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Comprehensive functional analysis of chymotrypsin C (*CTRC*) variants reveals distinct loss-of-function mechanisms associated with pancreatitis risk

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Abstract

Objective—The digestive enzyme chymotrypsin C (CTRC) protects against pancreatitis by promoting degradation of trypsinogen and thereby curtailing potentially harmful trypsinogen activation. Loss-of-function variants in *CTRC* increase the risk for chronic pancreatitis. The aim of the present study was to perform comprehensive functional analysis of all missense *CTRC* variants identified to date.

Design—We investigated secretion, activity and degradation of 27 published and 5 novel CTRC mutants. We also assessed the effect of 5 mutants on endoplasmic reticulum (ER) stress.

Results—None of the mutants exhibited a gain of function such as increased secretion or activity. In contrast, 11 mutants showed marked loss of function, 3 mutants had moderate functional defects, whereas 18 mutants were functionally similar to wild-type CTRC. The functional deficiencies observed were diminished secretion, impaired catalytic activity and degradation by trypsin. Mutants with a secretion defect caused ER stress that was proportional to

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COMPETING INTEREST STATEMENT
No competing interest to declare.

CONTRIBUTIONS
The study was designed by M.S.-T, S.B., J.Z. and A.S. The experiments were performed by S.B., J.Z. and A.S. Novel CTRC variants were provided by S.K., H.W. and G.R.C. The manuscript was written by S.B., J.Z. and M.S.-T.

the loss in secretion. ER stress was not associated with loss-of-function phenotypes related to catalytic defect or proteolytic instability.

Conclusion—Pathogenic *CTRC* variants cause loss of function by three distinct but mutually non-exclusive mechanisms that affect secretion, activity and proteolytic stability. ER stress may be induced by a subset of CTRC mutants but does not represent a common pathological mechanism of *CTRC* variants. This phenotypic dataset should aid in the classification of the clinical relevance of *CTRC* variants identified in patients with chronic pancreatitis.

Keywords

chronic pancreatitis; chymotrypsin C; trypsinogen; trypsin; endoplasmic reticulum stress

Idiopathic chronic pancreatitis in humans is a genetically determined condition; often associated with multiple mutations in various risk genes [1–3 and references therein]. Emergence of trypsin activity inside the pancreas seems to be a critical factor in disease pathogenesis and all susceptibility genes described to date regulate the development of trypsin activity. Mutations in the serine protease 1 (*PRSS1*) gene increase activation of human cationic trypsinogen and cause autosomal dominant hereditary pancreatitis or act as risk factors for sporadic disease. Conversely, the p.G191R variant in the anionic trypsinogen (*PRSS2)* gene stimulates autodegradation and protects against chronic pancreatitis. Mutations in the serine protease inhibitor Kazal-type 1 (*SPINK1*) gene decrease expression of a trypsin inhibitor protein and thereby increase trypsinogen activation and the risk for chronic pancreatitis. Cystic fibrosis transmembrane conductance regulator (*CFTR)* gene mutations might impair bicarbonate secretion and facilitate trypsinogen activation through altered intraductal pH and/or decreased ductal flushing. Association of *CFTR* mutations with chronic pancreatitis also suggests that pathological trypsinogen activation takes place in the ductal space.

More recently, mutations in the chymotrypsin C (*CTRC*) gene that diminish secretion or activity of the digestive enzyme CTRC were recognized as risk factors for chronic pancreatitis [4]. CTRC promotes proteolytic inactivation of trypsinogen and trypsin and is required to curtail intrapancreatic trypsinogen activation [5]. The importance of this protective mechanism is further highlighted by the recent finding that common *PRSS1* mutations that cause hereditary pancreatitis render trypsinogen resistant to CTRC-dependent degradation [6]. Since the publication of our original paper on *CTRC* variants in 2008 [4], six additional studies appeared that confirmed their association with chronic pancreatitis [7–12]. Five of these have been recently reviewed in detail [13]. Two new studies came out in 2012, one describing an European cohort that largely overlaps with the cohort published in 2008 [11] and another describing *CTRC* variants in a large Indian cohort [12]. All in all, the seven studies reported 54 *CTRC* variants, which included 26 missense variants, five nonsense or frame-shift variants, four synonymous variants, one in-frame microdeletion and 18 variants in non-coding regions. The most frequently found variant was the synonymous variant c.180C>T (p.G60=), which was present in 23–29% of the studied cohorts and increased the risk for chronic pancreatitis by about 2.5-fold in the heterozygous state and close to 10-fold in the homozygous state [12]. Considering non-synonymous variants and the microdeletion, only four exhibited statistically significant disease association (Tables 1–3). Variants p.A73T and p.V235I were mainly found in the Indian population, whereas variants p.R254W and the microdeletion p.K247_R254del were predominant in Europeans. The effect sizes of these variants in the heterozygous state, as expressed by the odds ratio, were 8.2-fold, 5.2-fold, 3.6-fold and 6.4-fold, and their frequency in the patient population were 3%, 3.2%, 2% and 0.9%, respectively (Tables 1 and 2). Thus, *CTRC* variants are relatively rare risk factors that increase the probability of pancreatitis by about 4- to 8-fold.

This becomes important when we consider rare *CTRC* variants which have been found not only in patients but also in healthy controls. The presence of a *CTRC* variant in a patient does not signify pathogenicity and, conversely, its presence in a healthy subject does not necessarily indicate harmless biological behavior. When the low frequency of a variant does not allow the determination of genetic association, its pathogenic nature can only be inferred from the biochemical or cell biological phenotype.

The primary aim of the present study was to catalog all missense *CTRC* variants according to their functional phenotype and thereby predict their clinical significance. Preliminary functional characterization was reported previously for a handful of *CTRC* variants, which indicated that both decreased secretion and loss of catalytic activity may be disease-relevant phenotypes. Furthermore, the p.A73T mutant was shown to elicit endoplasmic reticulum (ER) stress in pancreatic acinar cells, raising the possibility that other mutations may exert their pathogenic effect via a similar pathway [14]. Therefore, an additional objective of this study was to clarify whether or not ER stress is commonly associated with CTRC mutants.

METHODS

Nomenclature

Nucleotide numbering reflects coding DNA numbering with +1 corresponding to the A of the ATG translation initiation codon in *CTRC*. Amino acid residues are numbered starting with the initiator methionine of the primary translation product for human CTRC.

Novel CTRC variants

CTRC variants p.G18R, p.D35Y, p.Q178R and p.V250E were identified by Ambry Genetics, Inc (Aliso Viejo, CA) in subjects referred for diagnostic testing because of pancreatitis. All patients underwent sequence analysis of the *CTRC* gene including exons 1–8 with at least 20 nucleotides of flanking introns and 60 nucleotides upstream from the start codon. The novel p.G32V variant was identified by the Munich group as part of a recently published study on a well-characterized German cohort [11]. This variant was excluded from the previous publication because the subject tested was of Swedish origin.

