Pancreatology 25 (2025) 65–69

Contents lists available at ScienceDirect

Pancreatology

journal homepage: www.elsevier.com/locate/pan

Sequence analysis of the 5′ region of the chymotrypsin C (*CTRC*) gene in chronic pancreatitis

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ARTICLE INFO

Article history:
Received 23 December 2024
Received in revised form
30 December 2024
Accepted 30 December 2024
Available online 1 January 2025

Keywords:
Chronic pancreatitis
Chymotrypsin
Promoter variant
Genetic association

ABSTRACT

Background/objectives: Loss-of-function chymotrypsin C (*CTRC*) variants increase the risk for chronic pancreatitis (CP) by reducing protective pancreatic CTRC activity. Variants in the 5' upstream region that includes the promoter might affect *CTRC* expression but have not been investigated to date. The aim of the present study was to address this knowledge gap.

Methods: We analyzed ~1.4 kb of the 5' region of the *CTRC* gene in 293 patients with chronic pancreatitis of alcoholic and non-alcoholic etiology and 402 controls from the Hungarian National Pancreas Registry by direct Sanger sequencing.

Results: We identified 14 gene variants, which included 11 novel variants and 3 previously reported variants. When allele frequencies were considered, none of the variants were significantly over-represented in CP cases or controls. Genotype distribution of the frequently occurring variant c.-913A>G showed a statistically significant enrichment of the homozygous GG genotype (versus the AA genotype) in CP cases versus controls (OR 1.67, 95 % CI 1.2–2.4, *P* 0.0053). However, the disease association was driven by the linkage disequilibrium with the known *CTRC* risk variant c.180C>T.

Conclusions: We found no significant association between variants in the 5' region of the *CTRC* gene and CP risk.

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Chronic pancreatitis (CP), the persistent and progressive inflammatory disorder of the pancreas, develops in the background of genetic and/or environmental risk factors [1,2]. The majority of gene variants that increase risk for CP exert their effect by increasing the pathological, intrapancreatic activation of the digestive protease precursor trypsinogen to its active form trypsin [3]. Gain-of-function mutations in the *PRSS1* gene that codes for human cationic trypsinogen cause autosomal dominant hereditary CP by promoting trypsinogen autoactivation or preventing trypsinogen degradation. Anti-trypsin defenses of the pancreas include the trypsin inhibitor serine protease inhibitor Kazal type 1 (SPINK1) and the digestive protease chymotrypsin C (CTRC), which degrades trypsinogen. Accordingly, loss-of-function mutations in *SPINK1* or *CTRC* increase CP risk significantly. The central role of intrapancreatic trypsin activity in CP is also supported by the protective effect of gene variants that lower the levels of human anionic trypsinogen, encoded by the *PRSS2* gene. Thus, a loss-of-function mutation in *PRSS2* protects against CP by facilitating auto-degradation of human anionic trypsinogen [4]. Furthermore, a common genomic deletion in the trypsinogen locus reduces anionic trypsinogen expression and thereby protects against CP [5–7]. Finally, an inversion variant in the *CTRB1-CTRB2* locus (encoding chymotrypsins B1 and B2) increases expression of

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https://doi.org/10.1016/j.pan.2024.12.020
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CTRB2, resulting in more efficient degradation of anionic trypsinogen and protection against CP [8,9].

The protective effect of CTRC through degradation of trypsinogen has been extensively studied and the genetic, biochemical, and cell-biological effects of a large number of *CTRC* variants have been characterized [10–15]. A recent meta-analysis indicated that the p.Ala73Thr, p.Val235Ile, and p.Arg254Trp heterozygous missense *CTRC* variants and the p.Lys247_Arg254del microdeletion were found in 1.0–2.4 % of CP cases globally and they increased disease risk with odds ratio (OR) values that ranged from 2.6 to 6.5-fold [16]. *CTRC* mutations were shown to impair or abolish chymotrypsin function by various mechanisms that included reduced catalytic activity, diminished secretion from cells presumably due to misfolding, defective activation by trypsin, and increased susceptibility to degradation by trypsin [11,12,14,15]. A few CTRC variants also caused endoplasmic reticulum (ER) stress in transfected cells, which may contribute to CP risk as well [12,14]. Considering synonymous and intronic *CTRC* variants, a commonly occurring haplotype containing the exon 3 variant c.180C>T (p.Gly60=) and the intron 5 variant c.493+52G>A was detected with an allelic frequency of 14.2 % in CP cases and 8.7 % in controls (OR 2.2) as reported by a recent meta-analysis [17]. This haplotype increased CP risk by 1.9-fold and 5.3-fold in the heterozygous and homozygous states, respectively. Functional analysis indicated that the c.180C>T-associated haplotype reduced *CTRC* mRNA levels, and presumably CTRC protein expression, which can explain the increased CP risk.

