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ORIGINAL INVESTIGATION

Association of rare chymotrypsinogen C (*CTRC*) gene variations in patients with idiopathic chronic pancreatitis

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Abstract Extensive genetic studies of chronic pancreatitis over the past decade have highlighted the importance of a tightly regulated balance between activation and inactivation of trypsin within the pancreas to disease susceptibility and resistance. The recent identification of chymotrypsin C (CTRC) as enzyme Y, which was proposed to protect the pancreas by degrading prematurely activated trypsinogen within the pancreas 20 years ago, made *CTRC* an excellent candidate gene for disease-association studies. Here, we analyzed all eight exons of the *CTRC* gene for conventional genetic variants and copy number variations (CNVs) by direct sequencing and quantitative fluorescent multiplex PCR, respectively, in a total of 287 French white patients (idiopathic × 216; familial × 42; hereditary × 29). While no CNVs were found in any of the 287 subjects, 20 conventional variations including a nonsense mutation (p.W55X), a microdeletion mutation (p.K247_R254del) and nine missense mutations were found in the 216 patients with idiopathic chronic pancreatitis (ICP). Except for two common polymorphisms, all the remaining 18 mutational events represent rare variations, with a minor allele frequency of 0–0.3% in the control population. All these rare variants were always found more frequently in the ICP patients than in the controls, and their combined frequency in the ICP patients (26/216; 12.0%) is significantly different from that in the controls (4/350; 1.1%) (OR = 11.8 [3.9–40.6]), $\chi^2 = 31.58$, $P < 10^{-6}$). This genetic finding, when considered in the perceived role of CTRC in eliminating prematurely activated trypsin, indicated that *CTRC* is a new pancreatitis susceptibility gene.

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Introduction

Chronic pancreatitis was hypothesized to be an autodigestive disease resulting from inappropriate zymogen activation within the pancreas more than a century ago (Chiara 1896). Although subsequent findings from clinical observations and animal models suggested a pivotal role of premature trypsinogen activation in disease initiation (reviewed in Chen and Férec 2000), it is the genetic studies of chronic pancreatitis over the past decade that have provided the strongest support to Chiara's theory. Since the mapping and cloning of one gene for hereditary pancreatitis (HP; MIM #167800) in 1996 (Le Bodic et al. 1996; Pandya et al. 1996; Whitcomb et al. 1996a, b), four lines of complementary observations have firmly established the importance of a tightly regulated balance between activation and inactivation of trypsin within the pancreas to disease susceptibility and resistance: (1) gain-of-function missense mutations such as p.D19A, p.D22G, p.K23R (Chen et al. 2003a), p.N29I/T (Sahin-Tóth 2000) and p.R122H (Sahin-Tóth and Tóth 2000; Archer et al. 2006) in the *PRSS1* gene (encod-

ing cationic trypsinogen, the most abundant isoform of trypsinogen; MIM #276000) have been reported to cause the disease; (2) two loss-of-function mutations in the *PRSS1* gene (Chen et al. 2003b) and a degradation-sensitive missense mutation, p.G191R, in the *PRSS2* gene (encoding anionic trypsinogen, the second major isoform of trypsinogen; MIM #601564) have been reported to protect against the disease (Witt et al. 2006); (3) many loss-of-function variations including missense, splicing, frameshifting, nonsense mutations and large genomic deletions in the *SPINK1* gene (encoding trypsin's physiological inhibitor; MIM #167790) have been reported to be associated with idiopathic chronic pancreatitis (ICP), familial chronic pancreatitis (FCP), tropical calcific pancreatitis (TCP; MIM #608189), or even to cause HP (Chen et al. 2000, 2001; Pfutzer et al. 2000; Witt et al. 2000; Gaia et al. 2002; Bhatia et al. 2002; Chandak et al. 2002; Schneider et al. 2002; Le Maréchal et al. 2004; Masson et al. 2006, 2007; Boulling et al. 2007; Kiraly et al. 2007a, b); and (4) more recently, trypsinogen copy number gain mutations have also been found to cause ICP and HP through a gene dosage effect (Le Maréchal et al. 2006; Masson et al. 2008).

