No Significant Enrichment of Rare Functionally Defective CPA1 Variants in a Large Chinese Idiopathic Chronic Pancreatitis Cohort

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**ABSTRACT:** Rare functionally defective carboxypeptidase A1 (CPA1) variants have been reported to predispose to nonalcoholic chronic pancreatitis, mainly the idiopathic subtype. However, independent replication has so far been lacking, particularly in Asian cohorts where initial studies employed small sample sizes. Herein we performed targeted next-generation sequencing of the *CPA1* gene in 1112 Han Chinese idiopathic chronic pancreatitis (ICP) patients – the largest ICP cohort so far analyzed in a single population – and 1580 controls. Sanger sequencing was used to validate called variants, and the CPA1 activity and secretion of all newly found variants were measured. A total of 18 rare *CPA1* variants were characterized, 11 of which have not been previously described. However, no significant association was noted with ICP irrespective of whether all rare variants [20/1112 (1.8%) in patients vs. 24/1580 (1.52%) in controls; *P*=0.573] or functionally impaired variants [3/1112 (0.27%) in patients vs. 2/1580 (0.13%) in controls; *P*=0.410] were considered.

**KEY WORDS:** *CPA1* gene; idiopathic chronic pancreatitis; missense mutations; next-generation sequencing; rare variants
Since the identification of a gain-of-function missense mutation in the \textit{PRSS1} gene (encoding cationic trypsinogen; MIM\# 276000) as a cause of autosomal dominant hereditary pancreatitis (MIM\# 167800) 20 years ago [Whitcomb et al., 1996], heritable variants in multiple genes have been reported to cause, predispose to, or protect against several forms of chronic pancreatitis (see [Fjeld et al., 2015] and references therein). Some of these genetic associations have been consistently replicated in independent studies, and the underlying pathogenic mechanisms elucidated. In particular, findings from the analysis of four genes abundantly expressed in pancreatic acinar cells -- PRSS1 [Le Maréchal et al., 2006; Whitcomb et al. 1996; Whitcomb et al., 2012], PRSS2 (encoding anionic trypsinogen; MIM\# 601564) [Witt et al., 2006], \textit{SPINK1} (encoding pancreatic secretory trypsin inhibitor; MIM\# 167790) [Witt et al., 2000] and CTRC (encoding chymotrypsin C, which specifically degrades all human trypsinogen/trypsin isoforms [Szmola and Sahin-Tóth 2007]; MIM\# 601405) [Masson et al., 2008; Rosendahl et al., 2008] -- have established the importance of a balance between the activation and inactivation of trypsinogen within the pancreas, thereby defining a trypsin-dependent pathway as a key component of the pathogenesis of chronic pancreatitis.

Procarboxypeptidase A1, the inactive form of the digestive enzyme carboxypeptidase A1 (CPA1; MIM\# 114850), is the second most abundantly synthesized protein after trypsinogen in the pancreatic juice [Scheele et al., 1981]. In addition, procarboxypeptidase A1 is a component of the pancreatic zymogen activation cascade which, if prematurely activated within the pancreas, could potentially lead to pancreatic autodigestion [Chen and Férec, 2009]. Recently, a candidate gene approach [Witt et al., 2013] identified an overrepresentation of functionally impaired CPA1 variants (defined as exhibiting enzymatic activity <20\% of the wild-type in the conditioned medium of transfected cells) in German patients with nonalcoholic chronic pancreatitis (NACP) as compared to controls. Functionally impaired CPA1 variants were also found to be significantly overrepresented in NACP patients.
as compared to controls in a European replication cohort (mainly French, Czech and Polish) and two small Asian cohorts, albeit with much lower odds ratios (OR) and much higher $P$ values (see Table 1). It should be noted that (i) the NACP patients were mainly subjects with idiopathic chronic pancreatitis (ICP) along with a few cases with a family history of chronic pancreatitis and (ii) the <20% cut-off value for defining functionally defective $CPA1$ variants was set relative to p.Thr124Ile, a low-frequency variant (defined as having a minor allele frequency of 0.5%-5% in the control population as previously described [Manolio et al., 2009; Tennessen et al., 2012]) that was present with equal frequency in both German NACP patients and controls, and which exhibited 23% of the activity of the wild-type CPA1 enzyme [Witt et al. 2013]. Additionally, it is important to mention two other findings in the German discovery population. First, the association between defective $CPA1$ variants and NACP was strongest in those German patients aged ≤10 years, in whom the detection frequency was nearly 10% (22/228), with the corresponding OR reaching 84.0. Second, defective $CPA1$ variants were found in only 2/465 (0.4%) German patients with alcohol-related chronic pancreatitis, suggesting only a very minor role in this disease subtype [Witt et al. 2013].