Interestingly, the 5′ region of the *CTRC* gene containing promoter and regulatory sequences that affect gene transcription have not been studied in detail yet. Variants in the 5′ region may reduce CTRC expression levels, which would have a negative impact on its defensive function against CP. Derikx et al. (2009) reported the c.-1C>T variant that was detected in a case of tropical pancreatitis [18]. Masson et al. (2008) described variant c.-59C>T in 2.1 % of cases with non-alcoholic CP and in 0.6 % of controls [19]. The variant was also described by Tremblay et al. (2014) in a subject from a cohort of patients with lipoprotein lipase deficiency who were hospitalized with pancreatitis or abdominal pain [20] and by Litvinova et al. (2023) in a CP case and an unaffected control subject from Russia [21]. The Tremblay et al. study also reported variant c.-92C>T, which was found once, and variant c.-811G>A, which was detected frequently in cases and controls [20]. The effect of the 5’ *CTRC* variants on mRNA or protein expression has not been investigated by the reporting authors.

In the present study, we used direct sequencing to systematically analyze the 5’ region of the *CTRC* gene in non-alcoholic and alcoholic CP cohorts and control subjects with no pancreatic disease.

1. Methods

Nomenclature. Nucleotide numbering started from the first nucleotide (denoted as c.+1) of the ATG translation initiation codon of the *CTRC* reference sequence (genomic reference: NC_000001.11, Homo s*apiens* chromosome 1, GRCh38.p14 primary assembly). The 5’ upstream sequence was numbered starting from the nucleotide preceding the ATG codon, which was designated c.-1. Amino acids were numbered starting with the initiator methionine of the primary translation product.

Study subjects. The Hungarian cohort analyzed by direct DNA sequencing consisted of 293 CP patients (mean age at recruitment 55.8 ± 11.7 years), including 125 subjects with nonalcoholic CP (NACP) and 168 subjects with alcoholic CP (ACP), and 402 control subjects (mean age at recruitment 48.1 ± 13 years) with no pancreatic disease. Diagnosis of CP was based on the history of recurrent acute pancreatitis or recurrent abdominal pain typical for CP and/or pathological imaging findings consistent with CP, such as pancreatic calcifications, duct dilatation or irregularities, with or without exocrine pancreatic insufficiency or diabetes. Alcoholic CP was diagnosed when the patient's history included alcohol consumption of more than 80 g/day (men) or 60 g/day (women) for at least two years. De-identified genomic DNA samples were obtained from the Hungarian National Pancreas Registry (ethical approval: 22254-1/2012/EKU and TUKEB 36305-1/2016/EKU and NNK 17787-8/2020/EUIG, biobanking approval: IF702-19/2012). All participants gave informed consent according to the ethical guidelines of the Declaration of Helsinki.

DNA sequencing. Exon 1 and 1477 nt of the 5′ region of the *CTRC* gene was amplified with 2 primer pairs as follows: S1: 5′- ACT GAC CTT GAC ATT CAA GTT AC -3′, AS1: 5′- GAG TCT CAG CTC ACT ACA ACC -3′, amplicon size: 957 nt; and S2: 5′- CTG GTC CTT TCT CAT CTC ACC -3′, AS2: 5′- CTG GTT GGT AGC ATC TGA AC -3′, amplicon size 1087 nt. PCR amplicons were sequenced by the Sanger method. Primers S1 and AS2 were used as sequencing primers. Amplicons that contained the c.-314AAAT[5] repeated sequence variant were also sequenced with the following primers: RS: 5′- TGG TGG CAC ACA TCT GTA G – 3′, RAS: 5′ - TGG AGA TGG AGT CTT GCT C – 3’.

