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The hybrid allele 1 of carboxyl-ester lipase (*CEL-HYB1*) in Polish pediatric patients with chronic pancreatitis

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ABSTRACT

Objectives: It has previously been reported in a European case-control study with patients from Germany and France that *CEL-HYB1*, a hybrid allele of the carboxyl ester lipase (*CEL*) gene and its pseudogene *CELP*, increases susceptibility to chronic pancreatitis (CP). Here, we aimed to replicate this finding in Polish pediatric patients with CP.
Method: The distribution of the *CEL-HYB1* allele in a CP pediatric cohort (n = 147, median age at CP onset 7.6 years) with no history of alcohol/smoking abuse was compared with ethnically matched healthy controls (n = 500, median age 46 years). Screening was performed using long-range PCR followed by agarose gel-electrophoresis.
Results: We observed no significant difference in the carrier frequency of the *CEL-HYB1* allele between CP patients (7/147, 4.8%) and controls (12/500, 2.4%; $P = 0.16$).
Conclusions: This study found no statistically significant association between *CEL-HYB1* and chronic pancreatitis in a cohort of Polish pediatric CP patients.

Introduction

Chronic pancreatitis (CP) is a progressive inflammatory disorder resulting in destruction of the pancreas parenchyma and function. CP often develops in the background of genetic susceptibility. Cationic trypsinogen (*PRSS1*), carboxypeptidase A1 (*CPA1*), and additional genes such as *SPINK 1* (serum protease inhibitor Kazal type 1), *CFTR* (cystic fibrosis transmembrane conductance regulator), *CTRC* (chymotrypsin C), have been implicated in CP as either disease causing or risk modifiers of disease [1–6]. In general, CP seems to have a complex multigene and multifactorial cause, including gene–environment interactions between various pathogenic gene variants.

The carboxyl ester lipase (*CEL*) gene encodes a digestive enzyme which is secreted into the small intestine and activated by bile salts. The CEL protein is expressed in pancreas acinar cells and takes part in hydrolysis and absorption of cholesterol and lipid-soluble vitamins [7]. One of the most characteristic features of CEL is the presence of a variable number of tandem repeats (VNTR) region in the *C*-terminus of the protein [8]. The most common variant of CEL harbors 16 repeats [9]. A shorter and altered *C*-terminus of the protein is connected with different types of protein dysfunction. Frame shift deletions in the *CEL VNTR* region cause maturity-onset diabetes of the young (MODY) where the CEL-MODY protein contains 11 VNTR repeats of altered sequence [10]. Compared to normal CEL, the CEL-MODY protein has a strong tendency to form both intracellular and extracellular aggregates [11,12]. In 2015, a

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study by Fjeld et al. showed that non-allelic homologous recombination between *CEL* and its pseudogene *CELP* can create a hybrid allele (*CEL-HYB*, later named *CEL-HYB1*) that encodes a fusion protein. This variant consists of the proximal part of CEL and the distal part of CELP with only 3 VNTR repeats [13]. Screening a discovery cohort of 71 German patients with familial chronic pancreatitis, Fjeld et al. identified the *CEL-HYB* allele in 14% of patients compared to 1% of healthy controls. The significant overrepresentation of the allele was replicated in two German and one French cohort of non-alcoholic CP patients [13]. In 2016, Zou et al. attempted to replicate the association of *CEL-HYB* with CP in patient cohorts from China, Japan and India, but failed to detect the allele in any of these populations [14]. Interestingly, however, an alternative hybrid allele (*CEL-HYB2*) was identified, but was not associated with CP. Functional studies indicated that *CEL-HYB2* mRNA is less stable than *CEL-HYB1* due to the presence of a premature stop codon within the chimeric exon 10 leading to nonsense-mediated RNA decay [14].

The lack of *CEL-HYB1* in Asians suggested that the allele may be a European-specific disease risk factor, although this remains to be confirmed by replication in an independent cohort of European ancestry. Therefore, our goal was to determine the prevalence of the *CEL-HYB1* allele in Polish pediatric CP patients and to compare it to controls.

