biological approaches.

2. Methods

2.1. Nomenclature

Nucleotide numbering of the coding DNA starts with the first nucleotide of the translation initiation codon. Amino acid residues are numbered starting with the initiator methionine of the primary translation product. NM_007272.3, NCBI Reference Sequence for *Homo sapiens* chymotrypsin C (*CTRC*) mRNA.

2.2. Patients

This study was approved by the Cincinnati Children Hospital's institutional review board (2022-0055). See Table 1 for the characteristics of the 5 patients with the newly discovered *CTRC* variants. The age of onset for the first documented attack of acute pancreatitis ranged from 11 months to 11 years. With a single exception of ARP, all patients had CP. In 2 cases with CP the patients developed pre-diabetes. Exocrine pancreatic insufficiency was not detected in this cohort. Besides the novel *CTRC* variants of unknown significance, no established pathogenic variants were detected in the risk genes tested.

2.3. Next-generation sequencing

DNA was extracted from peripheral blood. Next-generation sequencing was performed on a defined panel of genes that included established risk genes (*CEL*, *CFTR*, *CLDN2*, *CPA1*, *CTRC*, *PRSS1*, and *SPINK1*), and putative susceptibility genes (*CASR*, *SBDS*, and *UBR1*). The coding regions with 20 bp flanking intronic sequences, and the 5′ and 3' untranslated regions were enriched/captured using either TruSeq Custom Amplicon technology (Illumina, Inc., San Diego, CA, USA) or SureSelect Target Enrichment System (Agilent Technologies Inc., USA). With TruSeq, the *CEL* gene was incompletely covered. *PRSS1* was sequenced with Sanger methodology due to its strong homology with multiple paralogs. The enriched targets were sequenced using next-generation technology on the Illumina instruments, Miseq and NextSeq 500 (Illumina Inc., USA) with 20- or 50-fold coverage. Regions with <20- or 50-fold coverage were filled in by Sanger sequencing. The sequence reads were aligned against the reference sequences using the BWA program, and variants were detected using the GATK software. Novel *CTRC* variants were confirmed by targeted Sanger sequencing.

2.4. CTRC expression plasmids

The pcDNA3.1(−) expression plasmids harboring the wild-type coding DNA for human CTRC with or without a 10His affinity tag were constructed previously [10,11]. *CTRC* mutants were generated by gene synthesis (GenScript, Piscataway, New Jersey) or by using the Q5 Site-Directed Mutagenesis kit (catalog number E0552S, New England Biolabs, Ipswich, MA, USA), and cloned into the pcDNA3.1(−) vector using *Xho*I and *Eco*RI restriction sites. The His-tagged versions of the constructs were used for purification and experiments with the purified enzymes. All other experiments used untagged constructs.

The 3′ untranslated region in our original *CTRC* expression plasmids contained 59 nucleotides between the stop codon and the *Eco*RI 3' cloning site. To study the p.Ser210Leufs*? frame-shift variant in the appropriate context, we extended this sequence to match the published cDNA clone of human *CTRC* [18]. According to this 1996 study, the polyA tail starts after the G nucleotide 69 nucleotides downstream of the stop codon. This corresponds to the first nucleotide of the *Eco*RI restriction site in the plasmid.

2.5. Cell culture and transfection

HEK 293T cells were cultured at 37 °C in six-well tissue culture plates at a density of 1.5×10^6 cells per well. The growth medium was DMEM (catalog number 10313039, Life Technologies, Carlsbad, CA) supplemented with 10 % fetal bovine serum, 4 mM glutamine and 1 % penicillin/streptomycin. Transfections were carried out using 4 μg expression plasmid with 5 μL Lipofectamine 2000 (Life Technologies) in 2 mL DMEM. After overnight incubation, cells were rinsed with 1 mL phosphate-buffered saline (pH 7.4) and covered with 1.5 mL OptiMEM (catalog number 11058021, Life Technologies). Conditioned medium and cells was harvested 48 h later [19].

2.6. Measurement of CTRC protein secretion

Aliquots (175 μL) of the conditioned media were precipitated with trichloroacetic acid (10 % final concentration). After centrifugation (16,000 g, 10 min, 4 °C), the protein pellet was resuspended in 25 μL Laemmli sample buffer containing 100 mM dithiothreitol, heat-denatured for 5 min at 95 °C, and electrophoresed on 15 % SDS-polyacrylamide gels. Gels were stained with Coomassie Blue. Densitometric quantitation of bands was carried out with the ImageJ (version 1.53e) and BioRad Image Lab 6.1 programs.

2.7. Measurement of CTRC activity from conditioned medium

The CTRC proenzyme in the conditioned medium was activated with human cationic trypsin for 1 h at 37 °C. The activation mix (110 μL) contained 44 μL medium, 0.1 M Tris-HCl (pH 8.0), 1 mM $CaCl_2$, 0.05 % Tween 20, and 50 nM trypsin (final concentrations). CTRC activity was measured in duplicate by mixing 50 μL activated medium with 150 μL of 200 μM Suc-Ala-Ala-Pro-Phe-*p*-nitroanilide substrate. Release of the yellow *p*-nitroaniline was followed at 405 nm for 2 min in a Spectramax Plus 384 microplate reader (Molecular Devices) at 22 °C. The rate of substrate cleavage was determined from the linear portion of the curves.

2.8. Purification of CTRC

His-tagged forms of CTRC variants were purified with nickel affinity chromatography from 200 mL conditioned media, as described previously [11]. Five-mL fractions were collected, and 200 μL of each fraction was analyzed by SDS-PAGE. Peak fractions were pooled (~20 mL), dialyzed against 2 × 3 L 20 mM Tris-HCl (pH 8.0), 100 mM NaCl, and concentrated to about 1 mL (range 0.6–1.5 mL) on 10 MWCO Vivaspin concentrators. Concentrations of purified proenzyme preparations were initially estimated from their UV absorbance at 280 nm using the extinction coefficient 64,565 M^{-1} cm^{-1}. These values ranged between 5 and 9.6 μM.