Polymerase chain reaction (PCR) was performed using 1.0 U HotStarTaq DNA Polymerase (Qiagen), 0.2 mM dNTP, 0.5 μM primers, 10x PCR buffer (Qiagen) and 10–50 ng of genomic DNA template in a volume of 20 μL. The reaction was started with a 15 min initial heat activation at 95 °C followed by 40 cycles of 30 s denaturation at 94 °C, 30 s annealing at 62 °C (for S1-AS1) or 60 °C (for S2-AS2) and 60 s extension at 72 °C; and was completed by a final extension step for 5 min at 72 °C. PCR products were verified by 1.5 % agarose gel electrophoresis. The PCR amplicons (5 μL) were treated with 1 μL FastAP Thermosensitive Alkaline Phosphatase and 0.5 μL Exonuclease I (Thermo Fisher Scientific) for 15 min at 37 °C, and the reaction was stopped by heating the samples to 85 °C for 15 min.

Statistical analysis. The significance of the differences in allele frequencies and genotype distribution between cases and controls was assessed by the MedCalc website's “Odds ratio calculator” (www.medcalc.org/calc/odds_ratio.php). $P < 0.05$ was considered statistically significant.

2. Results

DNA sequence analysis of the 5′ region of the *CTRC* gene. To

Table 1
Allele frequency of 5’ *CTRC* variants in chronic pancreatitis cases and controls. All low-frequency and rare variants were found in the heterozygous state. We also detected heterozygous variants c.27G>A (p.Ala9=, exon 1) and c.40+24G>A (intron 1) in 2 control subjects separately. CP, chronic pancreatitis. OR, odds ratio. CI, confidence interval.

Variant	CP	Controls	OR	95 % CI	*P*
c.-1331T>A	0/586	1/804 (0.12 %)	0.46	0.02–11.2	0.63
c.-999G>A	0/586	1/804 (0.12 %)	0.46	0.02–11.2	0.63
c.-993G>T	2/586 (0.38 %)	6/804 (0.75 %)	0.46	0.09–2.27	0.34
c.-913A>G	304/586 (51.9 %)	383/804 (47.6 %)	1.19	0.96–1.47	0.12
c.-811G>A	185/586 (31.6 %)	272/804 (33.8 %)	0.9	0.72–1.13	0.38
c.-755G>A	0/586	1/804 (0.12 %)	0.46	0.02–11.2	0.63
c.-590G>T	1/586 (0.17 %)	1/799 (0.1 %)	1.37	0.09–21.9	0.83
c.-379G>A	1/582 (0.17 %)	0/800	4.13	0.2–101.6	0.39
c.-314AAAT[5]	10/584 (1.7 %)	12/800 (1.5 %)	1.14	0.49–2.7	0.76
c.-296T>A	1/584 (0.17 %)	0/800	4.12	0.2–101.2	0.39
c.-265G>A	0/584	1/800 (0.13 %)	0.46	0.02–11.2	0.63
c.-92C>T	4/584 (0.7 %)	7/800 (0.9 %)	0.78	0.2–2.7	0.7
c.-59C>T	3/584 (0.51 %)	1/800 (0.13 %)	4.1	0.4–39.8	0.2
c.-55C>G	1/584 (0.17 %)	0/800	4.12	0.2–101.2	0.39

Table 2
Genotype distribution of *CTRC* variants c.-913A>G and c.-811G>A in chronic pancreatitis (CP) cases and controls. Genotypic odds ratio (OR) was calculated relative to the homozygous reference genotype. CI, confidence interval. The significant association is highlighted in bold. The genotype distribution followed the Hardy-Weinberg equilibrium in CP (P 0.62 for c.-913A > G and P 0.2 for c.-811G>A) but not in controls (P 0.001 for c.-913A > G and P 0.01 for c.-811G>A).

Variant	Genotype	CP	Controls	OR	95 % CI	*P*
c.-913A>G	AA	70/293 (23.9 %)	94/402 (23.4 %)			
	AG	142/293 (48.5 %)	233/402 (58 %)	0.97	0.68–1.4	0.87
	GG	**81/293 (27.6 %)**	**75/402 (18.7 %)**	**1.67**	**1.16–2.38**	**0.0053**
c.-811G>A	GG	142/293 (48.5 %)	165/402 (41 %)			
	GA	117/293 (40 %)	202/402 (50.3 %)	0.74	0.55–1	0.052
	AA	34/293 (11.6 %)	35/402 (8.7 %)	1.4	0.84–2.3	0.2

investigate the effect of the 5′ *CTRC* variants on CP risk, we sequenced a ~1.4 kb region upstream of the translation initiator ATG codon in a Hungarian cohort of 293 CP patients and 402 controls. We identified 14 gene variants, which included 11 novel variants and the 3 previously reported variants c.-59C>T, c.-92C>T, and c.-811G>A. Variant c.-1C>T, which was described in an Indian CP case [18], was not found in this cohort. With the exception of the repeated sequence variant c.-314AAAT[5], all variants were single nucleotide changes. When allele frequencies were compared between the CP cases and the controls, none of the 5' variants were significantly overrepresented in either group (Table 1). Similarly, subgroup analysis of ACP and NACP cases versus controls showed no significant association for any of the variants (not shown).