Expression plasmids, mutagenesis, adenovirus

Construction of the pcDNA3.1(-) human CTRC expression plasmid was reported previously [5]. CTRC mutants were created by overlap extension PCR and ligated into the pcDNA3.1(-) expression plasmid. The coding DNA for mutant p.K247_R254del with a C-terminal 10His affinity tag was custom synthesized (GenScript, Inc., Piscataway, NJ) and subcloned into pcDNA3.1(-). Recombinant adenovirus was generated by Viraquest, Inc. (North Liberty, Iowa). Adenovirus carrying wild-type CTRC and mutant p.A73T were described previously [14]. With the exception of mutant p.K247_R254del, all adenoviral CTRC constructs contained a GluGlu epitope tag at the C terminus.

Cell culture and transfection

HEK 293T cells were cultured in 6-well tissue culture plates (1.5×10^6 cells per well) in DMEM supplemented with 10% fetal bovine serum, 4 mM glutamine and 1% penicillin/streptomycin at 37°C. Transfections were carried out at 90% confluence, 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. The conditioned OptiMEM media were harvested after 24 h incubation. AR42J rat pancreatic acinar cells (ATCC #CRL-1492) were maintained in DMEM supplemented with 20% fetal bovine serum, 4 mM glutamine and 1% penicillin/streptomycin at 37°C. Prior to transfection, cells were plated in 6-well plates (10^6 cells per

well) and were grown in the presence of 100 nM concentration of dexamethasone for 48 h to induce differentiation [15]. Infections with adenovirus were performed using 2×10^8 plaque forming units (pfu) per mL final adenovirus concentrations in a total volume of 1 mL OptiMEM in the presence of dexamethasone (100 nM final concentration). Conditioned media were harvested after 24 h incubation.

Measurement of CTRC protein secretion

Proteins in the conditioned media (200 μL) were precipitated with 10% trichloroacetic acid (final concentration), resuspended in 20 μL Laemmli sample buffer containing 100 mM dithiothreitol, heat-denatured at 95°C for 5 min and electrophoresed on 15% SDS-polyacrylamide gels. Gels were stained with Coomassie Blue. Densitometric quantitation of bands was carried out with the GelDocXR+ gel documentation system and Image Lab software (Bio-Rad).

Measurement of CTRC activity

Conditioned media (37.5 μL from HEK 293T cells or 20 μL from AR42J cells) were incubated with 100 nM concentration of human cationic trypsin at 37°C for 1 h in 100 mM Tris-HCl (pH 8.0) and 10 mM $CaCl_2$ in 50 μL final volume. CTRC activity was measured at 22°C by adding 150 μL Suc-Ala-Ala-Pro-Phe-*p*-nitroanilide or Suc-Ala-Ala-Pro-Leu-*p*-nitroanilide substrate, as indicated, to 150 μM final concentration. The substrates were dissolved in 100 mM Tris-HCl (pH 8.0), 1 mM $CaCl_2$ and 0.05% Tween-20. Release of the yellow *p*-nitroaniline was followed at 405 nm for 1 min in a Spectramax Plus 384 microplate reader (Molecular Devices) and the rate of substrate cleavage was determined from the linear portion of the curves. Enzyme kinetic analysis of purified CTRC was performed in 100 mM Tris-HCl (pH 8.0), 1 mM $CaCl_2$ and 0.05% Tween-20 at 22°C.

Expression and purification of CTRC

Select CTRC mutants were purified from 80 mL conditioned media using ecotin affinity chromatography, as described previously [16]. Mutant p.K247_R254del did not bind to the ecotin column, therefore, a His-tagged form was purified using nickel-affinity chromatography [6]. Purified CTRC zymogen was activated with 20 nM human cationic trypsin in 100 mM Tris-HCl (pH 8.0) and 1 mM $CaCl_2$ (final concentrations) for 30 min at 37°C. Concentration of active CTRC was determined using active site titration with ecotin, as described recently [17]. The concentrations of mutants p.G217R, p.G217S, p.K247_R254del, which were degraded by trypsin during activation, were estimated from the ultraviolet (UV) absorption of the inactive zymogen form at 280 nm using the extinction coefficient 64,565 M^{-1} cm^{-1}.

Reverse transcriptase (RT)-PCR analysis and real-time PCR

Total RNA was extracted from AR42J cell lysates using Trizol reagent (Invitrogen). Following DNase I treatment (New England Biolabs), RNA was reverse-transcribed using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). X-box binding protein 1 (XBP1) splicing was studied by PCR using a primer set that flanked the spliced region and amplified both spliced and unspliced forms (Supplementary Table S1). PCR was carried out using the GoTaq Green Master Mix (Promega) with the following conditions: 3 min initial denaturation at 95°C followed by 35 cycles of 30 sec denaturation at 95°C, 30 sec annealing at 56°C, 30 sec extension at 68°C and a final extension at 68°C for 5 min. The PCR products were resolved on 2% agarose gels and stained with ethidium bromide. Quantification of mRNA expression was performed by real time PCR (7500 Real Time PCR System, Applied Biosystems). XBP1 expression was measured with SYBR Green (PCR Master Mix, Applied Biosystems) using different primer sets for the spliced, unspliced and

total mRNA (Supplementary Table S1). Levels of immunoglobulin-binding protein (BiP) and calreticulin mRNA were determined using TaqMan primers with TaqMan Universal PCR Mastermix (Applied Biosystems). Real time PCR conditions were as follows: 2 min equilibration at 50°C, 10 min denaturation and enzyme activation at 95°C followed by 40 two-step cycles of 15 sec at 95°C and 60 sec at 60°C. Gene expression was quantitated using the comparative C_T method ($\Delta\Delta C_T$ method). Threshold cycle (C_T) values were determined using the 7500 System Sequence Detection Software 1.3. Expression levels of target genes were first normalized to the GAPDH internal control gene (ΔC_T) and then to expression levels measured in cells infected with wild-type CTRC adenovirus ($\Delta\Delta C_T$). Results were expressed as fold changes calculated with the formula $2^{-\Delta\Delta CT}$.

RESULTS

CTRC variants studied

To date, seven published studies reported *CTRC* variants identified in patients with chronic pancreatitis or in controls without pancreatic disease [4, 7–12]. In the present study we included 26 missense variants and the in-frame deletion p.K247_R254del, which are expected to alter protein folding and/or function (Tables 1–3). The majority of these are rare variants. In addition to the 27 published variants, we present here 5 newly identified missense variants found in pancreatitis patients from Europe (p.G32V) and the United States (p.G18R, p.D35Y, p.Q178R and p.V250E) (Table 1). Thus, altogether 32 *CTRC* variants were studied.