The purified CTRC proenzyme (at 2 μM UV-based concentration) was activated with 50 nM human cationic trypsin in 0.1 M Tris-HCl (pH 8.0) and 0.05 % Tween 20 at 37 °C for 30 min, and the precise concentration of active CTRC (range 1.2–1.6 μM) was determined using active site titration with ecotin [20]. The concentration of the inactive variant p.Ser210Pro was estimated from the UV absorbance, and this value was then multiplied with a correction factor of 0.7, which represents the average ratio of ecotin-titrated versus UV-based concentration values of the active CTRC preparations.

2.9. Active site titration

Concentration of active CTRC was determined by titration with the pan-protease inhibitor ecotin. A two-fold serial dilution of 100 nM ecotin was prepared in 50 μL assay buffer (0.1 M Tris-HCl (pH 8.0), 1 mM $CaCl_2$, and 0.05 % Tween 20), and 50 μL CTRC diluted with assay buffer to 20 nM nominal concentration (based on UV absorbance) was added to each well. The 100 μL mixture was incubated at 22 °C for 30 min and the residual CTRC activity was measured with 100 μL 200 μM Suc-Ala-Ala-Pro-Phe-*p*-nitroanilide substrate (dissolved in enzyme buffer). Enzyme activity was plotted as a function of ecotin concentration, and the CTRC concentration was determined by extrapolating the linear portion of the inhibition curve to the *x* intercept.

2.10. Enzyme kinetic analysis

Measurements were performed in 0.1 M Tris-HCl (pH 8.0), 1 mM $CaCl_2$, and 0.05 % Tween 20 at 22 °C. The active CTRC concentration in the assay was 5 nM. The concentration of the Suc-Ala-Ala-Pro-Phe-*p*-nitroanilide substrate was varied between 1.8 and 114 μM. To determine K_M and k_{cat}, substrate cleavage rates were plotted as a function of the substrate concentration, and the data points (mean ± SD, n = 3) were fitted with the Michaelis-Menten hyperbolic equation.

2.11. Digestion of casein

Bovine β-casein (0.2 mg/mL concentration, catalog number C6905, Sigma) was digested with 5 nM CTRC in 0.1 M Tris-HCl (pH 8.0) and 1 mM $CaCl_2$, at 37 °C, in 100 μL final volume. The digestion mixtures contained 20 nM human SPINK1 to inhibit residual trypsin activity that may be present in the CTRC preparations. At the indicated times, 100 μL aliquots were withdrawn and the reactions were stopped by precipitation with trichloroacetic acid (10 % final concentration). The samples were analyzed by 15 % SDS-PAGE followed by Coomassie Blue staining and densitometric quantitation.

2.12. Preparation of cell lysates

The conditioned medium was harvested and the cells were rinsed with 1 mL phosphate-buffered saline (pH 7.4), suspended with 1 mL of the same buffer, and centrifuged at 850 g for 10 min at 4 °C. The cell pellets were resuspended in 500 μL ice-cold RIPA Buffer (catalog number R0278-50 ML, Sigma), supplemented with protease inhibitors (Halt Protease and Phosphatase Inhibitor Cocktail, catalog number 78444, Thermo Fisher Scientific, 100 × factory stock diluted to 1 ×). After 15 min incubation on ice, lysates were sonicated three times for 10 s.

2.13. Western blotting

Cell lysates (10 μL per lane) were electrophoresed on 15 % minigels and transferred to Immobilon-P membrane (catalog number IPVH00010, Sigma). Incubation with the primary and secondary antibodies was performed overnight at 4 °C and for 1 h at room temperature, respectively. Protein bands were detected using the SuperSignal West Pico PLUS Chemiluminescent Substrate (catalog number 34580, Thermo Fisher Scientific). Sheep polyclonal antibody against human CTRC (0.2 mg/mL stock, catalog number AF6907, R&D Systems, Minneapolis, MN) was used at 1:2000 dilution. Horseradish peroxidase (HRP)-conjugated donkey polyclonal anti-sheep IgG (catalog number HAF016, R&D Systems) was used at 1:2000 dilution.

2.14. Reverse-transcription quantitative PCR

Total RNA was extracted from transfected cells using the RNeasy Plus Mini Kit (catalog number 74136, Qiagen, Valencia, CA). Two μg RNA was reverse-transcribed using the High Capacity cDNA Reverse Transcription Kit (catalog number 4368814, Applied Biosystems). TaqMan Universal PCR Mastermix (catalog number 4364338, Life Technologies) and TaqMan Gene Expression Assays were used to determine mRNA expression levels for *HSPA5* (Hs00607129_gH) and *DDIT3* (Hs00358796_g1). *GAPDH* (Hs02758991_g1) served as the housekeeping reference gene. Expression was quantitated using the comparative cycle threshold method. CT values were normalized to those of GAPDH (ΔCT) and then to the average ΔCT of the empty vector (ΔΔCT). Results were expressed as fold changes calculated with the formula $2^{-\Delta\Delta CT}$.