Genotype distribution of variants c.-913A>G and c.-811G>A. While the rare and low-frequency variants were found only in the heterozygous state, the frequently occurring variants c.-913A>G and c.-811G>A were detected both in the heterozygous and homozygous states. Analysis of the genotype distribution of these 2 variants in the CP and control groups revealed a statistically significant enrichment of the homozygous GG genotype (versus the AA genotype) of the c.-913A>G variant in CP cases (OR 1.67, 95 % CI 1.2–2.4, P 0.0053) (Table 2). In subgroup analysis, the significant association of the GG genotype with CP was evident in both the NACP (OR 1.7, 95 % CI 1.1–2.7, P 0.03) and ACP (OR 1.6, 95 % CI 1.1–2.5, P 0.02) subgroups (Tables 3 and 4). Furthermore, in the ACP group the heterozygous AG genotype was also significantly overrepresented in cases (OR 1.7, 95 % CI 1.1–2.7, P 0.03). In contrast, the genotype distribution of the c.-811G>A variant showed no significant association with CP (Table 2) or the NACP and ACP subgroups (Tables 3 and 4).

Variant c.-913A>G is linked with the c.180C>T risk variant. The c.180C>T (p.Gly60=) synonymous variant is a commonly occurring, established risk factor of CP. It was recently reported that in the Hungarian cohort, the c.180C>T variant was found in 18.7 % of CP patient alleles and in 10.2 % control alleles (OR 2.04, 95 % CI 1.47–2.81) [17]. We investigated whether any of the 5′ *CTRC* variants identified in this study was on the same allele as the c.180C>T variant. With the sole exception of variant c.-913A>G, all 5' variants were on the c.180C reference allele. Variant c.-913A>G was detected both on the wild-type c.180C allele and the c.180T risk allele (Table 5). The G-T haplotype was observed in 17.9 % of CP alleles and in 9.95 % of control alleles (OR 1.97, 95 % CI 1.4–2.7, $P < 0.0001$) and showed significant association with CP. However, this association was driven by the c.180C>T variant with no apparent contribution form the c.-913A>G variant. This conclusion was supported by lack of association of variant c.-913A>G with CP when found on the wild-type c.180C allele. Thus, the G-C haplotype was detected in 30.9 % of CP alleles and 33.6 % of control alleles (OR 0.88, 95 % CI 0.7–1.1, P 0.3). Taken together, the results indicate that the c.-913A>G variant has no direct impact on CP risk and the significant association observed in the genotype analysis was due to its linkage with the c.180C>T risk variant.

3. Discussion

Variants in the 5′ region of pancreatitis susceptibility genes may

Table 3
Genotype distribution of *CTRC* variants c.-913A>G and c.-811G>A in chronic pancreatitis (CP) cases of alcoholic etiology and controls. Genotypic odds ratio (OR) was calculated relative to the homozygous reference genotype. CI, confidence interval. The significant association is highlighted in bold. The genotype distribution followed the Hardy-Weinberg equilibrium in alcoholic CP (P 0.28 for c.-913A>G and P 0.58 for c.-811G>A) but not in controls (P 0.001 for c.-913A>G and P 0.01 for c.-811G>A).

