The reversion variant (p.Arg90Leu) at the evolutionarily adaptive p.Arg90 site in CELA3B predisposes to chronic pancreatitis

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Abstract

A gain-of-function missense variant in the *CELA3B* gene, p.Arg90Cys (c.268C>T), has recently been reported to cause pancreatitis in an extended pedigree. Herein, we sequenced the *CELA3B* gene in 644 genetically unexplained French chronic pancreatitis (CP) patients (all unrelated) and 566 controls. No predicted loss-of-function variants were identified. None of the six low frequency or common missense variants detected showed significant association with CP. Nor did the aggregate rare/very rare missense variants (n=14) show any significant association with CP. However, p.Arg90Leu (c.269G>T), which was found in 4 patients but no controls, and affects the same amino acid as p.Arg90Cys, serves to revert p.Arg90 to the human elastase ancestral allele. Since p.Arg90Leu has previously been shown to exert a similar functional effect to that of p.Arg90Cys, our findings not only confirm the involvement of *CELA3B* in the etiology of CP but also pinpoint a new evolutionarily adaptive site in the human genome.

KEYWORDS

CELA3B, chronic pancreatitis, gain-of-function mutation, gene conversion, elastases, paralogues

Chronic pancreatitis (CP) is a complex disease that can be caused by genetic and/or environmental factors (Beyer, Habtezion, Werner, Lerch, & Mayerle, 2020; Hegyi et al., 2020; Kleeff et al., 2017). Since the mapping and cloning of the first gene found to underlie hereditary pancreatitis (i.e., *PRSS1*; MIM# 276000) more than 20 years ago (Le Bodic et al., 1996; Pandya et al., 1996; Whitcomb, Gorry, et al., 1996; Whitcomb, Preston, et al., 1996), multiple additional genes/loci associated with CP have been identified, either by means of candidate gene approaches (Cohn et al., 1998; Fjeld et al., 2015; Lasher et al., 2019; Le Maréchal et al., 2006; Masson, Chen, Scotet, Le Maréchal, & Férec, 2008; Rosendahl et al., 2008; Sharer et al., 1998; Witt et al., 2013; Witt et al., 2000; Witt et al., 2006) or hypothesis-free ('agnostic') approaches (Masamune et al., 2020; Moore et al., 2019; Rosendahl et al., 2018; Whitcomb et al., 2012; Zou et al., 2020).

CELA3B, encoding chymotrypsin-like elastase 3B (MIM# 618694), is one of the most recently identified CP-associated genes (Moore et al., 2019). Specifically, the whole-exome sequencing of a patient with CP, her affected daughter, unaffected brother and son, led to the identification of a missense variant in the *CELA3B* gene, p.Arg90Cys (c.268C>T), as the cause of the disease (Moore et al., 2019) in a large kindred which had originally been reported more than 50 years ago (Davidson, Costanza, Swieconek, & Harris, 1968). Transfection studies demonstrated that (i) p.Arg90Cys led to elevated CELA3B protein expression by upregulating translation and (ii) the mutant protein was more easily activated by trypsin. Moreover, CRISPR-Cas9-engineered homozygous *Cela3b* Arg89Cys mice developed more severe pancreatitis than did wild-type control animals (Moore et al., 2019).

Herein we report findings from the analysis of the *CELA3B* gene in 644 unrelated French CP patients and 566 controls. The patients comprised 73 cases with hereditary CP (HCP), 189 cases with familial CP (FCP) and 382 young cases (defined as either age of disease onset ≤20 years or diagnosis made at age ≤20 years, as previously described (Masson et al., 2008)) with