2.15. Measurement of XBP1 mRNA splicing

Splicing of the *XBP1* mRNA was analyzed by reverse transcription PCR and agarose gel electrophoresis. The following primers were used: *XBP1* sense primer, 5′-CCT TGT AGT TGA GAA CCA GG -3′; *XBP1* antisense primer, 5′-GGG CTT GGT ATA TAT GTG G -3′. The amplicons generated were 415 bp (spliced) and 441 bp (unspliced) long. *GAPDH* was amplified (352 bp) as the housekeeping control gene with the following primers: *GAPDH* sense primer, 5′- AAG GTC GGA GTC AAC GGA TTT -3′; *GAPDH* antisense primer, 5′- AGA TGA TGA CCC TTT TGG CTC -3′. The PCR products were resolved on 2.5 % agarose gels and stained with GreenGlo Safe DNA Dye (catalog number C788T73, Thomas Scientific, Swedesboro, NJ).

2.16. Statistics

Where indicated, the difference of means was analyzed by one-way ANOVA with Tukey's post-hoc analysis, respectively. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Novel CTRC variants in a pediatric cohort of acute recurrent pancreatitis and chronic pancreatitis

Children with ARP or CP routinely undergo genetic testing. Since 2016, the Cincinnati Children's Hospital Medical Center has used next-generation sequencing to screen nearly 700 pancreatitis patients for genetic alterations in a panel of verified (*CEL, CFTR, CLDN2, CPA1, CTRC, PRSS1,* and *SPINK1*) and presumed (*CASR, SBDS*, and *UBR1*) risk genes. In 5 patients with ARP or CP (see Table 1), we identified so far unreported *CTRC* variants (Table 2), including the frame-shift variant c.627dup (p.Ser210Leufs*?), and 4 novel missense variants: c.407C>T (p.Thr136Ile), c.550G>A (p.Ala184Thr), c.628T>C (p.Ser210Pro), and c.779A>G (p.Asp260Gly). The c.627dup variant results in a shift of the translational reading frame. Interestingly, the new reading frame contains no stop codon and translation presumably terminates at the end of the polyA tail. This uncertainty is reflected in the protein-level naming of the variant by a question mark. Variants p.Ser210Pro, p.Ser210Leufs*?, and p.Asp260Gly have not been reported in the gnomAD database (Table 2).

Among the amino acids affected by the genetic changes, Ser210 is notable, as this corresponds to Ser189 in the conventional chymotrypsin numbering biochemists and crystallographers use. Ser189 is located at the bottom of the primary substrate-binding pocket (the so-called S1 subsite), where the P1 residue of the substrate is inserted, using the Schechter-Berger nomenclature [21]. The AlphaMissense prediction program [22] classified the p.Ser210Pro variant as likely pathogenic, whereas variants p.Thr136Ile, p.Ala184Thr, and p.Asp260Gly were predicted as likely benign (Table 2).

3.2. Effect of CTRC variants on chymotrypsinogen secretion from transfected cells

We transiently transfected HEK 293T cells with expression plasmids carrying the *CTRC* coding DNA with the various variants. We measured secretion of CTRC by SDS-PAGE and Coomassie Blue staining (Fig. 1A). With the sole exception of p.Ser210Leufs*?, *CTRC* variants did not alter proenzyme secretion. In contrast, variant p.Ser210Leufs*? abolished CTRC secretion. Evaluation of multiple transfections (three independent transfections in duplicate each, n = 6) with densitometry confirmed that secreted CTRC variants were present in the medium in comparable concentrations relative to the wild-type proenzyme (Fig. 1B).

3.3. Effect of CTRC variants on chymotrypsin activity

To obtain an estimate of enzyme activity, we first measured chymotrypsin activity in the conditioned media after activation with trypsin (Fig. 1C). Variants p.Thr136Ile, p.Ala184Thr, and p.Asp260Gly exhibited similar activity as wild-type CTRC. No activity was detected in the conditioned medium of variant p.Ser210Leufs*?, which contained no secreted CTRC protein. Importantly, variant p.Ser210Pro had no measurable activity, in agreement with the critical role of Ser210 in substrate binding.

To obtain more quantitative data on the enzyme activity of secreted *CTRC* variants, we expressed His-tagged forms in HEK 293T cells, and purified the proenzymes using nickel-affinity chromatography. Purified CTRC was activated with human cationic trypsin, and the concentration of the active CTRC was determined by active-site titration using ecotin. Michaelis-Menten kinetic parameters were measured on the Suc-Ala-Ala-Phe-*p*-nitroanilide substrate (Table 3). Variants p.Thr136Ile, p.Ala184Thr, and p.Asp260Gly cleaved the small peptide substrate as well as wild-type CTRC with similar K_M and k_{cat} values. We were unable to measure kinetic parameters reliably with variant p.Ser210Pro due to its very low activity.

To test the activity of the CTRC variants on a large protein substrate, we digested bovine β-casein and analyzed the reaction by SDS-PAGE and Coomassie Blue staining (Fig. 2). The results were consistent with our previous findings on the peptide substrate. Thus, wild-type CTRC and variants p.Thr136Ile, p.Ala184Thr, and p.Asp260Gly digested casein comparably while variant p.Ser210Pro was inactive.

3.4. Variant p.Ser210Leufs*? causes misfolding and ER stress

To model the potential effects of the frame-shift variant

Table 1
Characteristics of patients with novel *CTRC* variants.

CTRC variant	Sex	Race/Ethnicity	Indication for testing	Age at first AP	DM and EPI status
c.407C>T	F	Hispanic	CP	11 years	unknown
c.550G>A	F	White, Hispanic	CP	4 years	preDM, no EPI
c.627dup	F	African American	CP	9 years	preDM, no EPI
c.628T>C	F	unknown	CP	6 years	unknown
c.779A>G	M	African American	ARP	11 months	no DM, no EPI

AP, acute pancreatitis, ARP, acute recurrent pancreatitis, CP, chronic pancreatitis, DM, diabetes, EPI, exocrine pancreatic insufficiency, preDM, prediabetes. Besides the novel *CTRC* variants, the patients carried no pathogenic variants in the risk genes tested.