Very recently, chymotrypsin C (CTRC) has been shown to be highly specific in degrading all human trypsin/trypsinogen isoforms (Szmola and Sahin-Tóth 2007). Interestingly, CTRC appears to be identical with the elusive enzyme Y that was identified by Rinderknecht et al. (1988) 20 years ago. The physiological role of enzyme Y had been perceived as a second line of defense against premature intrapancreatic activation of trypsinogen isoforms; enzyme Y activity may be generated when SPINK1, the first line of defense, is saturated with prematurely activated trypsin within the pancreas (Rinderknecht et al. 1988). This historical perception, when considered in the context of the aforementioned genetic findings, made the *CTRC* gene (located on chromosome 1p36.21) an excellent candidate for disease-association studies. This study represents such an attempt, in which we have systematically analyzed all eight exons of the *CTRC* gene for both conventional variations (i.e., point mutations and microinsertions/deletions) and copy number variations (CNVs) in a large cohort of French white patients with chronic pancreatitis.

Materials and methods

Patients

A total of 287 French white patients (ICP × 216; FCP × 42; HP × 29) participated in this study. Chronic pancreatitis was diagnosed as previously described (Masson et al. 2008). HP was defined as having three or more affected family members involving at least two generations (Le Maréchal et al. 2006). ICP was diagnosed when neither precipitating factors (e.g. alcohol abuse, infection, trauma, etc.) nor a positive family history was reported. The only difference between FCP and ICP is that the former did report a positive family history but did not meet the criteria we settled for HP (Masson et al. 2008). The inclusion criteria for ICP patients are that either the age of disease onset was known to be ≤20 years or the diagnosis was made at the age ≤20 years, irrespective of whether they had previously been found to carry known pancreatitis-associated variations in the *PRSS1*, *SPINK1* and *CFTR* genes or not. The 42 unrelated patients with FCP and 29 unrelated patients with HP were included regardless of their age of diagnosis or disease onset, but they had not been found to carry any known pancreatitis-associated variations in the three aforementioned genes. Three hundred and fifty unrelated healthy French white subjects served as controls. The University's ethical review committee approved this study, and all patients gave informed consent for genetic analysis.

Screening for conventional genetic variations by direct sequencing

Eight primer pairs (Table 1) were designed to PCR-amplify the eight exons and their immediate flanking sequences of the *CTRC* gene. PCR was performed in a 10-µl reaction mixture containing 200 µM of each dNTP, 1.5 mM $MgCl_2$, 0.25 U HotStarTaq DNA Polymerase (Qiagen, Courtaboeuf, France), 0.4 µM of each primer and 50 ng genomic DNA. The PCR program consists of an initial denaturation at 95°C for 15 min, followed by 26–32 cycles denaturation at 95°C for 30 s, annealing at 60°C for 30 s and extension at 72°C for 30 sc, and a final extension at 72°C for 10 min. PCR products were purified by ExoSAP-IT (GE Healthcare, Orsay, France) and then sequenced using the ABI PRISM™ BigDye™ Terminator Cycle Sequencing Kit v.1 (PE Applied Biosystems, Foster City, CA). *CTRC* variants found in patients were analyzed in controls by direct sequencing or by DHPLC (denaturing high performance liquid chromatography) using the Transgenomic Wave System.

Screening for CNVs by quantitative fluorescent multiplex PCR

Eight primer pairs targeting the eight exons of the *CTRC* gene (Table 2) were designed for screening CNVs in this locus, by means of quantitative fluorescent multiplex PCR (QFM-PCR). One primer of each pair was 5′-labeled with the HEX fluorochrome. All eight targeted regions, together with a control region (i.e., exon 7 of the *MGAM* gene located on chromosome 7; Table 2), were simultaneously amplified in a single reaction. QFM-PCR was

Table 1 Primer sequences and amplicon sizes pertaining to the conventional PCR method used for analyzing all eight exons of the *CTRC* gene

Exon	Primer sequence (5′ to 3′)	Amplicon size (bp)
1	Forward: CTAAGAAAGCCCTGTCTCCT Reverse: ACACAGCTGGTTGGTAGCAT	372
2	Forward: AGAGACCTGGGTGGCTTACC Reverse: AGGGCTGTGTGTCAGCCT	335
3	Forward: ACCTGCAGGCTGACACACA Reverse: GCTGGTTCCTGGCACATAAT	325
4	Forward: AGCTCCCTCTCTATCCCACT Reverse: GGGTATTTGTCTCCACTTAGCA	373
5	Forward: AAGAAGCTGGCAGTCAGGAT Reverse: GTGTCTGTCACATGGTATGTGCT	444
6	Forward: TGGGTCCTGTCCCAGGCAT Reverse: GTGATGGGCTTACCCTGAGC	390
7	Forward: GCAGGCTGAGGCCAAAT Reverse: TGAATGAGTGACTGAATAAGTG	463
8	Forward: ACAGTGGCCTGAAATGCTGA Reverse: ACCTGTCTAATGCAGGGTGG	377