Methods

Patients and controls

A total of 147 children with CP hospitalized at the Department of Gastroenterology, The Children's Memorial Health Institute, Warsaw, Poland, between 1988 and 2018 were enrolled. Data on presentation, diagnostic findings and molecular analysis of CP genes towards most frequent pathogenic mutations in *PRSS1* (exon 2,3), *CPA1* (exons 7–10), *CFTR* (exons 4,9,10) of *CTRC* (exon 2,3,7), *SPINK1* (exon 3) were reviewed. The inclusion criteria were: age <18 years and a diagnosis of CP verified by imaging methods (ultrasound scan, computed tomography [CT], magnetic resonance cholangiopancreatography, and/or endoscopic retrograde cholangiopancreatography). Children with acute pancreatitis, and patients with a history of alcohol/drug abuse, smoking were excluded.

The control group consisted of 500 ethnically matched, unrelated individuals with no history of pancreatic disease (mean age of 45 years; median age of 46 years, 338 females and 162 males) from Holy Cross region of Poland (the southern part of the central region of Poland). The study was approved by the local Committee on Bioethics. Characteristic of the control group has been also described in study by Koziel et al. [15]. Each patient or the patient's parent as well as the members of the control group gave their informed consent for genetic testing.

Molecular analysis

DNA was isolated from peripheral blood with the Genomic Maxi AX kit (A&A Biotechnology). Screening for the *CEL–HYB1* allele was performed by a long-PCR assay, using the same primers and conditions as previously described and including a positive control sample [13]. PCR reactions were prepared in total volumes of 10 μl containing 2x GC buffer, 0.2 mM each primer: L11F (5' - GTCCCTCACTCATTCTTCTATGGCAAC-3); IAR (5′-TCCAAAGCCCTAGCAGTAACGA-3′); CELP VNTR-rev (5′CTGTGGAGGGCATGGAACT-3), 0.4 mM each dNTP, 1 M betaine solution, 0.5 U LA Taq DNA polymerase (Takara; Biokom), and 25–30 ng genomic DNA. Amplification was performed by the same conditions as described by Fjeld et al. [13]. PCR screening products were analyzed by 1% agarose gel electrophoresis and compared to the positive control as depicted on Fig. 1A. Exon 10 of all *CEL-HYB1*-positive samples (both CP and controls) underwent Sanger DNA sequencing using the primers S10F (5′-AGTGAGCACCCTGCCTACTTGG-3′) and S10R (5′- TCAAACACACATGGATTCCGATG-3′) to exclude any *CEL-HYB2* carriers. Differences between the *CEL-HYB1* and *CEL-HYB2* alleles as observed by Sanger sequencing of exon 10 are described in Fig. 1B. The presence of the *CELP* VNTR within the *CEL-HYB1* allele was confirmed by sequencing with the primer K11F (5′- GGATGGCTCAGGCGTGC-3′). Prior to sequencing, PCR products underwent clean-up with ExoSAP IT (USB Corporation), and 2 μl of the treated products was used as template. Sequencing results were analyzed by Mutation Surveyor® DNA Analysis Software (Softgenetics).

All *CEL-HYB1* positive samples were confirmed by an independent screening and sequencing test at the Gade Laboratory for Pathology, Department of Clinical Medicine, University of Bergen, Norway. Sequencing was performed on *CEL* exons 8–11. We identified one *CEL-HYB2* carrier in the control group with a premature stop codon in exon 10 (Fig. 1B). This sample was excluded from further analyses. In addition, eight randomly selected *CEL-HYB1*-positive samples underwent sequencing of exons 1–7 to check for any mutations that would terminate protein translation in the proximal part of the gene. Primer sequences are available upon request.

Statistical analysis

Differences in the frequency of *CEL-HYB1* between cases and controls were determined with two-tailed Fisher's exact test. Statistical significance was set at a *P* value of <0.05. The statistical analysis were conducted using SPSS software (version 22; SPSS) and GraphPadPrism (version 4.03, GraphPad Software, Inc).

