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Chymotrypsin C (CTRC) variants that diminish activity or secretion are associated with chronic pancreatitis

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Chronic pancreatitis is a persistent inflammatory disease of the pancreas, in which the digestive protease trypsin has a fundamental pathogenetic role. Here we have analyzed the gene encoding the trypsin-degrading enzyme chymotrypsin C (*CTRC*) in German subjects with idiopathic or hereditary chronic pancreatitis. Two alterations in this gene, p.R254W and p.K247_R254del, were significantly overrepresented in the pancreatitis group, being present in 30 of 901 (3.3%) affected individuals but only 21 of 2,804 (0.7%) controls (odds ratio (OR) = 4.6; confidence interval (CI) = 2.6–8.0; $P = 1.3 \times 10^{-7}$). A replication study identified these two variants in 10 of 348 (2.9%) individuals with alcoholic chronic pancreatitis but only 3 of 432 (0.7%) subjects with alcoholic liver disease (OR = 4.2; CI = 1.2–15.5; $P = 0.02$). *CTRC* variants were also found in 10 of 71 (14.1%) Indian subjects with tropical pancreatitis but only 1 of 84 (1.2%) healthy controls (OR = 13.6; CI = 1.7–109.2; $P = 0.0028$). Functional analysis of the CTRC variants showed impaired activity and/or reduced secretion. The results indicate that loss-of-function alterations in CTRC predispose to pancreatitis by diminishing its protective trypsin-degrading activity.

Chronic pancreatitis is an inflammatory disorder characterized by permanent destruction of the pancreatic parenchyma, leading to maldigestion and diabetes mellitus as a result of exocrine and endocrine insufficiency. Insights into the pathological mechanism of the disorder have come from relatively recent studies investigating the genes encoding cationic trypsinogen (*PRSS1*; OMIM 276000), anionic trypsinogen (*PRSS2*; OMIM 601564) and the pancreatic secretory trypsin inhibitor (*SPINK1*; OMIM 167790). Gain-of-function variants in *PRSS1* have been linked to autosomal dominant hereditary pancreatitis and subsequently to idiopathic chronic pancreatitis as well[1–4]. Recently, triplication of the *PRSS1* locus has been observed in a subset of families with hereditary pancreatitis[5]. *In vitro* biochemical studies have indicated that the majority of disease-predisposing *PRSS1* variants increase autocatalytic conversion of trypsinogen to active trypsin and probably promote premature intrapancreatic trypsin activation *in vivo*[6,7]. Consistent with the central pathophysiological role of trypsin, p.N34S and other loss-of-function alterations in the trypsin inhibitor *SPINK1* predispose to idiopathic, tropical and alcoholic chronic pancreatitis[8–15]. In contrast to pathogenic *PRSS1* and *SPINK1* variations, the p.G191R *PRSS2* variant, which leads to rapid trypsin autodegradation, affords protection against chronic

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Table 1 ***CTRC*** **variants detected in German subjects with idiopathic or hereditary chronic pancreatitis and healthy controls**

Exon	Nucleotide change	Amino acid change	Affected individuals (ICP)	Affected individuals (HP)	Affected individuals (all)	Controls	*P* value	OR	95% CI
2	c.103G>C	p.D35H	0/758 (0%)	0/143 (0%)	0/901 (0%)	1/2689 (0.04%)	1.0	-	-
2	c.103G>A	p.D35N	0/758 (0%)	0/143 (0%)	0/901 (0%)	1/2689 (0.04%)	1.0	-	-
2	c.110G>A	p.R37Q	5/758 (0.7%)	1/143 (0.7%)	6/901 (0.7%)	10/2689 (0.4%)	0.25	-	-
3	c.143A>G	p.Q48R	0/758 (0%)	2/143 (1.4%)	2/901 (0.2%)	1/2689 (0.04%)	0.16	-	-
4	c.308delG	p.G103VfsX31	0/499 (0%)	1/122 (0.8%)	1/621 (0.2%)	0/614 (0%)	1.0	-	-
6	c.514A>G	p.K172E	0/499 (0%)	0/122 (0%)	0/621 (0%)	1/614 (0.2%)	0.5	-	-
7	c.649G>A	p.G217S	2/758 (0.3%)	0/143 (0%)	2/901 (0.2%)	1/2804 (0.04%)	0.15	-	-
7	c.652G>A	p.G218S	0/758 (0%)	0/143 (0%)	0/901 (0%)	1/2804 (0.04%)	1.0	-	-
7	c.659T>G	p.L220R	0/758 (0%)	0/143 (0%)	0/901 (0%)	1/2804 (0.04%)	1.0	-	-
7	c.674A>C	p.E225A	0/758 (0%)	0/143 (0%)	0/901 (0%)	1/2804 (0.04%)	1.0	-	-
7	c.703G>A	p.V235I	1/758 (0.1%)	0/143 (0%)	1/901 (0.1%)	1/2804 (0.04%)	0.43	-	-
7	c.760C>T	p.R254W	13/758 (1.7%)	6/143 (4.2%)	19/901 (2.1%)	18/2804 (0.6%)	0.0004[a]	3.3	1.7–6.4
7	c.738_761del24	p.K247_R254del	11/758 (1.5%)	0/143 (0%)	11/901 (1.2%)	3/2804 (0.1%)	0.00003[a]	11.5	3.2–41.5