In the previous study, most of the functionally impaired $CPA1$ variants were found only in patients. For those variants that were found in both patients and controls, or only in controls, their minor allele frequencies in the control population(s) were invariably <0.5% [Witt et al. 2013]. Therefore, all the functionally impaired $CPA1$ variants naturally fell into the category of rare variants [Manolio et al. 2009; Tennessen et al. 2012]. Biochemical studies revealed no detectable impact of CPA1 on trypsinogen activation, trypsin activity, or the degradation of trypsin and trypsinogen by CTRC, suggesting a trypsin-independent pathogenic mechanism underlying the functionally impaired CPA1 variants [Witt et al. 2013]. Importantly, the defective CPA1 variants were often characterized by significantly decreased secretion, intracellular retention and degradation, implying involvement of misfolding-induced
endoplasmic reticulum stress, as previously observed in a subset of rare PRSSI and CTRC variants [Beer et al., 2013; Kereszturi et al., 2009; Németh et al., 2016; Schnür et al., 2014]. Indeed, expression of the CPAI p.Asn256Lys variant, which was found in 7 NACP patients but not in controls, resulted in endoplasmic reticulum (ER) stress in AR42J rat acinar cells [Witt et al. 2013]. More recently, a novel CPAI variant, p.Ser282Pro, found in two Polish families with hereditary pancreatitis, has been shown to induce ER stress to a comparable degree as the p.Asn256Lys variant [Kujko et al., 2017].

Given the remarkable differences in the occurrence and distribution of rare functional CPAI variants among the different populations, and the small sizes of the two Asian cohorts analyzed to date (Table 1), further independent replication in larger studies is clearly warranted [MacArthur et al., 2014]. Herein we report the targeted sequencing of the CPAI gene in a large cohort of Han Chinese ICP patients. All 1112 Chinese ICP patients (776 males and 336 females) were of Han origin. A clinical diagnosis of ICP was made as previously described [Zou et al., 2016] (see also Supp. Methods). The 1580 healthy controls were unrelated Han Chinese blood donors. Informed consent was obtained from each patient and the study was approved by the Changhai Hospital’s Ethics Committee.

We designed a total of 16 target-specific primer pairs (Supp. Figure S1) for the coding sequence and exon/intron boundaries of the CPAI gene (human GRCh37/hg19 https://genome.ucsc.edu/; NM_001868.3) using Primer3 (http://www.genome.com/Primer3) [Untergasser et al., 2012]. The primers were synthesized with common adaptor sequences at their 5’ ends as previously described [Forshey et al., 2012]. The 16 primer pairs were divided into two multiplex primer pools. Pre-amplification of the tagged-CPAI amplicons, generation of a barcoded DNA library for multiplex high-throughput sequencing, quantification and clean-up of the DNA library and sequencing were performed essentially as previously described [Forshey et al. 2012]. The raw paired 150 bp-long reads were aligned to the human
reference genome (build hg19) using the Burrows-Wheeler Aligner (BWA version 0.5.9). The Genome Analysis Toolkit (GATK, version 3.1) was then used to perform local realignment and to recalibrate base quality scores, yielding a cleaned BAM file for each individual. The raw single nucleotide variants and small insertions or deletions were filtered based upon the variant quality score recalibration module in GATK. Had we added the step “QD < 2.0” to the SNV (single nucleotide variants) filtration process, most of the mutations located at the target-specific primer regions would have been missed, leading to false negative results. To avoid such a situation, the raw SNVs were simply filtered by the VariantFiltration module in GATK using the following steps: (i) MQ < 40.0, (ii) MQRankSum < -12.5 and (iii) ReadPosRankSum < -8.0; and the raw INDELs were only filtered by the VariantFiltration module in GATK using the following arguments: (i) ReadPosRankSum < -20.0 and (ii) FS>200.0. This approach was bound to generate false positive findings, which were then excluded by manual inspection of each called variant using the Integrative Genomics Viewer followed by validation by Sanger sequencing. Given that all the previously reported functionally defective CPA1 variants [Witt et al. 2013] fell into the category of rare variants, we only included variants for analysis which were absent or occurred with a minor allele frequency of <0.5% in the public database of the 1000 Genomes Project (http://www.1000genomes.org/) [Auton et al., 2015; Sudmant et al., 2015]. In case of SNVs, only those that occurred within canonical splice sites or were predicted to result in a premature stop codon or missense mutations were included for analysis. The types of variant under consideration followed the earlier report [Witt et al. 2013], and included (i) small deletions or insertions that occurred within the canonical splice sites and coding sequence and (ii) single nucleotide substitutions that altered either canonical splice sites or else resulted in missense or nonsense mutations. All called rare variants were subjected to validation by
Sanger sequencing. Sequences of the primers used to amplify the fragments of interest are provided in Supp. Figure S2.