Table 2
Novel *CTRC* variants analyzed in this study. The gnomAD database was queried on April 16, 2024 (https://gnomad.broadinstitute.org).

Exon	Nucleotide change	Amino-acid change	gnomAD alleles	AlphaMissense score
Exon 5	c.407C>T	p.Thr136Ile	1/628,782	0.296
Exon 6	c.550G>A	p.Ala184Thr	102/1,614,082	0.098
Exon 6	c.627dup	p.Ser210Leufs*?	not reported	not applicable
Exon 6	c.628T>C	p.Ser210Pro	not reported	0.956
Exon 7	c.779A>G	p.Asp260Gly	not reported	0.156

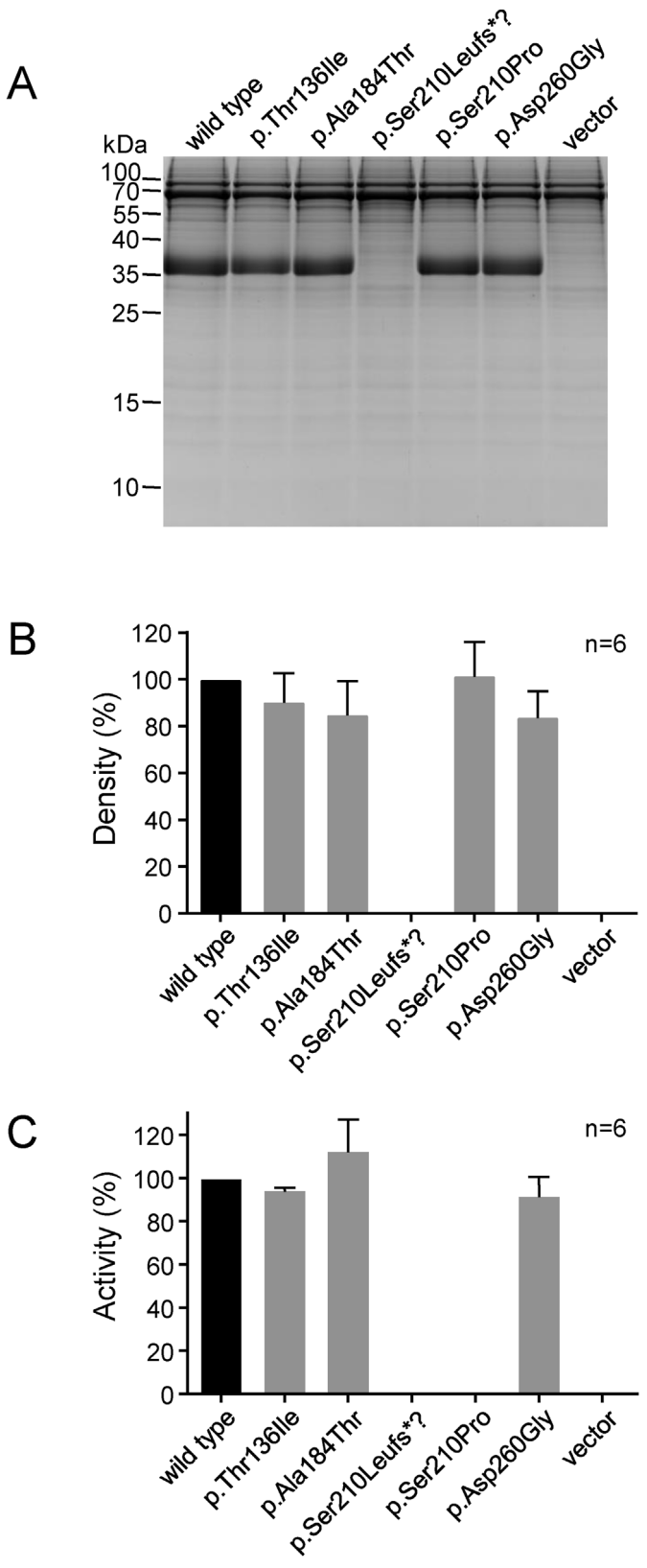

Fig. 1. Effect of *CTRC* variants on proenzyme secretion. HEK 293T cells were transiently transfected and conditioned medium was collected after 48 h. ***A***, Conditioned media were analyzed by SDS-PAGE and Coomassie Blue staining. Representative gel is shown. ***B***, Densitometric analysis of CTRC expression. Mean ± SD are shown, n = 6. ***C***, CTRC enzyme activity in the conditioned medium after activation with trypsin. Mean ± SD are indicated, n = 6. See *Methods* for experimental details.

Table 3
Enzyme kinetic parameters of wild-type CTRC and active CTRC variants on the Suc-Ala-Ala-Pro-Phe-*p*-nitroanilide substrate. See *Methods* for experimental details. The error of the hyperbolic fits are indicated.

	K_M (μM)	k_{cat} (s^{-1})	k_{cat}/K_M ($M^{-1}•s^{-1}$)
wild type	13.9 ± 1.2	7.7 ± 0.2	5.5×10^5
p.Thr136Ile	14.8 ± 0.9	7.6 ± 0.1	5.1×10^5
p.Ala184Thr	9.9 ± 1.3	7.7 ± 0.3	7.8×10^5
p.Asp260Gly	12.0 ± 1.0	8.3 ± 0.2	6.9×10^5