CTRC mutants with reduced secretion

First, we used HEK 293T cells which are relatively easy to culture and can be transfected using plasmid based methods with high efficiency and good reproducibility. Twenty-three of 32 mutants tested showed CTRC secretion close to wild-type levels (80–105%), including the clinically relevant variant p.V235I (~86% of wild type) (Fig 1). Complete loss of secretion with no detectable CTRC protein was observed with mutants p.G61R, p.C155Y and p.L220R; whereas mutants p.Q48R, p.A73T and p.G217R exhibited markedly reduced but still measurable secretion (20–35% of wild type). Moderately reduced secretion (60–65% of wild type) was noted with the clinically important p.R254W and p.K247_R254del mutants and with the p.P249L mutant (Figs 1 and 2A). Interestingly, however, mutant p.R254Q, in which the same amino-acid position is altered as in p.R254W, was secreted normally (~90% wild type).

Secretion of mutants p.G61R, p.Q48R, p.A73T, p.R254W and p.K247_R254del was also studied in the rat acinar cell line AR42J transfected with recombinant adenovirus. The five mutants selected include three clinically frequent variants and two representative mutants with moderate and severe secretion defect. As shown in Figs 2B and 2C, the secretion and activity pattern of the mutants tested in AR42J cells paralleled those observed in HEK 293T cells and mutants with significant secretion defects (p.G61R, p.Q48R and p.A73T) exhibited the same phenotype in both cell lines, although secretion levels were somewhat higher in AR42J cells. This may be cell-type specific variation or may be due to the more complex banding pattern of AR42J media which can interfere with densitometric evaluation. Secretion of mutant p.R254W was only slightly decreased (~80%) whereas mutant p.K247_R254del was secreted as well as wild-type CTRC from this cell line. Western blot analysis of AR42J cell lysates indicated that intracellular levels of mutants p.Q48R, p.G61R and p.A73T were lower than those of wild-type CTRC, whereas levels of mutants p.R254W and p.K247_R254del were comparable to wild type (reviewed but not shown). As noted in HEK 293T cells (Fig 1), mutant p.K247_R254del secreted from AR42J cells had no measurable enzyme activity (Fig 2C).

CTRC mutants with reduced catalytic activity

For the majority of mutants, CTRC enzyme activity in the conditioned medium correlated well with CTRC protein levels secreted, although enzyme activity tended to be slightly decreased relative to secretion levels. Seven mutants (p.G32V, p.Q178R, p.G217S, p.G217R, p.K247_R254del, p.P249L and p.V250E) had very low enzyme activity in the conditioned medium even though these mutants were secreted either normally or to lower but still significant levels. The clinically frequent p.V235I mutant also exhibited decreased activity (55%) relative to its secretion level (86%). There are two possibilities to explain a discrepancy between secretion and activity; the mutant either has a catalytic defect or it is prone to degradation by trypsin or by autolysis. To study these mechanistic aspects, we purified the seven inactive mutants as well as mutants p.G18R, p.D35Y, p.R37Q, p.Q48R, p.A73T, p.R254Q, p.R254W and p.V235I from conditioned media. The mutants were activated with trypsin and catalytic parameters were determined on a small peptide substrate (Table 4). Enzyme kinetic values for mutants p.G18R, p.D35Y, p.R37Q, p.Q48R, p.A73T, p.V235I, p.R254Q and p.R254W were comparable to that of wild type (k_{cat}/K_M 62–100%). In contrast, mutants p.G32V, p.Q178R, p.G217S, p.P249L and p.V250E had severe catalytic defects with 1.5%, 0.6%, 0.5%, 0.07% and 0.08% catalytic efficiency, respectively, relative to wild type. We could not detect any activity with mutants p.G217R and p.K247_R254del, therefore, catalytic parameters were not determined. Note that these mutants also suffered rapid degradation during activation by trypsin (see below) which hindered precise activity measurements.

Activity of the catalytically impaired mutants p.G32V, p.Q178R, p.G217S, p.P249L and p.V250E was also tested on the natural substrate human cationic trypsinogen (Table 4 and Supplementary Fig S1). The five mutants exhibited 6%, 25%, 8%, 3% and 12% activity in trypsinogen degradation, respectively, relative to the activity of wild-type CTRC. Surprisingly, the loss of activity was not nearly as pronounced as observed on the peptide substrates. Still, in physiological terms, these mutants can be considered inactive or markedly defective. Interestingly, mutant p.V250E cleaved trypsinogen not only at the Leu81-Glu82 peptide bond but also at other sites indicating that the mutation altered not only the activity but also the specificity of CTRC. Although data are not shown, the clinically relevant mutant p.V235I was found to exhibit ~50% activity in the trypsinogen degradation assay, which is slightly lower than observed with the small peptide substrate (~70%).

CTRC mutants degraded by trypsin

The inactive zymogen form of CTRC is converted to active CTRC by trypsin. Wild-type active CTRC is not degraded by trypsin nor does it suffer autolysis. However, mutations may introduce a new tryptic cleavage site or destabilize CTRC which would then undergo proteolytic degradation during trypsin-mediated activation with consequent loss of CTRC activity. To test for this possibility, we examined trypsin-mediated degradation of purified CTRC mutants. First, the seven low-activity mutants were studied on SDS-PAGE using a low trypsin-to-CTRC ratio (50 nM trypsin versus 1 μM CTRC concentrations). As shown in Fig 3A, mutants p.G217R, p.G217S and p.K247_R254del were almost completely degraded under these conditions within 60 min; whereas mutant p.P249L was degraded partially and mutants p.G32V, p.Q178R and p.V250E were stable. In a different set of experiments, trypsin-mediated degradation of mutants was followed by activity measurements (Fig 3B) using the same low trypsin-to-CTRC ratio. The majority of mutants tested (p.D35Y, p.R37Q, p.Q48R, p.A73T and p.V235I) were stable and only mutant p.G18R and the clinically frequent mutant p.R254W exhibited some loss of activity over the 60 min time course studied. When degradation was tested at a high trypsin-to-CTRC ratio (1 μM trypsin versus 100 nM CTRC concentrations), mutants p.G18R and p.R254W were almost

completely degraded while all other mutants tested were unaffected (Fig 3C). It is interesting to note that mutant p.R254Q, in which the same amino acid is altered as in p.R254W, showed no significant degradation (data not shown).

CTRC mutants and ER stress

Mutation-induced misfolding may result in ER stress, which may lead to acinar cell damage. Previously, we demonstrated that the p.A73T CTRC mutant elicited ER stress in acinar cells [14]. Here we extended these studies and besides p.A73T we tested the clinically common mutants p.R254W and p.K247_R254del as well as two rare mutants, p.Q48R and p.G61R. The rat acinar cell line AR42J was transfected with recombinant adenovirus carrying wild-type or mutant CTRC and ER stress was characterized by analyzing splicing of the X-box binding protein 1 (XBP1) mRNA and upregulation of mRNAs for the ER chaperons immunoglobulin-binding protein (BiP) and calreticulin. As shown in Figs 4A and 4B, at 24 h post-transfection, splicing of XBP1 was increased by mutants p.Q48R, p.G61R and p.A73T, whereas mutants p.R254W and p.K247_R254del had no such effect. The same XBP1 splicing pattern was found when RNA was isolated at 48 h after transfection (reviewed but not shown). All three mutants that stimulated XBP1 splicing were shown to have secretion defects in previous experiments (see Fig 2). Furthermore, the magnitude of the secretion defect correlated with the extent of XBP1 splicing (Fig 4C), suggesting a causal relationship. Messenger RNA levels for BiP and calreticulin were elevated by the same three mutants that stimulated XBP1 splicing (Fig 5). The results indicate that ER stress is only induced by mutants in which the loss of function is related to diminished secretion, whereas ER stress is not associated with other loss of function phenotypes, such as catalytic defect or proteolytic instability.