ICP, idiopathic chronic pancreatitis; HP, hereditary chronic pancreatitis. *P* values were determined by Fisher's exact test.
[a]All affected individuals against controls.

pancreatitis[16]. Taken together, genetic and biochemical evidence define a pathological pathway in which a sustained imbalance between intrapancreatic trypsinogen activation and trypsin inactivation results in the development of chronic pancreatitis (**Supplementary Fig. 1** online).

Because trypsin degradation serves as a protective mechanism against pancreatitis, we hypothesized that loss-of-function variants in trypsin-degrading enzymes increase the risk for pancreatitis. We recently demonstrated that chymotrypsin C (CTRC) degrades all human trypsin and trypsinogen isoforms with high specificity[17]. From these studies, *CTRC* emerged as a strong candidate for a pancreatitis-associated gene. We carried out direct DNA sequencing of all eight exons of the 8.2-kb *CTRC* gene in 621 individuals with idiopathic or hereditary chronic pancreatitis and in 614 control subjects of German origin. We found several *CTRC* variants, the large majority of which affected exons 2, 3 and 7. Therefore, we extended our analyses by sequencing an additional 280 affected individuals for these three exons and an additional 2,075 controls for exons 2 and 3 and 2,190 controls for exon 7. Altogether, we identified 11 missense and 2 deletion variants in *CTRC* (**Table 1**). The two most frequent variants, c.760C>T (p.R254W) and c.738_761del24 (p.K247_R254del), both located in exon 7, were found in affected individuals with a frequency of 2.1% and 1.2%, respectively. Taken together, the two alterations were significantly overrepresented in the pancreatitis group (30 of 901; 3.3%) compared to controls (21 of 2,804; 0.7%) (OR = 4.6; CI = 2.6–8.0; $P = 1.3 \times 10^{-7}$). Variant c.738_761del24, which causes an in-frame deletion of eight amino acids from Lys247 through Arg254 (p.K247_R254del), showed the strongest disease association (OR = 11.5; CI = 3.2–41.5; $P = 0.00003$). Subgroup analysis for these two heterozygous variants showed similar frequency in the hereditary (6 of 143; 4.2%; 6 × p.R254W) and idiopathic (24 of 758; 3.2%; 13 × p.R254W; 11 × p.K247_R254del) groups (**Table 1**). One individual with idiopathic disease was compound heterozygous for p.V235I (inherited from the mother) and p.R254W (inherited from the father).

To confirm our findings in an independent cohort affected with another inflammatory pancreatic disease, we sequenced all eight exons of *CTRC* in 96 German individuals affected with alcohol-related chronic pancreatitis. Subsequently, we sequenced exons 2, 3 and 7 in an additional 252 subjects (348 subjects in total) with alcoholic chronic pancreatitis. For controls, we analyzed exons 2, 3 and 7 in 432 German individuals with alcoholic liver disease but without chronic pancreatitis. Again, we found a significant enrichment of the two exon 7 variants, p.R254W and p.K247_R254del, in subjects with alcoholic pancreatitis (10 of 348; 2.9%) versus subjects with alcohol-related liver disease (3 of 432; 0.7%) (OR = 4.2; CI = 1.2–15.5; $P = 0.02$; **Table 2**).