accurately, we restored the full 3' untranslated sequence in our CTRC expression plasmid, using the information from the 1996 cloning paper [18], and studied the effect of the variant in this context (see *Methods*). When analyzed in transfected HEK 293T cells, variant p.Ser210Leufs*? abolished secretion of CTRC to the conditioned medium (Fig. 3A) but the proenzyme was detectable in cell lysates by Western blot (Fig. 3B). The variant migrated slower than wild-type CTRC due the frame-shifted extra sequence. Interestingly, densitometry of multiple blots demonstrated that intracellular levels of the p.Ser210Leufs*? variant were significantly higher than those of wild-type CTRC (Fig. 3C). Taken together, the lack of secretion and intracellular accumulation of the protein indicates that the variant promotes misfolding. Previously characterized misfolding CTRC variants were shown to elicit ER stress in cells [15,17]. To assess whether variant p.Ser210Leufs*? would induce ER stress, we measured mRNA levels for the ER-chaperone *HSPA5* (encoding BiP) and the ER-stress associated proapoptotic transcription factor *DDIT3* (encoding CHOP) (Fig. 3D). Both ER stress markers were significantly elevated in cells expressing the p.Ser210Leufs*? variant, relative to cells with wild-type CTRC or empty vector. Non-conventional mRNA splicing of the *XBP1* transcription factor was also increased in cells with variant p.Ser210Leufs*? versus cells with wild-type CTRC or empty vector (Fig. 3E).

4. Discussion

In the present study, we asked the question whether routine, real-world genetic testing of ARP and CP patients yields novel *CTRC* variants, and whether such variants are predominantly benign or pathogenic. To this end, we reviewed the genetic testing results of pediatric ARP and CP cases at a high-volume pediatric pancreatitis center in the USA, and identified 5 *CTRC* variants not reported in the literature before. We performed functional analysis and ascertained 2 variants as pathogenic, while the remaining 3 proved to be benign. Thus, we conclude that real-world genetic testing of CP cases can yield novel *CTRC* variants and establishing clinical significance necessitates functional analysis with biochemical and cell biological techniques. Recently, the computer program AlphaMissense was released as a state-of-the-art prediction tool for missense variants that supposedly outperforms all previous prediction algorithms [22]. We obtained scores for the 4 missense *CTRC* variants (see Table 2) and the predictions were accurate when compared with the results of the functional analysis. Thus, it is feasible to use AlphaMissense for preliminary prediction of pathogenic potential of missense variants, although validation against a larger set of *CTRC* variants is needed.

We previously established that pathogenic *CTRC* variants could cause loss of function by a variety of mechanisms [6,15–17]; all leading to increased trypsinogen autoactivation and higher intrapancreatic trypsin levels. In this study, the two variants classified as pathogenic either diminished enzyme activity by altering the

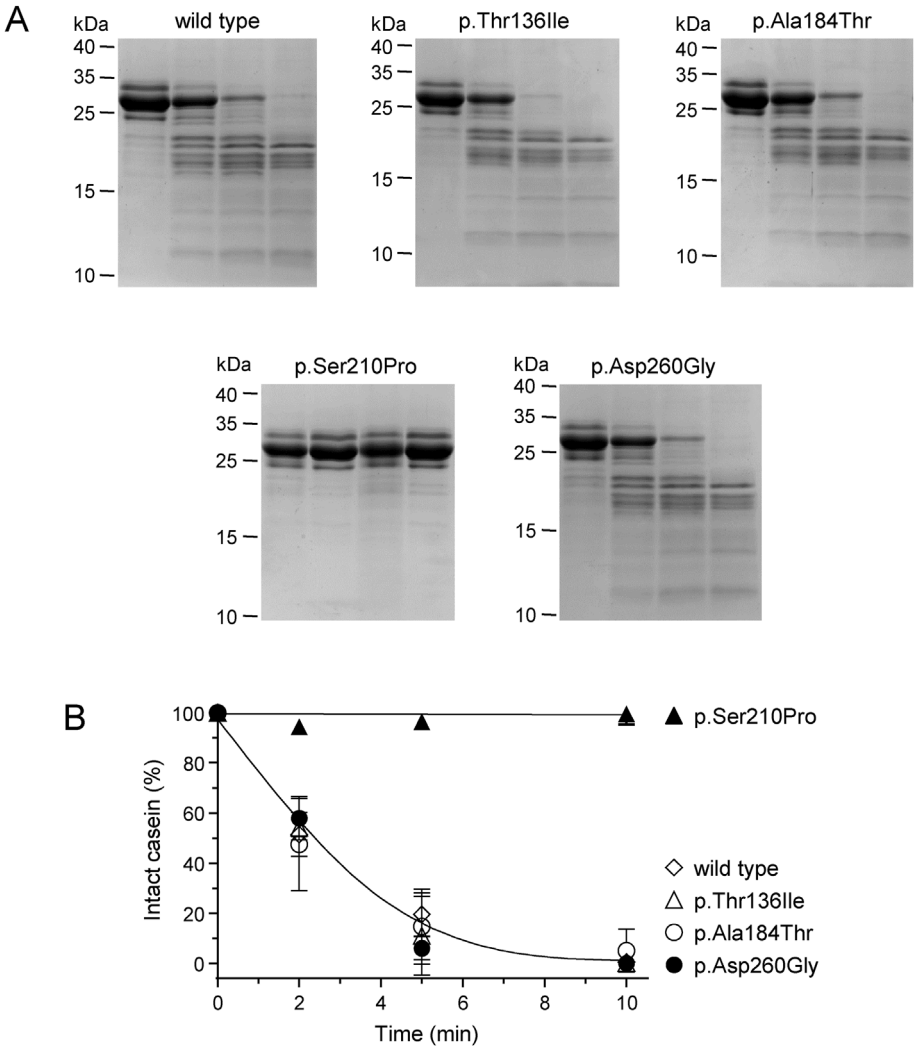

Fig. 2. Effect of *CTRC* variants on bovine β-casein degradation. ***A***, Reactions were analyzed by SDS-PAGE and Coomassie Blue staining. Representative gels are shown. ***B***, The rate of digestion was quantitated by densitometry. The relative amount of intact casein was plotted as a function of time. Mean ± SD are shown, n = 3. See *Methods* for experimental details.