DISCUSSION

In the present study we established a functional database for all missense *CTRC* variants identified to date. Because pathogenic *CTRC* variants are not causative but rather act as risk factors for chronic pancreatitis, they may be found both in patients and in healthy controls. Similarly, innocuous *CTRC* variants may be present in both populations. Consequently, clinical relevance of *CTRC* variants cannot be ascertained unless their frequency allows a statistically meaningful comparison between patient and control populations. To date, four variants have been classified as pathogenic on the basis of genetic association, p.A73T, p.V235I, p.R254W and p.K247_R254del (Tables 1–3). With respect to the other 28 rare *CTRC* variants, phenotypic resemblance, or the lack thereof, with the known pathogenic variants may support or rule out clinical relevance.

The results demonstrate that *CTRC* variants can cause loss of CTRC function by one or more of three mechanisms: reduced secretion, catalytic defect and increased degradation by trypsin (Fig 6, Table 5). Considering the clinically frequent mutants, a marked secretion defect was observed with p.A73T only, whereas mutants p.R254W and p.K247_R254del were secreted to slightly reduced levels and mutant p.V235I was secreted close to wild-type levels. With respect to catalytic activity, mutant p.K247_R254del was completely inactive, mutants p.A73T and p.V235I exhibited a small decrease in catalytic efficiency, whereas mutant p.R254W was as active as wild-type CTRC. The small catalytic defect of the p.V235I mutant was more prominent in the trypsinogen degradation assay. Finally, mutant p.K247_R254del was readily degraded by low concentrations of trypsin, mutant p.R254W was degraded by high concentrations of trypsin, while mutants p.A73T and p.V235I were resistant to degradation. Ten of the 28 rare mutants studied exhibited one or more forms of functional impairment; including secretion defect (p.G61R, p.C155Y, p.L220R; p.Q48R, p.G217R), catalytic deficiency (p.G32V, p.Q178R, p.G217S, p.P249L, p.V250E) and degradation by trypsin (p.G217R, p.G217S) (Table 5). High concentrations of trypsin also

degraded mutants p.P249L and p.G18R, however, the pathological significance of this phenotype is uncertain. Importantly, none of the mutants studied exhibited a gain of function such as increased secretion or higher activity, confirming that loss of CTRC activity is the disease-relevant phenotypic change caused by *CTRC* variants.

Preliminary functional characterization was reported previously for *CTRC* variants p.R37Q, p.Q48R, p.G61R, p.A73T, p.G217S, p.V235I, p.R254W, p.K247_R254del [4, 8]. In those studies a significant secretion defect was found with mutants p.Q48R, p.G61R, p.A73T and p.K247_R254del and a catalytic defect was observed with mutants p.G217S and p.K247_R254del. The present data confirm the previous findings to a large degree with some notable differences. Thus, mutant p.K247_R254del was secreted to much higher levels in this study than previously described, with levels reaching 60% of wild type in HEK 293T cells and 100% in AR42J cells. While the reason behind the conflicting results is not readily apparent, the newly discovered sensitivity of this mutant to proteolytic degradation may explain the lower values reported previously. Finally, mutant p.Q48R was reported earlier to undergo trypsin-mediated degradation which we were unable to reproduce here. On the other hand, trypsin-mediated degradation emerged as a new, so far underappreciated loss-of-function mechanism for a number of *CTRC* variants.

Intuitively, the extent of the loss of CTRC function should correlate with the clinical risk the mutation confers. In this respect, it is noteworthy that mutants p.V235I and p.R254W had a milder overall loss of function (circa 50%) than mutants p.A73T and p.K247_R254del. Accordingly, the OR values indicating clinical risk were somewhat smaller for variants p.V235I and p.R254W (5.2 and 3.6, respectively) compared to variants p.A73T and p.K247_R254del (8.2 and 6.4, respectively) (Tables 1 and 2). Despite this apparent trend, a clear quantitative correlation between genetic risk and CTRC activity cannot be drawn at this time and additional genetic studies are needed to obtain more precise OR values.

One of the most interesting questions of the present study was whether ER stress is a clinically relevant mechanism of action of *CTRC* variants. Previously, mutant p.A73T was characterized in this respect and we found that pancreatic acinar cells respond with ER stress and eventual apoptosis to high level expression of this CTRC mutant [14]. Here, we confirmed that p.A73T causes ER stress but also demonstrated that the clinically relevant p.R254W and p.K247_R254del mutants have no such effect. Therefore, we conclude, loss of CTRC activity rather than ER stress is the disease relevant mechanism of increased pancreatitis risk associated with *CTRC* variants. However, we cannot exclude the possibility that ER stress contributes to disease risk with a subset of *CTRC* variants (Fig 6). In this respect, the results indicated that only mutants with a secretion defect cause ER stress and the extent of ER stress seemed to correlate with the loss of secretion, suggesting that misfolding of CTRC underlies both phenomena. ER stress was previously suggested to cause chronic pancreatitis in patients carrying *PRSS1* variants p.R116C or p.C139S which cause trypsinogen misfolding [18].

In summary, we found 14 potentially pathogenic CTRC mutants that exhibit some form of functional defect (Table 5). We identified three different loss-of-function mechanisms: reduced secretion with associated ER stress, decreased catalytic activity and degradation by trypsin. The phenotypic dataset should aid in the classification of the clinical relevance of *CTRC* variants identified in patients with chronic pancreatitis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Summary box

What is already known about this subject

- Chymotrypsin C (CTRC) protects the pancreas by suppressing trypsinogen activation.
- Loss-of-function *CTRC* variants increase the risk for chronic pancreatitis.
- The p.A73T CTRC mutant elicits endoplasmic reticulum stress.

What are the new findings

- Less than half of known *CTRC* missense variants are functionally deleterious and can be considered pathogenic.
- *CTRC* variants impair function by three distinct mechanisms that affect secretion, catalytic activity and proteolytic stability.
- CTRC mutants with a secretion defect cause endoplasmic reticulum stress.

How might it impact on clinical practice in the foreseeable future?

- The functional database will facilitate clinical classification of *CTRC* variants as pathogenic versus neutral.