Under the conditions of our targeted sequencing approach, we initially discovered 30 rare CPA1 variants in 60 subjects. The various exons harboring these variants were then PCR amplified from the 60 carriers; subsequent Sanger sequencing of the resulting PCR products confirmed the presence of 18 variants in 44 subjects. The 18 validated variants spanned all ten exons of the CPA1 gene, were all detected in the heterozygous state and were all single nucleotide substitutions that were predicted to result in missense mutations. Nearly two thirds (N = 11) of the 18 variants had not been previously described (Table 2). It is possible that some rare variants were missed by this method. However, this is unlikely to be a major issue for two reasons. First, we found variants in all 10 exons of the CPA1 gene. Second, to avoid false negative results, we used rather relaxed parameters for variant calling, as evidenced by the high false positive rate. As indicated, false positive variants were eliminated by an independent PCR followed by Sanger sequencing.

The 18 rare CPA1 variants were present in 20 (1.80%) of the 1112 Chinese ICP patients and in 24 (1.52%) of the 1580 controls (Table 2), indicating no significant difference between the two groups (P = 0.573, 2-tailed Fisher’s exact test); Supp. Table S1). For comparison, we tested the association of all rare variants and NACP in the context of each previously published NACP cohort [Witt et al. 2013]: no association was observed in the European replication cohort whereas a positive association was observed in each of the German, Indian and Japanese NACP cohorts (Supp. Table S1). We then investigated whether there might be an association between rare functionally defective CPA1 variants and ICP in the Chinese cohort. To this end, we functionally characterized all 11 newly found rare variants in terms of their CPA1 activity and secretion as previously described [Witt et al. 2013] (for detailed description, see Supp. Methods). The activities and secretion levels determined for the new
CPA1 variants relative to the wild-type values are summarized in Table 2. Also shown are the CPA1 activity and secretion data for the 7 known variants taken from Witt et al. [Witt et al. 2013]. We designated CPA1 variants functionally defective if the carboxypeptidase activity in the conditioned medium measured after activation with trypsin and chymotrypsin C was lower than 20% of the wild-type value in accordance with [Witt et al. 2013]. This reduced activity could be the result of decreased secretion, impaired catalytic activity or degradation by the activating proteases. Functionally defective CPA1 variants were found in 3 (p.Gly225Ser × 1, p.His306Tyr × 1 and p.Glu380Lys × 1) of the 1,112 (0.27%) Chinese ICP patients and in 2 (p.Glu100Lys × 1, and p.Arg240Gln × 1) of the 1,580 (0.13%) controls (Table 2), again displaying no significant difference (P = 0.68; Table 1). As mentioned earlier, the association between defective CPA1 variants and NACP was at its strongest for the German patients aged ≤10 years, the corresponding detection frequency was nearly 10% [Witt et al. 2013]. The Chinese ICP cohort contained only 61 patients aged ≤10 years, but none of these individuals was found to harbor a functionally defective variant.

There is one additional point to make. Functionally impaired CPA1 missense variants have previously been reported to occur predominantly in the second half of the 419-amino acid-protein [Witt et al. 2013]. Consistent with this finding, four of five such variants found in the Chinese subjects were also located in the second half of the protein (Table 2). Evaluation of all the functionally impaired CPA1 missense variants in the context of the CPA1 structure (http://www.uniprot.org/uniprot/P15085) revealed that p.Arg237His, p.His306Tyr and p.Glu380Lys affected substrate binding or catalytic sites whereas p.Cys271Arg affected a disulfide bond-forming residue (Supp. Figure S3). It should be noted that all five variants occurred in the second half of the CPA1 protein sequence.
In summary, this is the first study to attempt to replicate the recently reported association of rare functional \textit{CPA1} variants with chronic pancreatitis [Witt et al. 2013] in the context of a large ICP cohort. This is also the largest ICP cohort to date used for the sequencing of the entire coding region and exon/intron boundaries of the \textit{CPA1} gene in a single population. Often, the OR of an association observed in a discovery cohort is much higher than in subsequent replication cohorts [Dumas-Mallet et al., 2016]; this was indeed the case for the rare functional \textit{CPA1} variants in the German, European replication and Asian cohorts (Table 1). However, no significant association with ICP was noted in our Chinese cohort, irrespective of whether all rare variants or functionally-deficient variants were considered. Differences in phenotype definition and ethnicity-specific disease risk factors as well as population stratification may account for the inconsistent findings between studies. For example, although both the present study and the previous study focused on non-alcoholic patients (Table 1), differences between the cohorts regarding alcohol consumption (even if not in causative dosage), smoking and age could conceivably have contributed to the observed differences in disease association. Additionally, given that the proposed disease mechanism is provoked ER stress [Witt et al. 2013], the possibility that \textit{CPA1} activity might not be the best proxy marker for the functional effect of the disease-associated \textit{CPA1} variants remains to be excluded.
REFERENCES