GenBank accession NC_000001.9 was used as the reference genomic DNA sequence

Table 2 Primer sequences, primer concentrations and amplicon sizes pertaining to the QFM-PCR method used for simultaneously analyzing the eight exons of the *CTRC* gene

Exon	Primer sequence (5′ to 3′)	Primer concentration (μM)	Amplicon size (bp)
1	Forward: CATCCCGATGGTCAGCCAGT Reverse: CCCGAGCTCACAGCCTGCTA	0.1	120
2	Forward: TGGTTCTTCTGGCCTCCTGTCT Reverse: TATCTGTACCTCCCAGCCCTACA	0.2	152
3	Forward: ACCTGCAGGCTGACACACA Reverse: ATGGCCAGGTCTCAGGGTATCAT	0.3	171
4	Forward: CCTTCCTCTGCCCAGCAACA Reverse: ATGGGCAGTCTGTCACTCA	0.5	161
5	Forward: ACTCACCCTCTCCACTTTGGAT Reverse: AAGGCTCTCAGGATTTGCCA	0.1	198
6	Forward: CTGGTCACTGCTCACTCTCT Reverse: TGCAGAACCTAGCCACTCACAT	0.1	192
7	Forward: TTATGCCCTCCCGGTCTGGT Reverse: TGCAGGGACAGCTGTGGA	0.1	207
8	Forward: AGCCCTGAGTCTCTCACACTGTT Reverse: ACAAATCAAGGATCCAGGTGGC	0.1	135
MGAM, exon 7[a]	Forward: TTTGCTGACCAGTTCTTGCAGCTC Reverse: GGGCCCATTTGAAAGCTTACTCCA	0.1	164

GenBank accession NC_000001.9 was used as the reference genomic DNA sequence

[a] Control gene located on chromosome 7

performed using the Qiagen Multiplex PCR kit (Qiagen, Courtaboeuf, France), with 100 ng genomic DNA in a 10-μl reaction mixture. The PCR program consists of an initial denaturation step at 95°C for 15 min, 25 cycles of denaturation at 95°C for 30 s, annealing at 58°C for 1 min and extension at 72°C for 90 s, and a final extension at 72°C for 10 min.

Amplified DNA fragments were separated on an ABI Prism 310 sequencer (Applied Biosystems, Foster City, CA), and data were analyzed with Genemapper v3.2 (Applied Biosystems) or Genescan v3.1 (Applied Biosystems) or both. Peak heights of the eight *CTRC* amplicons in a given sample were first normalized against that of the coamplified control *MGAM* amplicon. The normalized fluorescent profiles of a test sample were then superimposed upon those of a control sample. A twofold reduction in peak height was suggestive of a deletion.

Bioinformatics analysis

The *CTRC* promoter variant flanked by ±10 bp sequence was searched for transcription factor binding sites using the *TFSEARCH* program (Heinemeyer et al. 1998; http://mol-sun1.cbrc.aist.go.jp/research/db/TFSEARCH.html).

The effect of the three intron 1 variations and one intron 2 variation on splice site usage was assessed individually in the sequence tract going from c.-300 to c.230+300 (c.230 denotes the last nucleotide of exon 3), using the *NetGene2* program (http://www.cbs.dtu.dk/services/NetGene2/). The effect of the intron 5 variation on splice site usage was evaluated in the sequence tract going from c.231−300 to c.493+300 (c.231 and c.493 denote the first nucleotide of exon 4 and the last nucleotide of exon 5, respectively), also using the *NetGene2* program.

The effect of the two 3′ flanking region variations on the "local" secondary structure [i.e. ±100 bp sequence flanking each variant in accordance with Chen et al. (2006a)] of the *CTRC* pre-mRNA was assessed using the *mfold* program (Zuker 2003; http://frontend.bioinfo.rpi.edu/applications/mfold/cgi-bin/rna-form1.cgi).