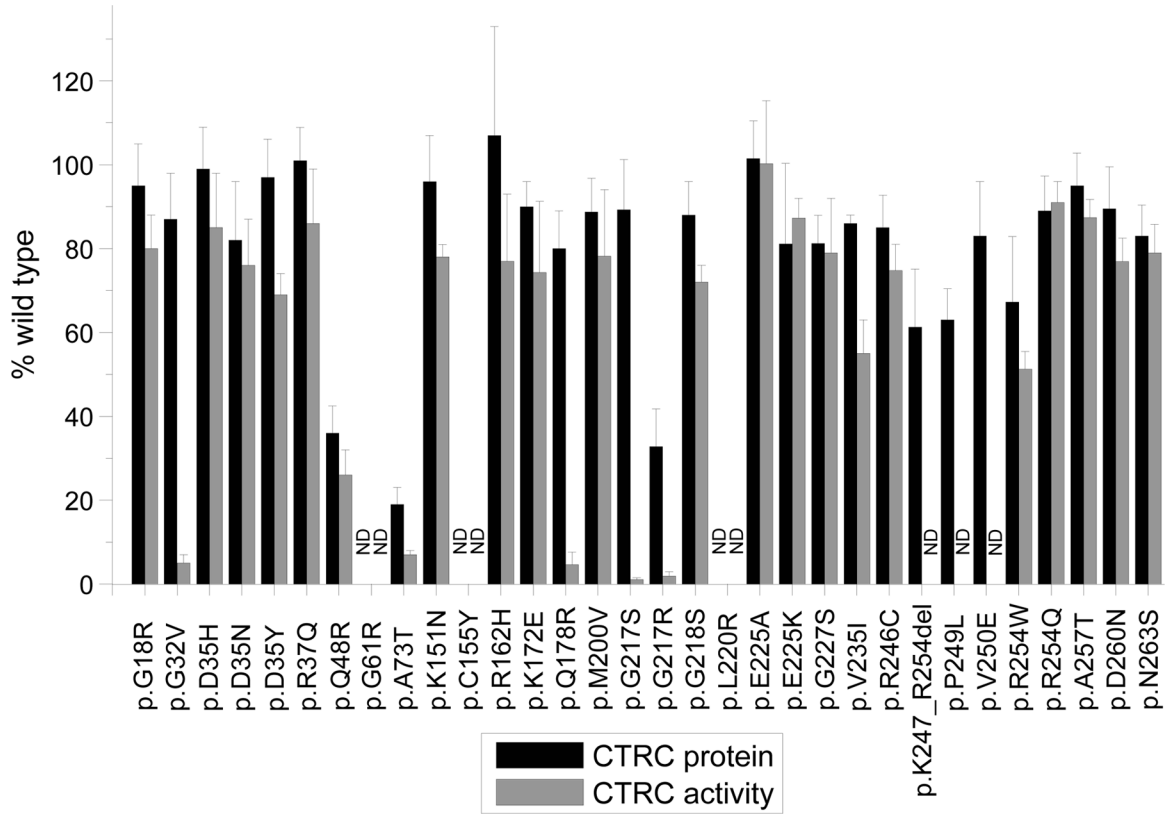

Figure 1.

CTRC protein content and enzyme activity in conditioned media of HEK 293T cells expressing CTRC mutants. Cells were transiently transfected with expression plasmids for wild-type CTRC and the indicated mutants and conditioned media were collected after 24 h, as described in *Methods*. CTRC protein levels (black bars) were determined by SDS-PAGE and densitometry and enzyme activity (gray bars) was measured after activation with trypsin using the Suc-Ala-Ala-Pro-Phe-*p*-nitroanilide substrate. See *Methods* for experimental details. CTRC protein content and activity were expressed relative to wild-type CTRC as percentage values. N.D., no protein or activity was detectable. The figure shows the average values for 3 independent experiments with the standard deviation.

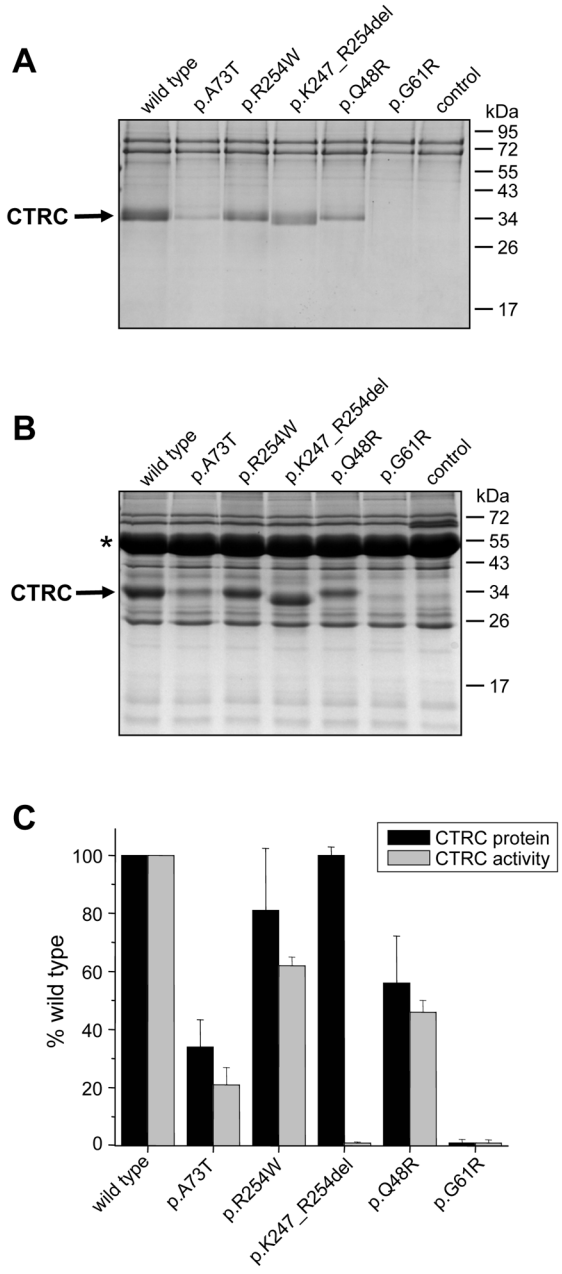

Figure 2.

Expression of select CTRC mutants in (**A**) HEK 293T cells and (**B, C)** AR42J cells. (**A, B**) Cells were transfected with the indicated wild-type and mutant expression plasmids (HEK 293T) or adenovirus vectors (AR42J) as given in *Methods*. Conditioned media were analyzed by SDS-PAGE and Coomassie blue staining. Representative gels are shown. Mutants presented from the HEK 293T cell experiments in panel **A** were selected to match those from the AR42J cell experiments in panel **B**. (**C**) Densitometric evaluation of CTRC protein content and enzyme activity in the conditioned medium from AR42J cells. CTRC protein content and activity were expressed relative to wild-type CTRC as percentage values (average of three experiments ± standard deviation). Enzyme activity was measured after activation with trypsin using the Suc-Ala-Ala-Pro-Leu-*p*-nitroanilide substrate, which is

poorly cleaved by endogenously expressed chymotrypsins. The asterisk indicates the characteristically strong amylase band. See *Methods* for experimental details. Note that, with the exception of the p.K247_R254del mutant, CTRC proteins expressed from adenovirus contained a GluGlu epitope tag, which slightly altered electrophoretic mobility.