Finally, to investigate the significance of *CTRC* variants in chronic pancreatitis in subjects of non-European descent, we analyzed 71 individuals affected with tropical pancreatitis and 84 controls of Indian origin. Notably, the frequency of *CTRC* alterations in subjects with pancreatitis was even higher in this cohort (**Table 3**). Overall, 14.1% of affected individuals but only 1.2% of controls carried a *CTRC* variant (OR = 13.6; CI = 1.7–109.2; $P = 0.0028$). Two relatively frequent variants found in Indians were absent in Germans: the c.217G>A (p.A73T) missense alteration and the c.190_193delATTG (p.I64LfsX69) frameshift deletion. On the other

Table 2 ***CTRC*** **variants detected in German subjects with alcohol-related chronic pancreatitis and controls with alcoholic liver disease without pancreatitis**

Exon	Nucleotide change	Amino acid change	Affected individuals	Controls	*P* value	OR	95% CI
2	c.110G>A	p.R37Q	0/348 (0%)	3/432 (0.7%)	0.26	–	–
7	c.649G>C	p.G217R	1/348 (0.3%)	0/432 (0%)	0.45	–	–
7	c.703G>A	p.V235I	1/348 (0.3%)	1/432 (0.2%)	1.0	–	–
7	c.746C>T	p.P249L	1/348 (0.3%)	0/432 (0%)	0.45	–	–
7	c.760C>T	p.R254W	8/348 (2.3%)	2/432 (0.5%)	0.03	5.1	1.1–24.0
7	c.738_761del24	p.K247_R254del	2/348 (0.6%)	1/432 (0.2%)	0.58	–	–

P values were determined by Fisher's exact test.

Table 3 ***CTRC*** **variants detected in subjects with tropical pancreatitis and healthy controls of Indian origin**

Exon	Nucleotide change	Amino acid change	Affected individuals	Controls	*P* value	OR	95% CI
3	c.190_193delATTG	p.I64LfsX69	2/71 (2.8%)	0/84 (0%)	0.21	–	–
3	c.217G>A	p.A73T	4/71 (5.6%)	0/84 (0%)	0.04	11.3	0.6–213
7	c.703G>A	p.V235I	1/71 (1.4%)	0/84 (0%)	0.46	–	–
7	c.760C>T	p.R254W	2/71 (2.8%)	1/84 (1.2%)	0.60	–	–
7	c.778G>A	p.D260N	1/71 (1.4%)	0/84 (0%)	0.46	–	–
All			10/71 (14.1%)	1/84 (1.2%)	0.0028	13.6	1.7–109.2

P values were determined by Fisher's exact test.

hand, the p.K247_R254del variant that was relatively frequent in affected individuals from Germany was not found in the Indian population, and the enrichment of the p.R254W variant in subjects with tropical pancreatitis did not reach statistical significance. However, because of the significantly smaller size of the Indian cohort relative to the German cohort, caution is warranted in the interpretation of these differences. Nonetheless, the data clearly indicate that *CTRC* variants increase the risk for tropical pancreatitis as well.