properties of the amino acid at the bottom of the substrate binding pocket of CTRC (p.Ser210Pro) or blocked secretion due to misfolding of the CTRC proenzyme (p.Ser210Leufs*?). Variants that cause reduced CTRC secretion due to misfolding (p.Gly61Arg, p.Ala73Thr, and to a lesser extent p.Gln48Arg) have been shown to induce ER stress [15,17], and this was also the case with the p.Ser210Leufs*? variant. It is unclear, however, whether ER stress contributes to the increased CP risk in carriers of misfolding CTRC variants. Misfolding variants of procarboxypeptidase A1 (*CPA1*) or human cationic trypsinogen (*PRSS1*) are associated with hereditary CP, and cell culture studies and animal modeling demonstrated that these variants cause ER stress [23,24]. A mouse model carrying the *Cpa1* p.Asn256Lys mutation developed slowly progressive CP [25]. Ultra rare misfolding variants in the pancreatic lipase gene (*PNLIP*) were also shown to elicit ER stress in cells [26,27]. A knock-in mouse model with a *Pnlip* variant developed progressive CP [28], however, association of *PNLIP* with CP in humans has not been demonstrated so far. A hybrid variant (*CEL-HYB1*) between the carboxyl ester lipase (*CEL*) gene and its adjacent pseudogene (*CELP*) was also shown to induce misfolding, cellular ER stress, and CP in transgenic mice [29,30]. It is noteworthy, however, that all these digestive enzymes are highly expressed, in fact, they represent the most abundant secretory proteins in the pancreas. In contrast, CTRC is a relatively minor chymotrypsin isoform [31], and heterozygous *CTRC* variants may not generate high enough levels of the toxic mutant protein that would result in maladaptive ER stress.

CTRC is essential for pancreas health and should be evaluated for genetic defects in all cases of ARP and CP. We expect that in the near future, loss of CTRC function would be readily restored via adeno-associated virus (AAV) based gene therapy introduced through the pancreatic duct [32]. This approach might work even in cases where the underlying genetic defects affect other genes. CTRC seems an attractive candidate for such gene therapy, because the proenzyme expressed from the virus would be inactive and become activated only under conditions of intrapancreatic trypsin activation, when its protective action is needed. Although AAV-based protein expression wanes with time, it is conceivable that breaking the vicious cycle of recurrent attacks of pancreatitis by a single CTRC treatment may bring long-lasting remission.