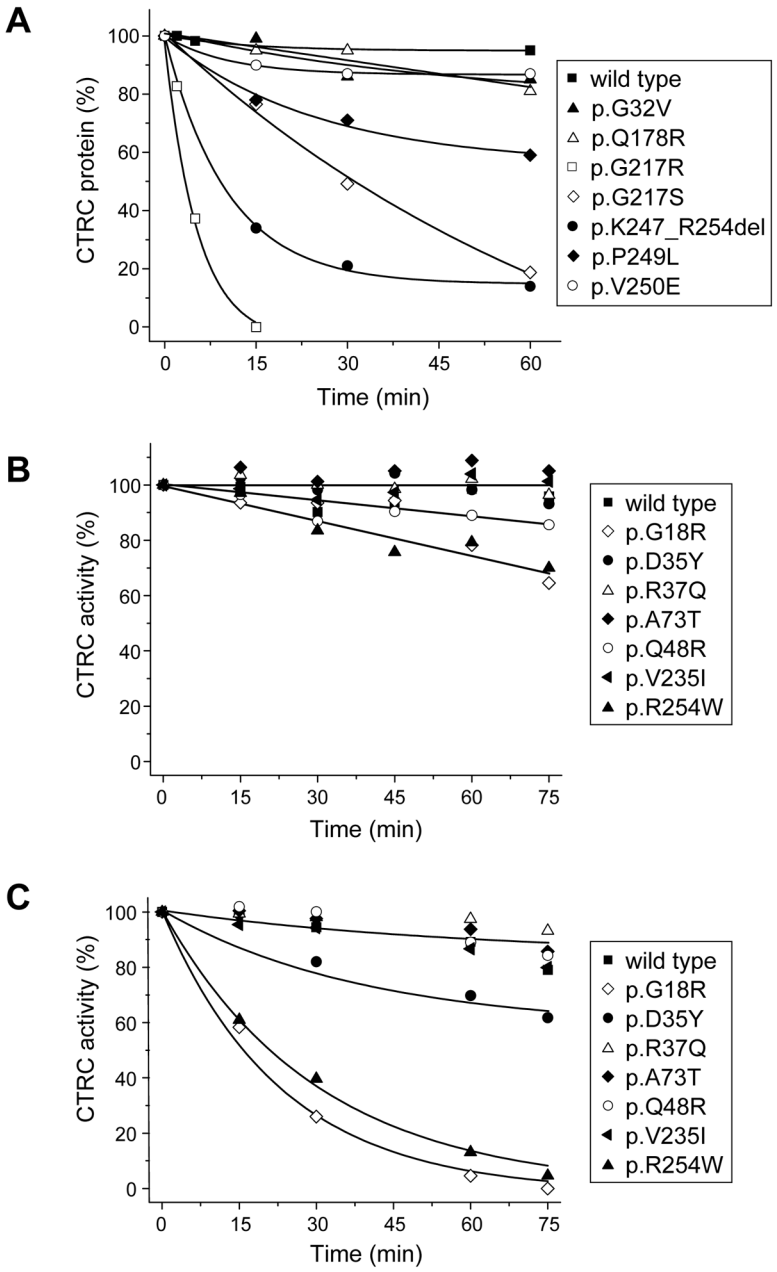

Figure 3.

Degradation of CTRC mutants by trypsin. Purified CTRC was incubated (**A, B**) at 1 μM concentration with 50 nM trypsin (low trypsin-to-CTRC ratio) or (**C**) at 100 nM concentration with 1 μM trypsin (high trypsin-to-CTRC ratio). Incubations were performed at 37°C in 100 mM Tris-HCl (pH 8.0), 10 mM $CaCl_2$ and 0.05 % Tween-20 (final concentrations). (**A**) Degradation of the low-activity CTRC mutants was analyzed by SDS-PAGE and densitometry. (**B, C**) Degradation of mutants with measurable activity was followed by activity assays. CTRC zymogen was first activated with trypsin for 5 min and the initial enzyme activity was determined. Activity was then measured at the indicated time points and expressed as percentage of the initial activity. The averages of two experiments are shown. Error bars were omitted for clarity, the error was within 15% of the mean.

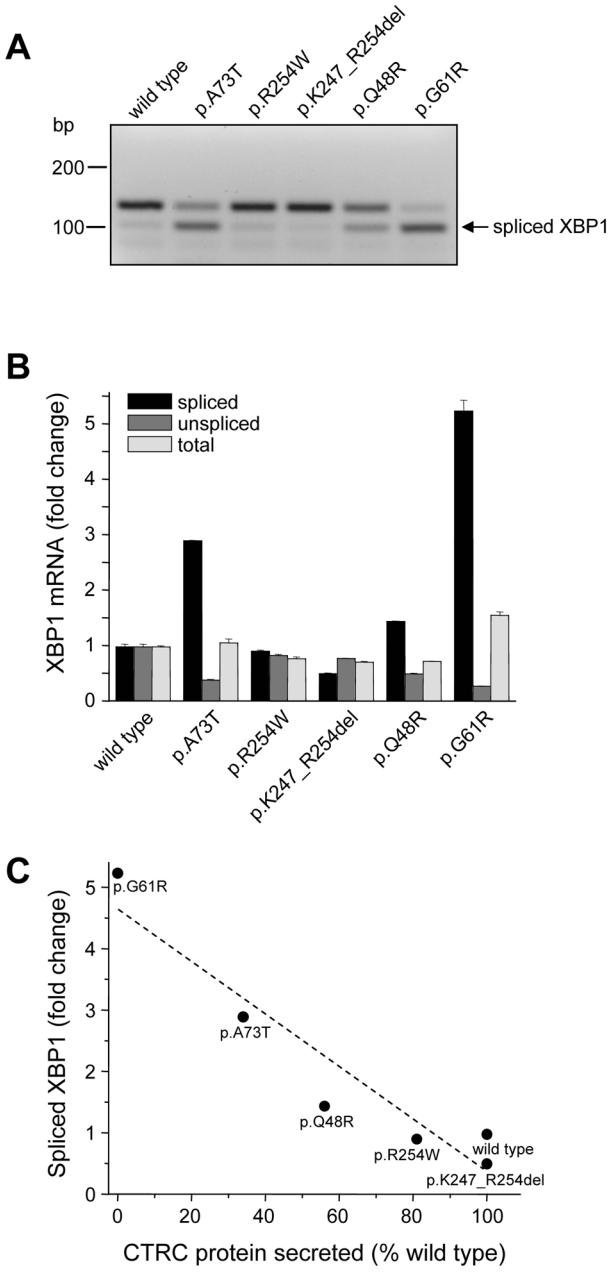

Figure 4.