Chronic pancreatitis is a complex, multigenic disease, and affected individuals often carry mutations in several disease-associated genes. To elucidate the relationship between *CTRC* alterations and *PRSS1* and *SPINK1* variants, we investigated all German subjects with idiopathic or hereditary pancreatitis for p.A16V, p.N29I and p.R122H in *PRSS1* and for p.N34S in *SPINK1*. In total, 52 of 901 of these individuals (5.8%) carried a heterozygous *PRSS1* variant, whereas 138 of 901 (15.3%) were positive for p.N34S (121 heterozygotes, 17 homozygotes). In the hereditary pancreatitis subgroup, 32 of 143 subjects (22.4%) carried a heterozygous *PRSS1* mutation, 30 of 143 (21%) carried a heterozygous p.N34S *SPINK1* mutation and 2 of 143 (1.4%) were homozygous for p.N34S. In the idiopathic pancreatitis subgroup, 20 of 758 individuals (2.6%) tested positive for a heterozygous *PRSS1* mutation, 91 of 758 (12%) were heterozygous for the p.N34S *SPINK1* mutation and 15 of 758 (2%) were homozygous for p.N34S. One subject with idiopathic disease was trans-heterozygous for the *PRSS1* p.R122H variant (inherited from the mother) and the *CTRC* p.G217S variant (inherited from the father). None of the 17 *SPINK1* p.N34S homozygotes carried a *CTRC* variant. On the other hand, 9 of 121 p.N34S heterozygotes (7.4%) were also heterozygous for one of two pancreatitis-associated *CTRC* variants (seven p.R254W, two p.K247_R254del). In contrast, only 21 of 763 affected individuals without p.N34S (2.8%) were heterozygous for p.R254W or p.K247_R254del ($P = 0.014$; p.N34S heterozygous versus p.N34S wild-type). Sixteen out of 902 control subjects (1.8%) were p.N34S heterozygous, but none of the 16 carried a *CTRC* variant. The alcoholic chronic pancreatitis cohort was analyzed for the *SPINK1* p.N34S variant. Twenty-two of 348 subjects (6.3%) were heterozygous for p.N34S, and one subject (1 of 22, 4.6%) was trans-heterozygous for *SPINK1* p.N34S and *CTRC* p.K247_R254del. The Indian subjects were screened for *PRSS1* (p.A16V, p.N29I and p.R122H) and *SPINK1* (p.N34S) variants as reported previously[12]. None of the affected individuals or control subjects carried a *PRSS1* variant. Three controls (3.6%) were heterozygous for p.N34S but carried no *CTRC* alterations. Among the subjects with tropical pancreatitis, 29 of 71 (40.9%) were positive for p.N34S (22 heterozygotes, 7 homozygotes). Notably, none of the homozygotes, but 6 of 22 of the heterozygotes (27.3%), carried a *CTRC* variant (two p.I64LfsX69, two p.A73T, two p.R254W and one p.D260N; one individual was compound heterozygous for p.A73T and p.D260N). In contrast, only 3 of 42 individuals (7.1%) with wild-type *SPINK1* were heterozygous for a *CTRC* variant (two p.A73T, one p.V235I) ($P = 0.051$; p.N34S heterozygous versus p.N34S wild type).

As has been observed previously for other pancreatitis-associated gene alterations, the majority of the *CTRC* variants identified do not alter the reading frame of the translated CTRC protein. The two exceptions are the c.190_193delATTG (p.I64LfsX69) and the c.308delG (p.G103VfsX31) variants, which cause a shift in the reading frame and may result in a truncated polypeptide chain. Alternatively, the mRNA of these variants may undergo nonsense-mediated decay and thus result in no CTRC protein whatsoever. In any event, these frameshift variants are expected to cause a complete loss of CTRC function.

To investigate the functional consequences of the *CTRC* missense alterations and the p.K247_R254del in-frame deletion, we expressed wild-type and variant CTRC proteins in human embryonic kidney (HEK) 293T cells through transient transfection. We found that secretion of the p.K247_R254del and p.A73T variant proteins was severely diminished relative to wild-type protein, as evidenced by the loss of secreted CTRC activity and the faint protein bands on gels (**Fig. 1a**,**b**, **Supplementary Fig. 2** and **Supplementary Table 1** online). When we determined the specific activity of the proteins encoded by these two variants, the p.K247_R254del variant protein proved completely inactive, whereas the p.A73T variant protein showed substantial protease activity (**Supplementary Table 1**). In contrast to the nearly complete loss of function observed with p.K247_R254del and p.A73T, CTRC activity secreted by cells expressing the p.R254W variant protein was reduced to about 50% of wild-type activity (**Fig. 1a**). SDS-PAGE of conditioned media showed that secreted amounts of p.R254W variant CTRC were about 40% that of wild-type CTRC, suggesting that the functional defect in this variant is related to decreased production rather than impaired catalytic activity (**Fig. 1b**, **Supplementary Fig. 2** and **Supplementary Table 1**). This conclusion was strengthened by enzyme kinetic analysis of purified wild-type and p.R254W variant CTRC. Kinetic parameters compared on two different chromogenic peptide substrates were essentially identical (**Supplementary Table 2** online), indicating that reduced function of p.R254W variant CTRC is solely due to decreased production.