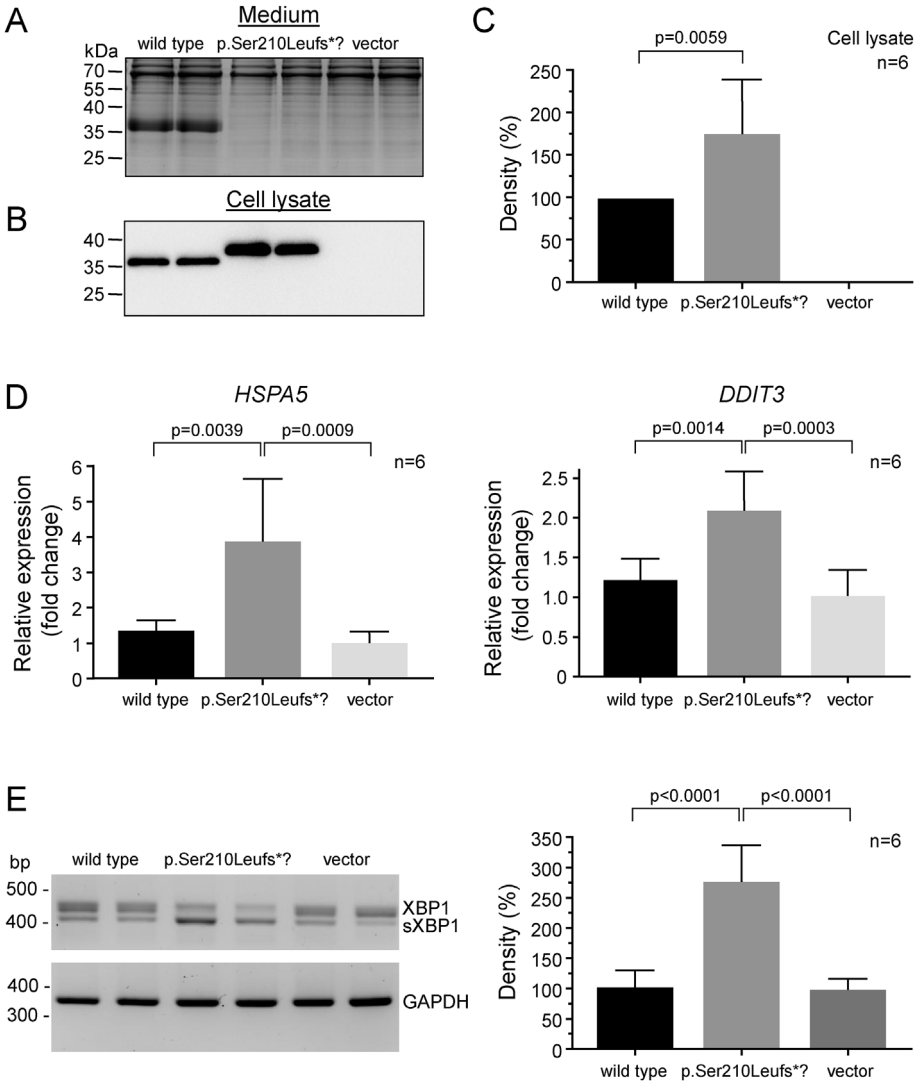

Fig. 3. Effect of *CTRC* variant p.Ser210Leufs*? on proenzyme expression, secretion, and endoplasmic reticulum stress. HEK 293T cells were transiently transfected, and conditioned medium and cell lysates were collected after 48 h. ***A***, Lack of secretion of *CTRC* variant p.Ser210Leufs*? in the conditioned medium. ***B***. Expression levels of the p.Ser210Leufs*? variant in cell lysates. Representative Western blot is shown. ***C***, Densitometric analysis of the expression of variant p.Ser210Leufs*? in cell lysates. Mean ± SD are shown, n = 3. ***D***, Messenger RNA levels of *HSPA5* (encoding BiP) and *DDIT3* (encoding CHOP) in cells transfected with wild-type CTRC, variant p.Ser210Leufs*?, or empty vector. Data were measured by reverse-transcription quantitative PCR. Mean ± SD are shown, n = 6. ***E***, Splicing of *XBP1* mRNA in cells transfected with wild-type CTRC, variant p.Ser210Leufs*?, or empty vector. Representative agarose gel is shown (left panel). The faint topmost bands represent heteroduplexes. Densitometric evaluation of *XBP1* splicing. Mean ± SD are shown, n = 6 (right panel). See *Methods* for experimental details. Variant p.Ser210Leufs*? is indicated as p.S210Lfs. The difference of means between the groups was analyzed by one-way ANOVA followed by Tukey's post-hoc test.

Author contributions

MST, BCN, WZ, and MAH conceived and directed the study. RS, SM, MST, and BCN designed the experiments. RS, SM, GB, AD, and MST performed the experiments and analyzed the data. MST wrote the manuscript, RS and SM prepared the figures. All authors contributed critical revisions and approved the final manuscript.

Declaration of competing interest

The authors have declared that no conflict of interest exists.

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References

[1] Kumar S, Ooi CY, Werlin S, Abu-El-Haija M, Barth B, Bellin MD, Durie PR, Fishman DS, Freedman SD, Gariepy C, Giefer MJ, Gonska T, Heyman MB, Himes R, Husain SZ, Lin TK, Lowe ME, Morinville V, Palermo JJ, Pohl JF, Schwarzenberg SJ, Troendle D, Wilschanski M, Zimmerman MB, Uc A. Risk factors associated with pediatric acute recurrent and chronic pancreatitis: lessons from INSPPIRE. JAMA Pediatr 2016;170:562–9.

[2] Vitale DS, Abu-El-Haija M. Genetic testing in children with recurrent and chronic pancreatitis. J Pediatr 2017;191:10–1.

[3] Xiao Y, Yuan W, Yu B, Guo Y, Xu X, Wang X, Yu Y, Yu Y, Gong B, Xu C. Targeted