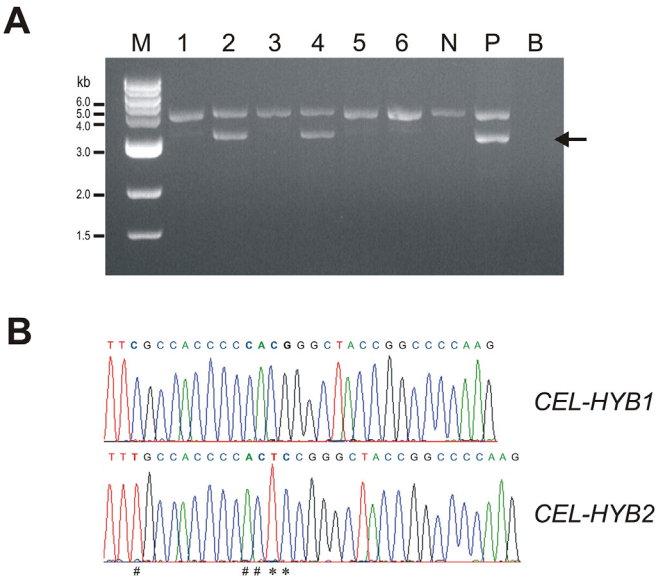

Fig. 1. The analysis of *CEL-HYB* allele and discrimination between *CEL-HYB1* and *CEL-HYB2* allele.
A. Screening for *CEL-HYB* alleles by long-PCR is shown for six selected CP samples (1–6). The lower band of around 3.2 kb (indicated by arrow) is specific for *CEL-HYB*. Samples 2 and 4 are positive; M, molecular weight standard; N, negative control; P, positive control; B, blank. B. **B**. Differences between the *CEL-HYB1* and *CEL-HYB2* alleles as observed by Sanger sequencing of exon 10. *CEL-HYB2* has two additional nucleotides inserted (asterisks), leading to a predicted translation termination 40 bp downstream of the insertion. The hashtags denote other differences between *CEL-HYB1* and *CEL-HYB2*, not changing the reading frame.

Results

Patients

The study included 147 CP patients with a median age of disease onset at 7.6 years (mean = 8.5, range 0.2–17). Two of 147 (1.3%) cases carried a heterozygous *PRSS1* mutation (p.Glu79Lys; *PRSS1_PRSS3P2* hybrid), 2/147 (1.3%) cases carried a heterozygous *CPA1* mutation (p.Tyr318Ter, p.Arg382Trp), 12/147 (8.2%) carried a heterozygous *CFTR* mutation (p.Phe508del), 41/147 (27.8%) had a *SPINK1* mutation (p.Asn34Ser or IVS3+2T > C in one or two alleles), and 9/147 carried a heterozygous *CTRC* mutation (p.Lys247_Arg254del or p. Arg254Trp). In 42/147 (28.5%) and 17/147 (11.5%) of the cases heterozygous and homozygous c.180 C > T *CTRC* variant (p.Gly60Gly) was detected, respectively. Anatomic anomalies (*pancreas divisum or ansa pancreatica*) were present in 19/147 (13%), lipid disturbances in 13/147 (8.8%), biliary tract disorders in 7/147 (4.7%) and autoimmune pancreatitis in 3/147 (2%) of the cases. Forty one cases had a positive family history for acute or chronic pancreatitis (at least one additional case in the family). Parts of these data have previously been published [2,16–19].

Analysis of CEL-HYB1 carrier frequency

We detected the *CEL-HYB1* allele in 4.8% (7/147) of the CP patients and in 2.4% (12/500) of the controls (OR = 2.03; 95% CI: 0.78–5.2; $P = 0.16$). The *CEL-HYB1* allele was detected in a heterozygous state only. We also analyzed the frequency of the allele in the subgroup of 20 CP patients with a positive family history and no known genetic risk factors (defined as the absence of pathogenic mutations in tested CP genes with exclusion of the low penetrant heterozygous c.180C > T variant in *CTRC* gene). The *CEL-HYB1* allele was found in 3/20 (15%) of these cases.

Table 1 shows the characteristics of the seven *CEL-HYB1*-positive patients. Three of them had other known CP risk factors such as genetic variants or trauma. In three additional positive cases, a family history of CP or recurrent acute pancreatitis was present. In one of the positive families, the index patient (no. 5, Table 1) had inherited the *CEL-HYB1* allele from an affected mother. Also five other affected family members carried the allele, whereas most of the unaffected individuals (6/7) available for testing were negative. In the other two familial cases (no. 1 and 4, Table 1), the index patients had inherited the allele from a healthy parent.

Discussion

Genetic risk in chronic pancreatitis may be mediated not only by trypsin activity but also by trypsin –independent mechanism that involve misfolding and endoplasmic reticulum stress. The best characterized misfolding variants are *PRSS1* and *CPA1* variants which strongly increase CP risk or cause hereditary pancreatitis [2,3,20,21]. The study by Fjeld et al. showed that also a hybrid allele *CEL-HYB* (later named *CEL-HYB1*) is associated with CP in patients from Germany and France [13] and suggested that the pathogenic effect could be due to intracellular retention and impaired secretion of the CEL-HYB protein which may be related to misfolding [13]. Since Zou WB et al. failed to detect the presence of the *CEL-HYB1* allele in Asian patients, it has been postulated that the allele may be a CP risk factor specific for European ethnicities [14]. Therefore, our aim was to evaluate the risk of CP associated with the *CEL-HYB1* allele in Polish pediatric patients. In our study, however, we did not find any significant difference in the frequency distribution of the allele between CP patients and controls (4.8% vs 2.4%, $P = 0.16$).