In addition to the frequent CTRC alterations mentioned above, we also analyzed variants p.R37Q, p.Q48R, p.G217S and p.V235I through transient transfections in HEK 293T cells (**Supplementary Fig. 2** and **Supplementary Table 1**). Variant p.R37Q protein showed essentially normal activity and secretion (~82–88% that of wild type). In contrast, no CTRC activity was measurable from conditioned media of cells transfected with variant p.Q48R, and SDS-PAGE revealed a secretion defect (~30% that of wild type). We also found that variant p.Q48R protein underwent degradation during trypsin-mediated activation, presumably because the alteration introduced a new trypsin-sensitive site (data not shown). Variant p.G217S protein

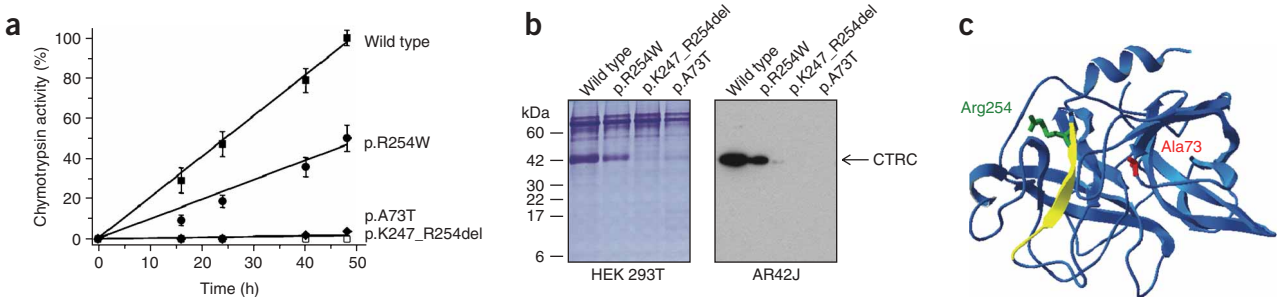

Figure 1 Effects of pancreatitis-associated *CTRC* variants on the secretion of chymotrypsinogen C. (**a**) We transfected HEK 293T cells with the indicated constructs and withdrew aliquots of conditioned media at the given times. CTRC activity was determined after activation with trypsin and expressed as percentage of the maximal activity, which corresponded to 14.7 μM *p*-nitroaniline released per minute. (**b**) Aliquots (0.15 ml) of conditioned media from transfected HEK 293T cells were precipitated with 10% trichloroacetic acid (final concentration) and analyzed by SDS-PAGE and Coomassie Blue staining (left). Alternatively, AR42J cells were transfected with Glu-Glu–tagged versions of wild-type and variant *CTRC* constructs and stimulated with 1 nM cerulein. We then precipiated aliquots (0.1 ml) of the conditioned media with trichloroacetic acid and analyzed them by protein blotting using an HRP-conjugated goat polyclonal antibody against the Glu-Glu tag (Abcam). As a control, the cerulein-induced endogenous chymotrypsin secretion was also measured from 0.1 ml medium with 0.1 mM Suc-Ala-Ala-Pro-Phe–*p*-nitroaniline substrate (final concentration). We determined activity values of 24, 23, 21 and 21 μM *p*-nitroaniline per min for cells expressing wild-type, p.R254W, p.K247_R254del or p.A73T *CTRC*, respectively. (**c**) Ribbon diagram of chymotrypsinogen C. The bovine chymotrypsinogen C molecule is shown; this was crystallized as part of a ternary complex with proproteinase E and procarboxypeptidase A. The position of Ala73 is shown in red and that of Arg254 is shown in green. The yellow peptide segment is deleted in variant p.K247_R254del. The image was rendered using DeepView/Swiss-PdbViewer v. 3.7 (see URLs section in Methods).

showed significant catalytic impairment (specific activity only ~7% that of wild-type) and a modest decrease in secretion (~70% that of wild type). Finally, CTRC activity secreted by cells transfected with variant p.V235I was moderately reduced (~65% that of wild type), which seemed to be the result of a combination of slightly lower specific activity and slightly reduced secretion of CTRC protein. Michaelis-Menten kinetic parameters determined for variant p.V235I CTRC were not significantly different from those of wild-type CTRC (**Supplementary Table 2**). Thus, from the four relatively rare *CTRC* variants analyzed, p.Q48R and p.G217S are loss-of-function *CTRC* alterations, whereas p.R37Q and p.V235I result in normal or slightly diminished function, respectively.