Acquisition of mammalian CTRC amino acid sequences

Searching with the keyword "CTRC" in the UniProt Knowledgebase (http://www.pir.uniprot.org/database/knowledgebase.shtml) yielded four mammalian CTRC orthologs (i.e., human, Q99895; cattle, Q7M3E1; rat, P55091; mouse, Q3SYP2). Then blasting the protein database with the human sequence using *Blastp* (protein–protein BLAST; http://www.ncbi.nlm.nih.gov/blast/Blast.cgi) yielded additional three mammalian orthologs viz. chimpanzee (XP_001149762.1), rhesus monkey (XP_001089102.1) and dog (XP_852179.1). Amino acid sequence alignment was performed using the ClustalW program at http://www.ebi.ac.uk/Tools/clustalw/index.html.

Statistic analysis

Comparison of variation frequency between cases and controls was made using either χ^2 test or Fisher's exact test. In cases of significant differences ($P < 0.05$), the strength of the association was assessed by calculating the odds-ratio (OR) with 95% confidence intervals.

Results and discussion

Recent revelation of CTRC as the elusive enzyme Y identified a potential pancreatitis susceptibility gene

The recent biochemical characterization of CTRC (Szmola and Sahin-Tóth 2007) resolved a 20-year-old enigma (Rinderknecht et al. 1988), shed new lights on a potential pathological mechanism and, at the same time, identified a potential pancreatitis susceptibility gene. Indeed, as stated by Szmola and Sahin-Tóth M (2007), "the Rinderknecht's theory that enzyme Y protects the pancreas by decreasing trypsinogen concentrations during inappropriate zymogen activation might be valid, and our present study should stimulate further research in this direction." In this study, we pursued this direction by initially screening for both *CTRC* conventional variations and CNVs in our well-characterized ICP patients (age of disease onset was known to be ≤20 years or the diagnosis was made at the age of ≤20 years). This strategy was based upon previous observations in which disease-predisposing genetic variations were more easily identified in children and adolescents than in adults (Witt et al. 1999, 2000; Chen et al. 1999, 2001; Masson et al. 2008). However, unlike our previous study that aimed at finding copy number gain mutations in a major disease locus (i.e. the trypsinogen locus; Masson et al. 2008), here we did not exclude those patients who had been previously found to carry pancreatitis-associated mutations/variations in the *PRSS1*, *SPINK1* and *CFTR* genes. This treatment was meant not to miss those *CTRC* variations that may not be sufficient to cause the disease by themselves but may well modify a known disease-associated variant or cause the disease through cooperating with other disease-associated variants.

Significant enrichment of rare *CTRC* variations in ICP

Direct sequencing of the *CTRC* gene in our 287 patients found a total of 21 conventional variations. Except for the two common polymorphisms (i.e., c.180C > T and c.285C > T, minor allele frequencies of 11.9% and 4.3% in the control population, respectively), of which both heterozygotes and homozygotes were identified (Table 3), all the remaining 19 variations were invariably found in the heterozygous state (Table 4).

Genotype distribution of the two common polymorphisms in the different patient groups and controls were detailed in Table 3; a positive association was only found between the genotype CT of the c.180C > T variation and FCP (genotype CC as reference; OR = 2.46 [1.14−5.27], ($\chi^2 = 6.52$, $P = 0.011$). Interestingly, all the 19 rare variations (minor allele frequencies of 0–0.3% or carrier frequencies of 0–0.6% in the control population) were always found more frequently in the patients than in the controls (Table 4). Individually, only the variation (i.e., p.R254W) that was observed most frequently in the ICP patients was found to be significantly associated with the disease ($P = 0.032$). However, the occurrence of these rare variations (excluding the c.40+24G > A in intron 1 that was found only in a single case with FCP), when considered collectively, is significantly different between the ICP patients and controls (OR = 11.8 [3.9–40.6]), $\chi^2 = 31.58$, $P < 10^{-6}$).