Effect of expression of CTRC mutants on the splicing of XBP1 mRNA in AR42J cells. (**A**) XBP1 splicing was assessed by RT-PCR and agarose gel electrophoresis with ethidium bromide staining. (**B**) Levels for spliced (black bars), unspliced (dark gray bars) and total (light gray bars) XBP1 mRNA were measured by quantitative real-time PCR as described in *Methods*. Expression was normalized to GAPDH mRNA levels and then expressed as fold changes relative to levels measured in cells transfected with the wild-type CTRC adenovirus. Error bars represent standard deviation (n=3). (**C**) Correlation between XBP1 splicing and CTRC protein secretion. Changes in spliced XBP1 mRNA from Fig 4B were plotted against secretion data from Fig 2C. The correlation coefficient (R value) of the linear fit was −0.95.

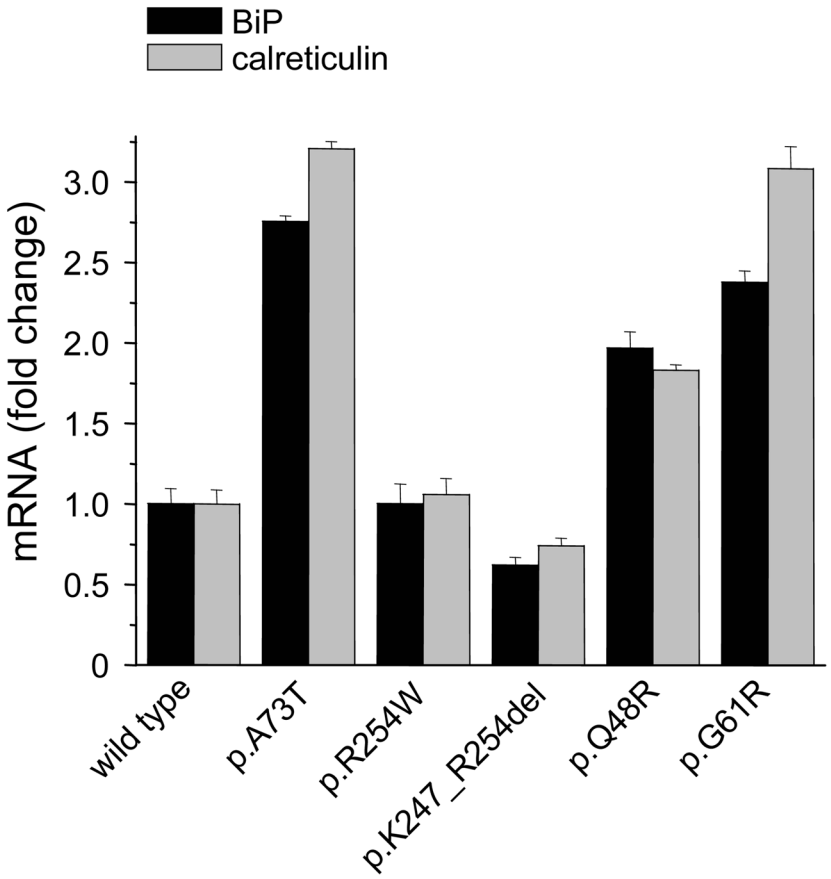

Figure 5.

Effect of expression of CTRC mutants on the mRNA levels for immunoglobulin binding protein (BiP) and calreticulin in AR42J cells. Cells were infected for 24 h with adenovirus carrying wild-type CTRC or the indicated mutants using 2×10^8 pfu per mL virus concentration. Quantitative real-time PCR measurement of BiP (black bars) and calreticulin (gray bars) mRNA with TaqMan probes was performed as described in *Methods*. Expression was normalized to GAPDH mRNA levels and then expressed as fold changes relative to levels measured in cells transfected with the wild-type CTRC adenovirus. Error bars represent standard deviation (n=3).

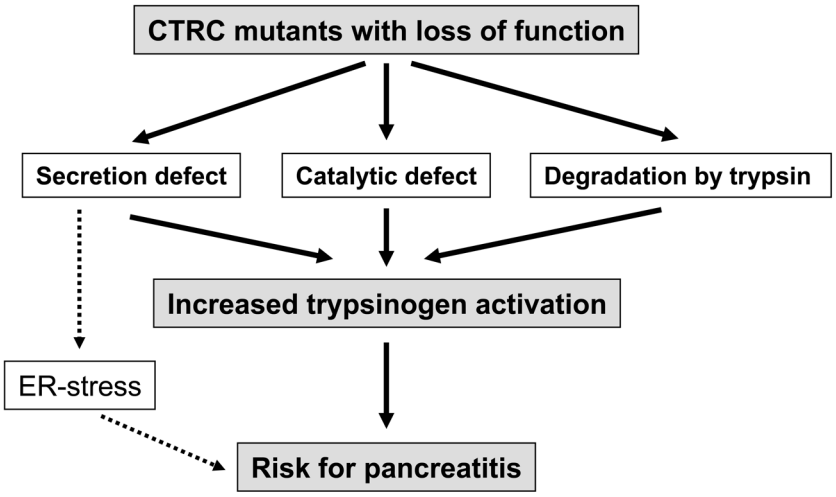

Figure 6.
Mechanism of genetic risk for chronic pancreatitis associated with CTRC mutations. See text for discussion.

Table 1

Chymotrypsin C variants in individuals of European origin. The table shows compiled data from four studies [4, 7, 10, 11]. Note that duplicate records were removed from the partially overlapping cohorts reported by Rosendahl et al. [4, 11]. The five novel mutations indicated without frequency values were identified by Ambry Genetics (p.G18R, p.D35Y, p.Q178R and p.V250E) and by the Munich laboratory (p.G32V). Homozygous (hm) individuals are listed separately. Synonymous, nonsense, frame-shift, intronic and other non-coding variants were excluded. OR, odds ratio; CI, confidence interval. The *p* values determined by Fisher's exact test were 1.0×10^{-4} for p.K247_R254del and 4.1×10^{-6} for p.R254W.