To demonstrate that the clinically significant CTRC variants are also poorly secreted in cells that more closely resemble pancreatic acinar cells, we carried out transfection experiments with the AR42J rat acinar cell line[18]. Wild-type CTRC and the p.A73T, p.K247_R254del and p.R254W variant proteins were tagged with a Glu-Glu affinity tag to allow specific detection in the background of the native chymotrypsinogens secreted by AR42J cells. Stimulation of transfected cells with the cholecystokinine analog cerulein resulted in the secretion of immunoreactive wild-type protein and somewhat less of the p.R254W variant protein, whereas p.A73T and p.K247_R254del variant proteins were not secreted at detectable amounts (**Fig. 1b**).

Molecular modeling indicates that p.K247_R254del eliminates the last of the six β-strands of the C-terminal antiparallel β-barrel domain (**Fig. 1c**). A change of this magnitude in a structurally conserved region is expected to cause a folding defect, which might lead to the observed loss of catalytic function and the diminished secretion. Notably, p.R254W is also located within this deleted peptide segment. The mechanism underlying the decreased CTRC production caused by the p.A73T alteration is not readily apparent but may also involve misfolding. It is intriguing to note that alterations at different positions in the protein structure can lead to similar secretion problems. Possible explanations for this phenomenon are that local misfolding exposes hydrophobic portions of the protein or that the alteration itself renders the hydrophilic surface more hydrophobic. The hydrophobic surfaces may in turn interact with various chaperones resident in the endoplasmic reticulum, resulting in retention and eventual degradation.

In summary, the genetic and functional data presented in this study identify *CTRC* as a new pancreatitis-associated gene. Our observations provide further support for the trypsin-dependent pathogenic model of chronic pancreatitis in humans (**Supplementary Fig. 1**) by demonstrating that trypsin-trypsinogen degradation by CTRC is an important mechanism for maintaining the physiological protease-antiprotease balance in the pancreas.

METHODS

Study population. This study was approved by the medical ethical review committees of the University of Leipzig and the Charité University Hospital. All affected individuals gave informed consent. We enrolled 1,320 unrelated individuals with the diagnoses of hereditary (n = 143) or idiopathic chronic pancreatitis (n = 758) or alcoholic chronic pancreatitis (n = 348), all originating from Germany. In addition, we also investigated subjects affected with tropical calcific pancreatitis originating from India (n = 71). The diagnosis of chronic pancreatitis was based on two or more of the following findings: presence of a typical history of recurrent pancreatitis, pancreatic calcifications and/or pancreatic ductal irregularities revealed by endoscopic retrograde pancreaticography or by magnetic resonance imaging of the pancreas, and pathological sonographic findings. Hereditary chronic pancreatitis was diagnosed when one first-degree relative or two or more second-degree relatives suffered from recurrent acute or chronic pancreatitis without apparent precipitating factors. Affected individuals were classified as having idiopathic chronic pancreatitis when precipitating factors, such as alcohol abuse, trauma, medication, infection, metabolic disorders or a positive family history consistent with hereditary pancreatitis, were absent. Alcohol-induced chronic pancreatitis was diagnosed in patients who consumed more than 60 g (females) or 80 g (males) of ethanol per day for more than 2 years. Control subjects free of pancreatic diseases were recruited from Germany (n = 2,804) and India (n = 81). The German controls included parents of children recruited to the German Multicenter Allergy Study, healthy controls recruited for a genetic study on type 2 diabetes at the University Hospital at Leipzig, blood donors, medical students, and staff and subjects recruited to the Berlin Aging Study. In addition, 432 German subjects with alcoholic liver disease but without pancreatic disease were recruited as controls for the alcoholic chronic pancreatitis group.

Mutation screening. Oligonucleotide sequences, PCR and cycle sequencing conditions are available online in **Supplementary Methods**. We digested the PCR products with shrimp alkaline phosphatase and exonuclease I and carried out cycle sequencing using BigDye terminator mix (Applied Biosystems). The reaction products were purified with ethanol precipitation or Sephadex G-50 and loaded onto an ABI 3100-Avant or an ABI 3730 sequencer (Applied Biosystems).