Based upon the current understanding of CTRC's physiological role, disease-predisposing variations in the *CTRC* gene must be loss-of-function mutations. In this

Table 3 Two common synonymous *CTRC* variations detected in this study

Region	Nucleotide change	Amino acid change	Genotype	Frequency in ICP (%)[a]	Frequency in FCP (%)[a]	Frequency in HP (%)[a]	Frequency in all patients (%)[a]	Frequency in controls (%)[a]
Exon 3	c.180C > T	p.G60G	CC	164/216 (75.9)	25/42 (59.5)	20/29 (69.0)	209/287 (72.8)	277/350 (79.1)
			CT	44/216 (20.4)	14/42 (33.3)	7/29 (24.1)	65/287 (22.7)	63/350 (18.0)
			TT	8/216 (3.7)	3/42 (7.2)	2/29 (6.9)	13/287 (4.5)	10/350 (2.9)
Exon 4	c.285C > T	p.D95D	CC	200/216 (92.6)	39/42 (92.9)	28/29 (96.6)	267/287 (93.0)	321/350 (91.7)
			CT	16/216 (7.4)	3/42 (7.1)	1/29 (3.4)	20/287 (7.0)	28/350 (8.0)
			TT	0/216 (0)	0/42 (0)	0/29 (0)	0/287 (0)	1/350 (0.3)

Nomenclature follows the guidelines recommended by the Human Genome Variation Society (http://www.hgvs.org/); that is, cDNA-based numbering with the A of the ATG translational initiation codon ascribed as +1. GenBank accessions NM_007272.2 and NC_000001.9 were used as the reference mRNA and genomic DNA sequences, respectively

[a] Carrier frequency

Table 4 Rare *CTRC* variations detected in this study

Region	Nucleotide change	Amino acid change	Frequency in ICP (%)[a]	Frequency in FCP (%)[a]	Frequency in HP (%)[a]	Frequency in controls (%)[a]
Promoter variation						
Promoter	c.−59C > T	NA[b]	4/216 (1.9)	1/42 (2.4)	1/29 (3.4)	2/350 (0.6)
Intronic variations						
Intron 1	c.40+24G > A	NA	0/216 (0)	1/42 (2.4)	0/29 (0)	0/350 (0)
Intron 1	c.40+66G > A	NA	1/216 (0.5)	0/42 (0)	0/29 (0)	0/350 (0)
Intron 1	c.41−50G > A	NA	1/216 (0.5)	0/42 (0)	0/29 (0)	0/350 (0)
Intron 2	c.133−19C > G	NA	1/216 (0.5)	0/42 (0)	0/29 (0)	0/350 (0)
Intron 5	c.494−10C > T	NA	1/216 (0.5)	0/42 (0)	0/29 (0)	0/350 (0)
3′ flanking region (3′-FR)						
3′-FR	c.807+83T > C	NA	1/216 (0.5)	0/42 (0)	0/29 (0)	0/350 (0)
3′-FR	c.807+86A > G	NA	1/216 (0.5)	0/42 (0)	0/29 (0)	0/350 (0)
Missense mutations						
Exon 3	c.217G > A	p.A73T	1/216 (0.5)	0/42 (0)	0/29 (0)	0/350 (0)
Exon 5	c.464G > A	p.C155Y	1/216 (0.5)	0/42 (0)	0/29 (0)	0/350 (0)
Exon 5	c.485G > A	p.R162H	1/216 (0.5)	0/42 (0)	0/29 (0)	0/350 (0)
Exon 6	c.514A > G	p.K172E	1/216 (0.5)	0/42 (0)	0/29 (0)	0/350 (0)
Exon 6	c.598A > G	p.M200V	1/216 (0.5)	0/42 (0)	0/29 (0)	0/350 (0)
Exon 7	c.649G > A	p.G217S	1/216 (0.5)	0/42 (0)	0/29 (0)	0/350 (0)
Exon 7	c.649G > C	p.G217R	1/216 (0.5)	0/42 (0)	0/29 (0)	0/350 (0)
Exon 7	c.703G > A	p.V235I	1/216 (0.5)	0/42 (0)	0/29 (0)	0/350 (0)
Exon 7	c.760C > T	p.R254W	5/216 (2.3)	0/42 (0)	0/29 (0)	1/350 (0.3)
Nonsense mutation						
Exon 3	c.164G > A	p.W55X	1/216 (0.5)	0/42 (0)	0/29 (0)	0/350 (0)
Microdeletion mutation						
Exon 7	c.738_761del24	p.K247_R254del	2/216 (1.0)	0/42 (0)	0/29 (0)	1/350 (0.3)

Nomenclature follows the guidelines recommended by the Human Genome Variation Society (http://www.hgvs.org/); that is, cDNA-based numbering with the A of the ATG translational initiation codon ascribed as +1. GenBank accessions NM_007272.2 and NC_000001.9 were used as the reference mRNA and genomic DNA sequences, respectively. All variations were found invariably in the heterozygous state