idiopathic CP (ICP). The classification of patients as HCP (having three or more affected members spanning at least two generations), FCP (having a positive family history but not satisfying a diagnosis of HCP) and ICP (having a negative family history) is in accordance with our previous publications (Chen & Férec, 2009; Masson et al., 2008). All participating patients had remained genetically unexplained after sequence analysis of the coding regions and flanking splice junctions of the *PRSS1*, *SPINK1*, *CTRC*, *CFTR* (Masson, Chen, Audrézet, Cooper, & Férec, 2013), *CPA1* (Witt et al., 2013), *CEL-HYB1* (Fjeld et al., 2015) and *TRPV6* (Masamune et al., 2020) genes. The entire coding and proximal intronic regions of the *CELA3B* gene were amplified using three primer pairs (see Supp. Table S1 for primer sequences and Supp. Methods for PCR conditions). PCR products were purified by Illustra™ ExoProStar™ (Dominique Dutscher, Brumath, France) and then sequenced using the BigDye™ Terminator v1.1 Cycle Sequencing Kit (ThermoFisher Scientific, Waltham, MA). Sequencing primers are provided in Supp. Table S2. We focused our analysis on (i) deletions or insertions that affected canonical GT-AG splice sites and/or coding sequence and (ii) single nucleotide substitutions that altered either canonical GT-AG splice sites or resulted in missense or nonsense variants. Variant nomenclature followed HGVS recommendations (den Dunnen et al., 2016). NM_007352.4 (https://www.ncbi.nlm.nih.gov/nuccore/NM_007352.4) was used as the reference mRNA sequence. The Ethical Review Committee of Brest University approved this study. All patients gave their informed consent for genetic analysis.

We identified a total of 20 variants, which were classified into (i) low frequency or common (n = 6; Table 1) and (ii) rare or very rare (n = 14; Table 2) in accordance with their allele frequencies in the 566 controls. The classification of variants as very rare (allele frequency of <0.001), rare (allele frequency from 0.001 to <0.005), low frequency (from 0.005 to 0.05) and common (allele frequency of >0.05) followed Manolio and colleagues (Manolio et al., 2009).

All 20 variants were predicted to result in either single or multiple missense substitutions. Thus, no obvious loss-of-function (pLoF) variants such as nonsense, canonical splice-site or frameshifting variants (in accordance with the gnomAD definition of pLoF variants (Lek et al., 2016)) were found in any patient. This is consistent with two observations. First, the previously reported CP-causing p.Arg90Cys is a gain-of-function variant by virtue of its upregulatory effect on translation (Moore et al., 2019). Second, the pLI score for *CELA3B* in genomAD (http://gnomad.broadinstitute.org/; as of 13 November 2020) is 0, suggesting that the gene is completely tolerant of heterozygous loss-of-function variants. In this regard, it is pertinent to mention that a *CELA3B* intronic variant, c.643-7G>T (rs61777963), manifests an association with alcoholic CP with a small protective effect (allele frequency: 13.8% in patients vs. 21.3% in controls; odds ratio (OR) = 0.59, 95% confidence interval (CI) 0.39 to 0.89; $P = 0.01$) (Parniczky et al., 2016). However, as acknowledged by the original authors, the number (n = 120) of alcoholic CP patients analyzed was small, and no association was found in a small cohort (n = 105) of non-alcoholic CP (allele frequency: 18.6% in patients vs. 21.3% in controls; OR = 0.84, 95% CI 0.56 to 1.26; $P = 0.4$) (Parniczky et al., 2016). We extracted corresponding data from our patients and controls, showing no significant association (allele frequency: 17.2% (222/1288) vs. 17.1% (194/1132); OR = 1.01, 95% CI 0.81 to 1.24; $P = 1.0$). Therefore, the aforementioned protective association is most likely spurious.

Three variants, namely the common c.[71G>A;73C>T;91A>C], rare c.[529G>C;536T>G] and very rare c.736_742delACCCGCAinsTTCATCT, involved ≥2 closely spaced single nucleotide substitutions. The ≥2 single nucleotide substitutions in each case were confirmed to be in *cis* by a newly developed next-generation sequencing method (detailed method will be published elsewhere), with the original sequencing data being deposited in the NCBI Sequence Read Archive (SRA) database (https://www.ncbi.nlm.nih.gov/sra) under accession