Plasmid construction and mutagenesis. We constructed the pcDNA3.1(–)-CTRC expression plasmid using the IMAGE clone 5221216 (ref. 17). CTRC mutants and the Glu-Glu–tagged CTRC constructs were generated by overlap extension PCR mutagenesis and ligated into pcDNA3.1(–). The Glu-Glu tag sequence (EYMPME) is derived from the polyoma virus medium T antigen.

Transfection of HEK 293T and AR42J cells. Human embryonic kidney (HEK) 293T cells were cultured and transfected as described[14]. Approximately 10^6 cells per well were plated in six-well tissue culture plates in DMEM culture medium (Invitrogen) supplemented with 10% FBS and 4 mM glutamine. We carried out transfections using 4 μg pcDNA3.1(–)-CTRC plasmid and 10 μl Lipofectamine 2000 (Invitrogen) in 2 ml OptiMEM medium (Invitrogen) supplemented with 2 mM glutamine. After 5 h incubation at 37 °C, 2 ml DMEM with 20% FBS and 4 mM glutamine was added to each well, and cells were incubated for an additional 24 h. We then washed the cells with 1 ml OptiMEM and 2 mM glutamine and replaced the transfection medium with 2 ml OptiMEM and 2 mM glutamine. Time courses of expression were measured starting from this medium change and were followed for 48 h. AR42J cells were maintained as subconfluent cultures in DMEM containing 20% FBS, 4 mM glutamine and 1% penicillin/streptomycin solution. Cells (10^6) were plated into 35-mm wells and grown in the presence of 100 nM dexamethasone for 48 h. We carried out transfections using the Glu-Glu–tagged CTRC constructs, as described above. After 48 h, we replaced the medium with fresh OptiMEM and 2 mM glutamine, and we added 1 nM cerulein (final concentration) to stimulate secretion. After 15 min of incubation the medium was collected and analyzed by protein blotting.

Chymotrypsin C activity assay. The conditioned medium was supplemented with 0.1 M Tris-HCl (pH 8.0) and 10 mM $CaCl_2$ (final concentrations), and CTRC was activated with 100 nM human cationic trypsin (final concentration) for 20 min at 37 °C. All CTRC variants were fully activated by trypsin within this time period (data not shown). We measured CTRC activity with Suc-Ala-Ala-Pro-Phe–*p*-nitroanilide or Glt-Ala-Ala-Pro-Leu–*p*-nitroanilide (0.15 mM final concentration) in 0.1 M Tris-HCl (pH 8.0), 1 mM $CaCl_2$, at 22 °C.

Protein blotting. We ran samples on 15% Tris-glycine gels under reducing conditions and transferred them onto an Immobilon-P membrane (Millipore) at 300 mA for 1 h. The membrane was blocked with 5% milk powder solution overnight and incubated with a horseradish peroxidase (HRP)-conjugated goat polyclonal antibody against the Glu-Glu tag (Abcam) at a concentration of 0.1 μg/ml for 1 h at room temperature. HRP was detected using the Super-Signal West Pico Chemiluminescent Substrate (Pierce).

Statistics. The significance of the differences between mutation frequencies in affected individuals and controls was tested by two-tailed Fisher's exact test. Additional odds ratios were calculated using SAS/STAT software (v. 9.1) and GraphPad Prism (v. 4.03).

Accession codes. Entrez Nucleotide: sequences for chymotrypsin C (*CTRC*) are available with accession codes NT_004873 (chromosome 1 genomic contig), NM_007272 (human CTRC mRNA sequence) and BI832476 (IMAGE clone used for plasmid construction). Protein Data Bank: chymotrypsinogen C crystal structure, 1PYT.

URLs. DeepView/Swiss-PdbViewer v. 3.7, http://www.expasy.org/spdbv/.

Note: Supplementary information is available on the Nature Genetics website.

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AUTHOR CONTRIBUTIONS

M.S.-T., N.T. and H.W. conceived, designed and directed the study. J.R. and H.W. designed, performed and interpreted genetic analyses. R.S., M.S.-T. and B.O. carried out functional characterization of CTRC variants. M.S.-T. wrote the manuscript with significant contributions from R.S., H.W. and J.R. O.L. provided oligonucleotides. N.T., J.R., H.W. and all other coauthors recruited patients and control subjects, collected clinical data and provided genomic DNA samples. All authors approved the final manuscript and contributed critical revisions to its intellectual content.

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