[a] Carrier frequency

[b] Not applicable

regard, identification of the p.W55X nonsense mutation (see Fig. 1 for its location within the protein) is of paramount importance, because it will undoubtedly lead to a complete functional loss of the involved allele. The microdeletion mutation, p.K247_R254del, is almost certain to result in a defective protein: of the eight amino acids

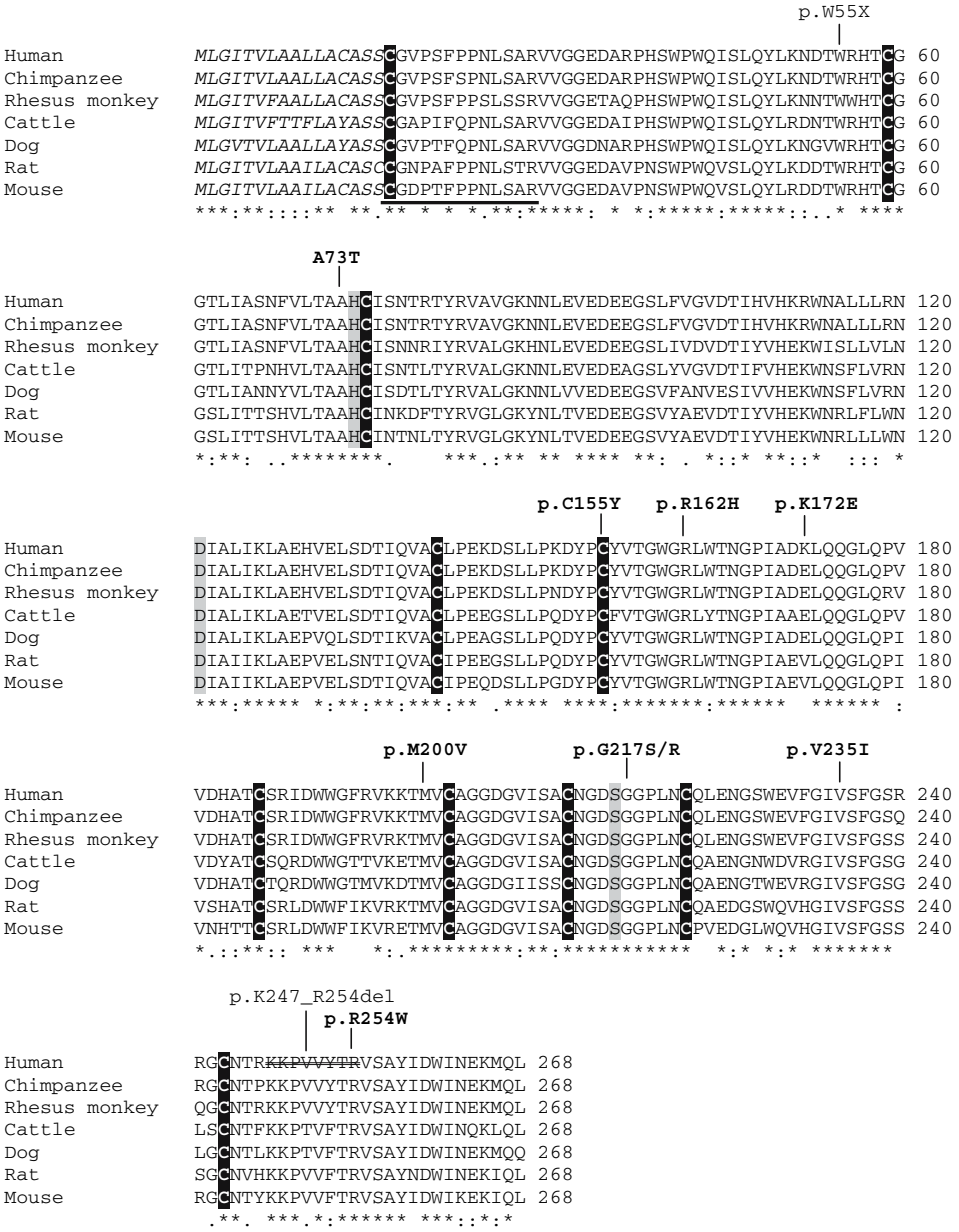

Fig. 1 A survey of the *CTRC* missense mutations (as well as the nonsense and microdeletion mutations) found in this study in the context of the mammalian CTRC orthologs. Signal peptides are in *italics* and activation peptides are *underlined*. The catalytic triad residues are shaded in *gray* and the cysteine residues are shaded in *black*