In the previous study by Fjeld et al., the combined carrier frequency observed in non-alcoholic German and French CP cohorts (3.7%) is similar to that found in our Polish CP group (4.8%). For comparison purpose, we also analyzed the subgroup of our patients with a positive family history and no genetic risk factors present. The carrier frequency in this subgroup (3/20; 15.0%) is similar to that observed in the discovery cohort of 71 German patients with familial CP without known genetic etiology (14.1%) [13]. Furthermore, since it has been suggested that *CEL-HYB1* is inherited in an autosomal dominant manner with incomplete penetrance [13], we performed co-segregation analysis of the allele with CP in the three families positive for *CEL-HYB1*. We found co-segregation in one of the 3 positive families. This, however, might be a coincidental finding taking into account the relatively high frequency of the allele in the control group. Surprisingly, the *CEL-HYB1* allele was detected in 2.4% (12/500) of our controls which is higher than in German (0.7%–1%), French (0.7%) and also Norwegian (0.3%) controls [13,22].

The limitation of our study is that the control group was recruited by a single study center in Holy Cross region of Poland (the southern part of the central region of Poland), whereas CP patients were recruited by reference hospital in Warsaw admitting the patients from different regions of Poland. However, taking into account that Poland is a relatively ethnically homogeneous country, we find the possibility of an ethnical bias unlikely. One possible scenario could be that the *CEL-HYB1* originates from Eastern parts of Europe and that the observed frequency differences between Eastern and Western Europe might be due to a genetic drift. Also, at the current point, we cannot rule out that there is another, yet unknown, genetic variant, presumably on the same chromosome as *CEL* (chromosome 9), which might have arisen in the West European population and is responsible for *CEL-HYB1* pathological effect in this region.

In summary, we found that the *CEL-HYB1* allele is frequent in our Polish control group (around 2.4%) and is not significantly associated with pediatric CP. We note that the frequency in the CP cases was twice as high as in the controls suggesting a trend for an

Table 1
Characteristics of the *CEL-HYB1*-positive patients with chronic pancreatitis.

Patient No.	Gender	Age at onset (years)	CP or RAP family history	Susceptibility gene mutation[a]	Other risk factors
1	M	3.8	RAP (mother and grandfather)	No	
2	F	9.5	No	*CTRC*: Gly60Gly/Gly60Gly	
3	M	5.3	No	No	
4	M	14.8	CP (father)	No	Trauma
5	M	2.8	CP (7 family members)[b]	No	
6	F	5.7	No	No	
7	F	6.3	No	*CTRC*:Arg254Trp/-	

The *CEL-HYB1* allele was detected in the heterozygous state only.
CP, chronic pancreatitis; RAP, recurrent acute pancreatitis.
[a] All patients had undergone genetic screening for mutations in major CP susceptibility genes (see Methods).
[b] Fulfilling the criteria of hereditary pancreatitis by means of positive family history towards CP (with no *PRSS1* mutation).

approximately two-fold effect size. Even if significant, however, such a low effect size would have limited clinical value comparable with a heterozygous p.Gly60Gly variant of *CTRC* [16]. On the other hand, taking into account that CP often results from complex gene-gene/environmental interactions, we cannot exclude that *CEL-HYB1* may be a low-penetrance risk factor which in conjunction with other risk factors influences susceptibility to CP. Further studies are warranted to answer the question whether the *CEL-HYB1* allele is truly a European-specific CP risk variant.

Conflicts of interest

The authors are unaware of any conflicts of interest.

Author Contributions

Contributors: AMR: conception and design of the study, the study supervision, statistical analysis of the data, wrote the original draft; GO: patients enrolment to the study, clinical data collection and analysis, revision of the manuscript; AAK – molecular data collection and analysis, assistance in writing of the part of original draft; KWT– analysis of molecular data, revision of the manuscript; SJS -molecular re-analysis of the *CEL-HYB1* allele positive samples; KF, AM - assistance in the study design and revision of the manuscript, AB - DNA isolation from the control group, DK, SG, - recruitment of the control group. All authors participated in approval of the final manuscript.

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