Variant	Genotype	Alcoholic CP	Controls	OR	95 % CI	*P*
c.-913A>G	AA	45/168 (26.8 %)	94/402 (23.4 %)			
	AG	**77/168 (45.8 %)**	**233/402 (58 %)**	**1.7**	**1.1–2.7**	**0.03**
	GG	**46/168 (27.4 %)**	**75/402 (18.7 %)**	**1.6**	**1.1–2.5**	**0.02**
c.-811G>A	GG	83/168 (49.7 %)	165/402 (41 %)			
	GA	68/168 (40.8 %)	202/402 (50.3 %)	0.7	0.5–1	0.07
	AA	17/168 (9.5 %)	35/402 (8.7 %)	1.2	0.64–2.2	0.6

Table 4
Genotype distribution of *CTRC* variants c.-913A>G and c.-811G>A in chronic pancreatitis (CP) cases of non-alcoholic etiology and controls. Genotypic odds ratio (OR) was calculated relative to the homozygous reference genotype. CI, confidence interval. The significant association is highlighted in bold. The genotype distribution followed the Hardy-Weinberg equilibrium in non-alcoholic CP (P 0.6 for c.-913A>G and P 0.19 for c.-811G>A) but not in controls (P 0.001 for c.-913A>G and P 0.01 for c.-811G>A).

Variant	Genotype	Non-alcoholic CP	Controls	OR	95 % CI	*P*
c.-913A>G	AA	25/125 (20 %)	94/402 (23.4 %)			
	AG	65/125 (52 %)	233/402 (58 %)	1.22	0.74–2	0.43
	GG	**35/125 (28 %)**	**75/402 (18.7 %)**	**1.7**	**1.1–2.7**	**0.03**
c.-811G>A	GG	59/125 (47 %)	165/402 (41 %)			
	GA	49/125 (40.2 %)	202/402 (50.3 %)	0.78	0.52–1.17	0.22
	AA	17/125 (12.8 %)	35/402 (8.7 %)	1.65	0.89–3.1	0.11

Table 5
CTRC variant c.-913A>G is linked with the c.180C>T risk variant. Allele frequencies of the four haplotypes with respect to positions c.-913 and c.180. The c.-913G/c.180T haplotype, which is significantly enriched in chronic pancreatitis (CP) cases, is highlighted in bold. CP, chronic pancreatitis. OR, odds ratio. CI, confidence interval.

	CP	Controls	OR	95 % CI	*P*
c.-913G/c.180C	30.9 %	33.6 %	0.88	0.7–1.1	0.3
c.-913G/c.180T	**17.9 %**	**9.95 %**	**1.97**	**1.4–2.7**	**<0.0001**
c.-913A/c.180C	48.1 %	52.6 %	0.8	0.65–1.0	0.051
c.-913A/c.180T	0.7 %	0 %	11.9	0.64–221.4	0.097

contribute to disease risk by altering expression of key proteins such as digestive enzymes or the SPINK1 trypsin inhibitor. Curiously, genetic screening in the clinical setting often ignores the 5′ region and discovery studies focusing on promoter and upstream regulatory variants are scarce relative to those investigating the coding regions. The *SPINK1* gene is an interesting exception, where multiple variants in the 5′ region were described and functionally analyzed [22–24]. Some variants are linked to the pathogenic p.N34S-associated haplotype and there is evidence suggesting that a 5′ variant might be responsible for the pathogenic effect [25–27]. In contrast, there is almost no data on the significance of 5' variants in the *CTRC* gene in CP risk. A handful of variants were identified in various studies with no clear disease association [18–21].

To fill this knowledge gap, in the present study we analyzed CTRC 5' region by Sanger sequencing in a cohort of Hungarian CP cases and unaffected controls. We found 14 variants, that included 3 previously reported and 11 new variants. Most variants were rare, 7 variants were found on only 1 subject, and the allele frequencies of 5 other variants ranged from 0.17 % to 1.7 %. Two common variants were identified, c.-913A>G and c.-811G>A. When allele frequencies were considered, none of the variants was enriched in cases or controls significantly. Interestingly, genotype analysis of the c.-913A>G variant showed that the GG homozygous genotype was significantly enriched in patients versus controls (OR 1.67). However, haplotype analysis revealed that the c.-913A>G variant was linked with the c.180C>T pathogenic variant and the association with CP was purely driven by this linkage disequilibrium. When cases and controls carrying the c.-913A>G variant without the c.180C>T variant were compared, no association was evident.

In summary, we found no significant association between variants in the 5' region of the *CTRC* gene and CP risk. A limitation of our study is the lack of functional analysis, which may help to identify rare risk variants.

Financial support

This work was supported by the EU's Horizon 2020 research and innovation program under grant agreement No. 739593 to BCN.

Acknowledgements

We are grateful to Anett Dancs, Zsófia Varga and Ottóné Árva Miklós for their help in laboratory work.

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