deleted, three (i.e., K247, K248 and R254) are positively charged and strictly conserved among the mammalian orthologs (Fig. 1). The c.-59C > T promoter variation was found in a total of six patients (4 × ICP, 1 × FCP and 1 × HP) but only twice in the 350 controls. Interestingly, this variation eliminates a potential binding site for the ubiquitously expressed transcription factor E47 (Longo et al. 2007), as revealed by *TFSEARCH*. The two 3′ flanking region variations, which are located just 13 bp (c.807+83T > C) and 16 bp (c.807+86A > G) downstream the pre-mRNA cleavage site and occurred within TG-rich sequences, are potentially of functional significance. For example, the c.807+83T > C variant was shown to occur within a TG-rich loop (AUUUGU > AUUUGC), using the method of Chen et al. (2006a). This pattern of change has been previously predicted to be an indicator of functionality of 3′ UTR variations (Chen et al. 2006a). Thus, it is highly possible that the decrease of a motif-defining nucleotide in the loop would affect the motif's affinity for its cognate binding factor(s), thereby resulting in an reduced rate of mRNA 3′ end formation (for a recent review, see Chen et al. 2006b). Of the four intronic variations detected in ICP, two (c.133−19C > G and c.494−10C > T) were predicted by *NetGene2* (http://www.cbs.dtu.dk/services/NetGene2/) to slightly reduce the confidence values (with a difference of 0.02–0.03) of the donor splice site of intron 3 and the acceptor splice site of intron 5, respectively.

Missense mutations account for 50% of the 18 rare variants found in the ICP patients (Table 4). To obtain useful

insights into their putative functional consequences, we evaluated these variations in the context of the aligned mammalian CTRC orthologs. Except for p.K172E, all the remaining eight missense mutations occurred in strictly conserved residues (Fig. 1). By analogy to the *SPINK1* missense mutations, whose effect on protein secretion correlates well with the conservation status of the involved residues in the context of the mammalian SPINK1 orthologs (Boulling et al. 2007), perhaps most of the *CTRC* missense mutations may also cause reduced secretion of their respective mutant proteins. For example, p.C155Y, which removes the strictly conserved cysteine at residue 155 that normally interacts with cysteine at position 222 to form one of the five sulfide bonds of the mature peptide (Fig. 1), is likely to cause significant changes of the protein's conformation, thereby leading to reduced protein secretion. In addition, two missense mutations occurred in residue G217, which is located immediately after the serine residue at position 216. Because serine at 216 is one of CTRC's catalytic triad residues (Fig. 1), it is possible that these two missense mutations may also modify the catalytic activities of their respective mutant proteins (provided that the mutant proteins could be expressed, fully or partially). In short, most of the *CTRC* missense mutations are likely to be functional, causing reduced secretion and/or affecting catalytic activities of their respective mutant proteins. The overall frequency of the missense, nonsense and microdeletion mutations in the ICP patients is significantly different from that in the controls (OR = 9.9 [2.2–63.2], $\chi^2 = 13.62$, $P = 0.00022$).

As shown is Table 5, patients PC2228 and PC2336 were found to carry two different *CTRC* variations whilst patient PC536 was found to carry three *CTRC* variations. In addition, up to five patients are *trans* or complex heterozygotes, carrying one *CTRC* variant plus one or two *SPINK1* variants. This high frequency of compound, *trans* or complex genotypes suggested that *CTRC* variations, like *SPINK1* variations, often represent low-penetrance factors. In support of this view, *CTRC* rare variations appear not to play an important role in either FCP or HP.

Our findings concur with those just reported in an advance online publication (Rosendahl et al. 2007), in the context of missense mutations and clearly loss-of-function mutations: (1) frame-shifting or nonsense mutations were found rarely in patients [c.308delG in a single German patient, c.190_193delATTG in two Indian patients with tropical pancreatitis (Rosendahl et al. 2007) and p.W55X in a single patient in our study]; (2) all the identified variations are rare in the control populations; (3) the most frequently found variation in patients is p.R254W; and (4) *CTRC* variations were much less frequently found in HP patients. In addition, Rosendahl et al. (2007) functionally characterized several *CTRC* missense mutations. For example, while secretion of the p.R254W mutant protein was reduced to about 50% of normal, that of the p.A73T and p.K247_R254del mutant proteins was almost undetectable. Finally, it is important to point out that Rosendahl et al. did not report any variations that occurred in the promoter region, intronic regions and the 3′ flanking region. They did not report the two common polymorphisms that occurred within exons 3 and 4 (see Table 3), either.