numbers SAMN16675587, SAMN16675586 and SAMN1667558. c.736_742delACCCGCAinsTTCATCT has previously been shown to represent a gene conversion event (Parniczky et al., 2016). c.[71G>A;73C>T;91A>C] and c.[529G>C;536T>G] probably also arose via gene conversion (Chen, Cooper, Chuzhanova, Férec, & Patrinos, 2007) as, in each case, a putative donor sequence is present at the aligned positions of the highly homologous and tandemly linked *CELA3A* gene on human chromosome 1p36.12. It should be noted that gene conversion events involving ≥2 nucleotide substitutions constitute a subtype of simultaneously generated multiple nucleotide variants (Chen, Férec, & Cooper, 2009, 2013).

The carrier frequencies of each of the six low frequency or common missense variants are broadly similar between the HCP, FCP and ICP patients (Table 1). We therefore combined the three clinical datasets for the purposes of analysis at the individual variant level. None of the variants were found to be associated with CP in terms of a significantly different allele frequency between patients and controls. As for the rare or very rare variants (Table 2), we combined the three clinical datasets in order to perform an aggregate association analysis. 22 (3.4%) of the 644 patients and 16 (2.8%) of the 566 controls harbored rare/very rare variants, a difference which was not significant (OR = 1.22, 95% CI 0.63 to 2.34; $P = 0.56$).

The above notwithstanding, p.Arg90Leu (c.269G>T; Supp. Figure S1A), which affected the same amino acid as the CP-causing p.Arg90Cys, was found in 4 patients [two FCP (each having an (as yet unanalyzed) affected first-degree relative) and two ICP] but in none of the controls (OR = 7.96, 95% CI 0.43 to 148.17; $P = 0.1275$). p.Arg90Leu was also found to be absent from the 574 French subjects in the public dataset of the French Exome (FrEx) project (Génin et al., 2017) and is extremely rare in gnomAD (allele frequency 0.0008097 in all populations; accessed November 16, 2020). Most importantly, this variant has been previously subjected to functional characterization together with the disease-causing *CELA3B*

p.Arg90Cys variant; these variants were remarkably similar in terms of all their measured biochemical and functional parameters as well as mouse phenotypes (Moore et al., 2019). It should be noted that the p.Arg90Leu variant had not been found in any patient in the original Moore study; it was analyzed functionally because, of the six human elastases, only CELA3B has an arginine at position 90 whereas all the others have a leucine (Moore et al., 2019). In this regard, we constructed the phylogenetic tree of the human elastase paralogues by means of NGPhylogeny.fr [https://ngphylogeny.fr/] (Lemoine et al., 2019), thereby formally confirming that p.Leu90 represents the ancestral allele whereas p.Arg90 is the derived allele (Supp. Figure S1B). Interestingly, replacement of p.Leu90 of the human wild-type CELA3A by arginine was found to reduce protein expression (Moore et al., 2019). The constellation of these genetic, functional and evolutionary data therefore argues that p.Arg90 in CELA3B was an evolutionarily adaptive change and that reversion to the ancestral allele predisposes to CP.

In summary, on the basis of sequencing a large French cohort of CP patients and controls, we provide new evidence to support the involvement of the *CELA3B* gene in the etiology of CP. Moreover, our identification of the p.Arg90Leu variant in multiple CP patients has revealed a new instance in which a genetic study has helped to pinpoint an evolutionarily adaptive site (Chen, Montier, & Férec, 2001; Chimpanzee & Analysis, 2005). Larger genetic and functional studies are however required to determine whether other variants of CELA3B occurring beyond the p.Arg90 site might also confer risk of CP.

DATA AVAILABILITY STATEMENT

All data relevant to the study are included in the article or uploaded as Supplementary information.

WEB RESOURCES

dbSNP: http://www.ncbi.nlm.nih.gov/SNP

genomAD: http://gnomad.broadinstitute.org/

NCBI Sequence Read Archive (SRA) database: https://www.ncbi.nlm.nih.gov/sra

NM_007352.4: https://www.ncbi.nlm.nih.gov/nuccore/NM_007352.4

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