gene next-generation sequencing in Chinese children with chronic pancreatitis and acute recurrent pancreatitis. J Pediatr 2017;191:158–63.
[4] Palermo JJ, Lin TK, Hornung L, Valencia CA, Mathur A, Jackson K, Fei L, Abu-El-Haija M. Genophenotypic analysis of pediatric patients with acute recurrent and chronic pancreatitis. Pancreas 2016;45:1347–52.
[5] Abu-El-Haija M, Valencia CA, Hornung L, Youssef N, Thompson T, Barasa NW, Wang X, Denson LA. Genetic variants in acute, acute recurrent and chronic pancreatitis affect the progression of disease in children. Pancreatology 2019;19:535–40.
[6] Rosendahl J, Witt H, Szmola R, Bhatia E, Ozsvári B, Landt O, Schulz HU, Gress TM, Pfützer R, Löhr M, Kovacs P, Blüher M, Stumvoll M, Choudhuri G, Hegyi P, te Morsche RH, Drenth JP, Truninger K, Macek Jr M, Puhl G, Witt U, Schmidt H, Büning C, Ockenga J, Kage A, Groneberg DA, Nickel R, Berg T, Wiedenmann B, Bödeker H, Keim V, Mössner J, Teich N, Sahin-Tóth M. Chymotrypsin C (CTRC) variants that diminish activity or secretion are associated with chronic pancreatitis. Nat Genet 2008;40:78–82.
[7] Masson E, Chen JM, Scotet V, Le Maréchal C, Férec C. Association of rare chymotrypsinogen C (CTRC) gene variations in patients with idiopathic chronic pancreatitis. Hum Genet 2008;123:83–91.
[8] Zhou J, Sahin-Tóth M. Chymotrypsin C mutations in chronic pancreatitis. J Gastroenterol Hepatol 2011;26:1238–46.
[9] Hegyi E, Sahin-Tóth M. Genetic risk in chronic pancreatitis: the trypsin-dependent pathway. Dig Dis Sci 2017;62:1692–701.
[10] Szmola R, Sahin-Tóth M. Chymotrypsin C (caldecrin) promotes degradation of human cationic trypsin: identity with Rinderknecht's enzyme Y. Proc Natl Acad Sci USA 2007;104:11227–32.
[11] Szabó A, Sahin-Tóth M. Increased activation of hereditary pancreatitis-associated human cationic trypsinogen mutants in presence of chymotrypsin C. J Biol Chem 2012;287:20701–10.
[12] Jancsó Z, Sahin-Tóth M. Tighter Control by chymotrypsin C (CTRC) explains lack of association between human anionic trypsinogen and hereditary pancreatitis. J Biol Chem 2016;291:12897–905.
[13] Takáts A, Berke G, Gede N, Németh BC, Witt H, Głuszek S, Rygiel AM, Hegyi P, Sahin-Tóth M, Hegyi E. Risk of chronic pancreatitis in carriers of loss-of-function *CTRC* variants: a meta-analysis. PLoS One 2022;17:e0268859.
[14] Berke G, Beer S, Gede N, Takáts A, Szentesi A, Hegyi P, Rosendahl J, Sahin-Tóth M, Németh BC, Hegyi E. Risk of chronic pancreatitis in carriers of the c.180C>T (p.Gly60=) *CTRC* variant: case-control studies and meta-analysis. Pancreatology 2023;23:481–90.
[15] Beer S, Zhou J, Szabó A, Keiles S, Chandak GR, Witt H, Sahin-Tóth M. Comprehensive functional analysis of chymotrypsin C (CTRC) variants reveals distinct loss-of-function mechanisms associated with pancreatitis risk. Gut 2013;62:1616–24.
[16] Szabó A, Ludwig M, Hegyi E, Szépeová R, Witt H, Sahin-Tóth M. Mesotrypsin signature mutation in a chymotrypsin C (CTRC) variant associated with chronic pancreatitis. J Biol Chem 2015;290:17282–92.
[17] Szmola R, Sahin-Tóth M. Pancreatitis-associated chymotrypsinogen C (CTRC) mutant elicits endoplasmic reticulum stress in pancreatic acinar cells. Gut 2010;59:365–72.
[18] Tomomura A, Akiyama M, Itoh H, Yoshino I, Tomomura M, Nishii Y, Noikura T, Saheki T. Molecular cloning and expression of human caldecrin. FEBS Lett 1996;386:26–8.
[19] Tóth A, Demcsák A, Zankl F, Oracz G, Unger LS, Bugert P, Laumen H, Párniczky A, Hegyi P, Rosendahl J, Gambin T, Płoski R, Koziel D, Gluszek S, Lindgren F, Löhr JM, Sahin-Tóth M, Witt H, Rygiel AM, Ewers M, Hegyi E. Loss-of-function variant in chymotrypsin like elastase 3B (*CELA3B*) is associated with non-alcoholic chronic pancreatitis. Pancreatology 2022;22:713–8.
[20] Szabó A, Héja D, Szakács D, Zboray K, Kékesi KA, Radisky ES, Sahin-Tóth M, Pál G. High affinity small protein inhibitors of human chymotrypsin C (CTRC) selected by phage display reveal unusual preference for P4' acidic residues. J Biol Chem 2011;286:22535–45.
[21] Schechter I, Berger A. On the size of the active site in proteases. I. Papain. Biochem Biophys Res Commun 1967;27:157–62.
[22] Cheng J, Novati G, Pan J, Bycroft C, Žemgulytė A, Applebaum T, Pritzel A, Wong LH, Zielinski M, Sargeant T, Schneider RG, Senior AW, Jumper J, Hassabis D, Kohli P, Avsec Ž. Accurate proteome-wide missense variant effect prediction with AlphaMissense. Science 2023;381:eadg7492.
[23] Sahin-Tóth M. Genetic risk in chronic pancreatitis: the misfolding-dependent pathway. Curr Opin Gastroenterol 2017;33:390–5.
[24] Sándor M, Sahin-Tóth M. Functional predictors of pathogenicity of missense *CPA1* variants in chronic pancreatitis. Gut 2023 Oct 19. Epub ahead of print.
[25] Hegyi E, Sahin-Tóth M. Human *CPA1* mutation causes digestive enzyme misfolding and chronic pancreatitis in mice. Gut 2019;68:301–12.
[26] Szabó A, Xiao X, Haughney M, Spector A, Sahin-Tóth M, Lowe ME. A novel mutation in *PNLIP* causes pancreatic triglyceride lipase deficiency through protein misfolding. Biochim Biophys Acta 2015;1852:1372–9.
[27] Toldi V, Kassay N, Szabó A. Missense *PNLIP* mutations impeding pancreatic lipase secretion cause protein misfolding and endoplasmic reticulum stress. Pancreatology 2021;21:1317–25.
[28] Zhu G, Wilhelm SJ, George LG, Cassidy BM, Zino S, Luke CJ, Hanna M, Stone S, Phan N, Matiwala N, Ballentine SJ, Lowe ME, Xiao X. Preclinical mouse model of a misfolded *PNLIP* variant develops chronic pancreatitis. Gut 2023;72:1340–54.
[29] Mao XT, Zou WB, Cao Y, Wang YC, Deng SJ, Cooper DN, Férec C, Li ZS, Chen JM, Liao Z. The *CEL-HYB1* hybrid allele promotes digestive enzyme misfolding and pancreatitis in mice. Cell Mol Gastroenterol Hepatol 2022;14:55–74.
[30] Fjeld K, Gravdal A, Brekke RS, Alam J, Wilhelm SJ, El Jellas K, Pettersen HN, Lin J, Solheim MH, Steine SJ, Johansson BB, Njølstad PR, Verbeke CS, Xiao X, Lowe ME, Molven A. The genetic risk factor *CEL-HYB1* causes proteotoxicity and chronic pancreatitis in mice. Pancreatology 2022;22:1099–111.
[31] Geisz A, Jancsó Z, Németh BC, Hegyi E, Sahin-Tóth M. Natural single-nucleotide deletion in chymotrypsinogen C gene increases severity of secretagogue-induced pancreatitis in C57BL/6 mice. JCI Insight 2019;4:e129717.
[32] Wang YC, Mao XT, Sun C, Wang YH, Zheng YZ, Xiong SH, Liu MY, Mao SH, Wang QW, Ma GX, Wu D, Li ZS, Chen JM, Zou WB, Liao Z. Pancreas-directed AAV8-hSPINK1 gene therapy safely and effectively protects against pancreatitis in mice. Gut 2024 Mar 29. Epub ahead of print.