CNVs were not found in any of the 287 French white patients

We also systematically searched for *CTRC* CNVs in the 216 ICP patients, 42 FCP patients and 29 HP patients. No CNVs were found in any of these subjects. It seems unlikely that our QFM-PCR method has inherent problems, because we have used the same technique to identify large genomic deletions in the *CFTR* (Audrézet et al. 2004; Férec et al. 2006) and *SPINK1* (Masson et al. 2006, 2007) genes as well as the trypsinogen locus duplication and triplication mutations (Le Maréchal et al. 2006; Masson et al. 2008). In this regard, the heterozygous *CTRC* microdeletion mutation served as an internal control show-

Table 5 ICP patients carrying more than one variation

Patient	First *CTRC* variation	Second *CTRC* variation	Third *CTRC* variation	*SPINK1* variation(s)
PC2180	c.-59C > T	No	No	N34S
PC1196	c.494-10C > T	No	No	c.1A > T (p.0?) plus N34S[a]
PC536	p.K172E[a]	c.807+83T > C[a]	c.807+86A > G[a]	No
PC2228	p.G217S[a]	p.R254W[a]	No	No
PC2336	p.G217R[a]	p.R254W[a]	No	No
PC548	p.V235I	No	No	N34S (homozygote)
PC836	p.K247_R254del	No	No	N34S
PC1246	p.R254W	No	No	N34S

Variations were present in the heterozygous state unless specified

[a] Whether they are located in the same allele or not is not determined

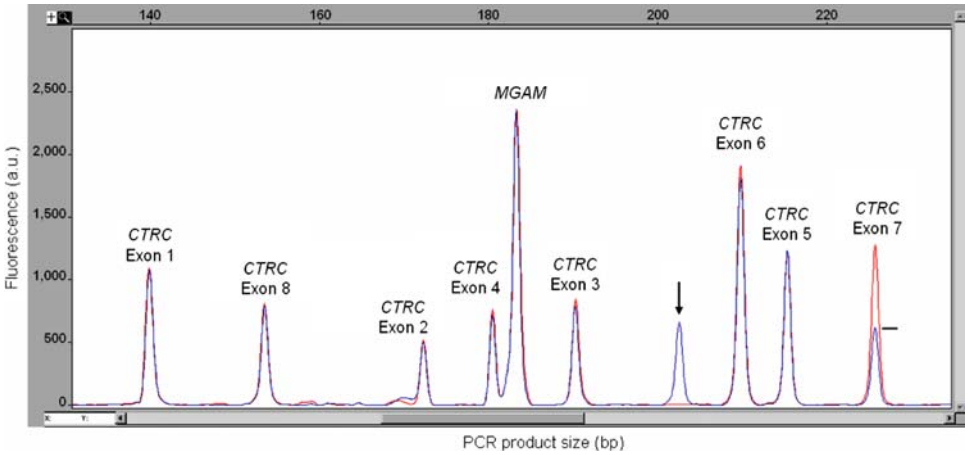

Fig. 2 The QFM-PCR electropherogram of the p.K247_R254del mutation carrier (*blue*) superimposed on that of a normal control (*red*) after normalization against the control *MGAM* amplicon. The deletional allele appeared as a new peak (*downward arrow*) whilst the wild-type allele (exon 7) manifested as a "pseudodeletion"

ing that our current QFM-PCR is capable of detecting *CTRC* deletions (Fig. 2).

In summary, stimulated by the study of Szmola and Sahin-Tóth (2007), we have analyzed the *CTRC* gene for both conventional genetic variations and CNVs in a large cohort of French white patients with chronic pancreatitis. We have found a significant enrichment of rare *CTRC* variations including a nonsense mutation, a microdeletion mutation and nine missense mutations in the ICP patients. Our findings, together with those independently obtained from German and Indian patients (Rosendahl et al. 2007), demonstrated that *CTRC* is a new pancreatitis-predisposing gene.

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