	Nucleotide change	Amino acid change	Affected individuals	Controls	OR	95% CI
Exon 2	c.52G>A	p.G18R	1	-		
	c.95G>T	p.G32V	1	-		
	c.103G>C	p.D35H	0 / 1708	1 / 3471 (0.03%)		
	c.103G>A	p.D35N	0 / 1708	1 / 3471 (0.03%)		
	c.103G>T	p.D35Y	1	-		
	c.110G>A	p.R37Q	6 / 1708 (0.35%)	13 / 3471 (0.37%)		
Exon 3	c.143A>G	p.Q48R	2 / 1708 (0.12%)	1 / 3471 (0.03%)		
	c.217G>A	p.A73T	1 / 1708 (0.06%)	0 / 3471		
Exon 5	c.453G>C	p.K151N	1 / 1176 (0.09%)	0 / 1396		
	c.464G>A	p.C155Y	1 / 1176 (0.09%)	0 / 1396		
	c.485G>A	p.R162H	1 / 1176 (0.09%)	0 / 1396		
Exon 6	c.514A>G	p.K172E	1 / 1176 (0.09%)	1 / 1396 (0.07%)		
	c.533A>G	p.Q178R	3	-		
	c.598A>G	p.M200V	1 / 1176 (0.09%)	0 / 1396		
Exon 7	c.649G>A	p.G217S	3 / 1708 (0.18%)	1 / 3586 (0.03%)		
	c.649G>C	p.G217R	2 / 1708 (0.12%)	0 / 3586		
	c.652G>A	p.G218S	0 / 1708	1 / 3586 (0.03%)		
	c.659T>G	p.L220R	0 / 1708	1 / 3586 (0.03%)		
	c.674A>C	p.E225A	0 / 1708	1 / 3586 (0.03%)		
	c.703G>A	p.V235I	3 / 1708 (0.18%)	2 / 3586 (0.06%)		
	c.738_761del24	p.K247_R254del	15 / 1739 (0.86%)	5 / 3686 (0.14%)	6.4	2.3–17.5
	c.746C>T	p.P249L	1 / 1708 (0.06%)	0 / 3586		
	c.749T>A	p.V250E	1	-		

Nucleotide change	Amino acid change	Affected individuals	Controls	OR	95% CI
c.760C>T	p.R254W	36 / 1739 (2.07%)	21 / 3686 (0.57%)	3.6	2.1–6.2
c.760C>T	p.R254W (hm)	1 / 1739 (0.06%)	0 / 3686		

Table 2

Chymotrypsin C variants in individuals of Indian origin. The table combines data from three studies [4, 8, 12]. Homozygous (hm) individuals are listed separately. Synonymous, nonsense, frame-shift, intronic and other non-coding variants were excluded. OR, odds ratio; CI, confidence interval. The *p* values determined by Fisher's exact test were 2.3×10^{-5} for p.A73T and 1.5×10^{-4} for p.V235I.

	Nucleotide change	Amino acid change	Affected individuals	Controls	OR	95% CI
Exon 3	c.143A>G	p.Q48R	1 / 801 (0.12%)	0 / 826		
	c.181G>A	p.G61R	1 / 801 (0.12%)	0 / 826		
	c.217G>A	p.A73T	24 / 801 (3%)	3 / 826 (0.36%)	8.2	2.5–27.5
	c.217G>A	p.A73T (hm)	2 / 801 (0.25%)	0 / 826		
Exon 6	c.514A>G	p.K172E	4 / 795 (0.5%)	6 / 828 (0.72%)		
Exon 7	c.679G>A	p.G227S	0 / 794	1 / 832 (0.12%)		
	c.703G>A	p.V235I	25 / 794 (3.2%)	5 / 832 (0.6%)	5.2	2.0–13.8
	c.703G>A	p.V235I (hm)	5 / 794 (0.63%)	1 / 832 (0.12%)		
	c.736C>T	p.R246C	0 / 794	2 / 832 (0.24%)		
	c.760C>T	p.R254W	5 / 794 (0.63%)	2 / 832 (0.24%)		
	c.769G>A	p.A257T	1 / 794 (0.13%)	0 / 832		
	c.778G>A	p.D260N	1 / 794 (0.13%)	0 / 832		

Table 3

Chymotrypsin C variants in individuals of Chinese origin [9]. Synonymous, nonsense, frame-shift, intronic and other non-coding variants were excluded. Note that p.E225K and p.R254Q were found in the same subject.

	Nucleotide change	Amino acid change	Affected individuals	Controls
Exon 7	c.673G>A	p.E225K	1 / 126 (0.79%)	0 / 90
	c.761G>A	p.R254Q	1 / 126 (0.79%)	0 / 90
	c.788A>G	p.N263S	1 / 126 (0.79%)	0 / 90

Table 4

Enzymatic activity of chymotrypsin C variants. Michaelis-Menten kinetic parameters were determined using purified CTRC and Suc-Ala-Ala-Pro-Phe-*p*-nitroanilide substrate as described in *Methods*. ND, no activity was detectable. Select mutants were also tested in trypsinogen degradation assays and their activity was expressed as percentage of the wild-type CTRC activity (see also Supplementary Fig S1).

	k_{cat} s^{-1}	K_M µM	k_{cat} / K_M s^{-1} M^{-1}	k_{cat} / K_M (% wild type)	Trypsinogen degradation (%)
wild type	15	13.5	1.1×10^6	100	100
p.G18R	15.7	17	9.2×10^5	84	-
p.G32V	10	590	1.7×10^4	1.5	6
p.D35Y	11.7	17.1	6.8×10^5	62	-
p.R37Q	14.4	20.4	7.1×10^5	64	-
p.Q48R	15.1	16.2	9.3×10^5	85	-
p.A73T	16.6	24.4	6.8×10^5	62	-
p.Q178R	3.5	495	7.1×10^3	0.6	25
p.G217R			ND	ND	-
p.G217S	2.7	482	5.5×10^3	0.5	8
p.V235I	12.6	15.8	8.0×10^5	73	52
p.K247_R254del			ND	ND	-
p.P249L	0.6	761	7.9×10^2	0.07	3
p.V250E	1.4	1539	9.1×10^2	0.08	12
p.R254Q	15.5	15.2	1.0×10^6	91	-
p.R254W	16.6	15.1	1.1×10^6	100	-

Table 5

Clinically relevant *CTRC* variants classified on the basis of functional phenotype. Effective activity reflects the combined effects of the mutations on secretion, catalytic activity and degradation by trypsin. This corresponds to the CTRC activity measured in Fig 1.

	Nucleotide change	Amino acid change	Loss of function mechanism
High risk CTRC variants (effective activity <10% wild type)			
Exon 2	c.95G>T	p.G32V	catalytic
Exon 2	c.181G>A	p.G61R	secretion
Exon 3	c.217G>A	p.A73T	secretion
Exon 5	c.464G>A	p.C155Y	secretion
Exon 6	c.533A>G	p.Q178R	catalytic
Exon 7	c.649G>A	p.G217S	catalytic, degradation
Exon 7	c.649G>C	p.G217R	secretion, degradation
Exon 7	c.659T>G	p.L220R	secretion
Exon 7	c.738_761del24	p.K247_R254del	catalytic, degradation
Exon 7	c.746C>T	p.P249L	catalytic
Exon 7	c.749T>A	p.V250E	catalytic
Moderate-to-low risk CTRC variants (effective activity 36–55% wild type)			
Exon 3	c.143A>G	p.Q48R	secretion
Exon 7	c.703G>A	p.V235I	catalytic
Exon 7	c.760C>T	p.R254W	secretion, degradation