Association between calcium sensing receptor gene polymorphisms and chronic pancreatitis in a US population: Role of serine protease inhibitor Kazal 1type and alcohol

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Abstract

AIM: To test the hypothesis that calcium sensing receptor (CASR) polymorphisms are associated with chronic pancreatitis (CP), and to determine whether serine protease inhibitor Kazal 1type (SPINK1) N34S or alcohol are necessary co-factors in its etiology.

METHODS: Initially, 115 subjects with pancreatitis and 66 controls were evaluated, of whom 57 patients and 21 controls were predetermined to carry the high-risk SPINK1 N34S polymorphism. We sequenced CASR gene exons 2, 3, 4, 5 and 7, areas containing the majority of reported polymorphisms and novel mutations. Based on the initial results, we added 223 patients and 239 controls to analyze three common nonsynonymous single nucleotide polymorphisms (SNPs) in exon 7 (A986S, R990G, and Q1011E).

RESULTS: The CASR exon 7 R990G polymorphism was significantly associated with CP (OR, 2.01; 95% CI, 1.12-3.59; P = 0.015). The association between CASR R990G and CP was stronger in subjects who reported moderate or heavy alcohol consumption (OR, 3.12; 95% CI, 1.14-9.13; P = 0.018). There was no association between the various CASR genotypes and SPINK1 N34S in pancreatitis. None of the novel CASR polymorphisms reported from Germany and India was detected.

CONCLUSION: Our United States-based study confirmed an association of CASR and CP and for the first time demonstrated that CASR R990G is a significant risk factor for CP. We also conclude that the risk of CP with CASR R990G is increased in subjects with moderate to heavy alcohol consumption.

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Key words: Calcium sensing receptor; Serine protease inhibitor Kazal 1type; Chronic pancreatitis; Alcohol

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INTRODUCTION

Chronic pancreatitis (CP) is a debilitating, inflammatory disease of the pancreas, characterized by progressive organ destruction and fibrosis. CP results in profound exocrine and endocrine insufficiency and, in many cases, intractable, chronic pain. As a complex disorder, CP can develop from a variety of etiologies with multiple pathological pathways\(^{[1]}\). For several years, alcohol abuse has been considered the most likely causative agent for CP in Western countries, although etiologies including toxic, metabolic (hypercalcemia, hyperlipidemia), genetic mutations, autoimmune, and duct obstruction, have also been implicated\(^{[2],[3]}\).

Consistent experimental evidence links elevated acinar cell calcium levels with acute pancreatitis in association with premature trypsinogen activation to trypsin\(^{[4]}\). Recurrent acute pancreatitis (RAP), as illustrated in patients with hereditary and sporadic pancreatitis, can lead to CP\(^{[5-7]}\). Hypercalcemia itself has been associated with the development and complications of CP\(^{[2]}\). Recent studies from Germany and India have reported that novel calcium sensing receptor (CASR) gene mutations in combination with the presence of serum protease inhibitor Kazal Type (SPINK1) N34S increased the risk of CP\(^{[8-10]}\). The SPINK1 N34S “high-risk haplotype” is strongly associated with CP, but only a limited portion of mutation carriers develop CP during their life time, suggesting that additional factors are necessary to develop this complex disorder\(^{[11,13]}\).

CASR is a member of the G-protein-coupled receptor (GPCR) superfamily\(^{[3]}\). CASR plays an important role in calcium homeostasis, as is reflected in its expression by cells of the parathyroid gland and renal tubules that are involved in calcium metabolism. CASR has been identified in both human pancreatic acinar and ductal cells, as well as in various non-exocrine cells\(^{[14]}\), although its functional significance in the pancreas has not been determined.

The human CASR gene is located on chromosome 3q 13.3-21\(^{[15,16]}\). CASR possesses a coding region of 3234 base pairs (bp) which is contained within 6 of the seven exons that make up the gene. One hundred and twelve functional mutations (40 activating and 72 inactivating) have been described in the CASR mutation database related to familial hypocalciuric hypercalcemia (FHH), neonatal severe primary hyperparathyroidism (NSHPT), and autosomal dominant hypocalcemia (ADH) families as well as in de-novo disease\(^{[17]}\). In addition, single activating or inactivating CASR mutations may cause hypercalcemic or hypocalcemic disorders\(^{[18,19]}\).

We hypothesized that CASR polymorphisms are associated with the development of CP and that SPINK1 N34S mutations or alcohol may be important co-factors in its etiology. We tested this hypothesis by evaluating subjects with RAP, CP and healthy controls with known SPINK1 genotypes and alcohol intake for common and novel CASR polymorphisms in exons 2, 3, 4, 5 and 7.

MATERIALS AND METHODS

Study population
Subjects were recruited from the North American Pancreatic Study 2 (NAPS2). The NAPS2 study is a multicenter, molecular epidemiology study designed to evaluate the genetic and environmental factors predisposing to recurrent acute pancreatitis (RAP) and CP. Detailed description of methods are presented elsewhere\(^{[20]}\). The subjects were stratified into alcohol categories based on self-reported average number of drinks consumed per week during the period of heaviest lifetime drinking. Alcohol categories were defined based on the drinking pattern as: (1) abstainers: no alcohol use or < 20 drinks in lifetime; (2) light drinkers: \(≤ 3\) drinks/week; (3) moderate drinkers: 4-7 drinks/week for females; 4-14 drinks/week for males; (4) heavy drinkers: 8-34 drinks/week for females; 15-34 drinks/week for males; (5) very heavy drinkers: \(≥ 35\) drinks/week for both males and females. For analysis, alcohol drinking categories were combined into 3 groups based on their risk for causing CP: (1) abstainers and light drinkers were considered very low risk, (2) moderate and heavy drinkers were considered moderate risk, and (3) very heavy drinkers were considered substantial risk\(^{[20]}\).

One hundred and fifteen affected individuals and 66 controls were selected initially from four sites of the NAPS2 cohort. These subjects were selected based on the presence or absence of SPINK1 N34S mutations, of which 57 patients and 21 controls were determined by previous genetic analysis to carry the high-risk SPINK1 mutation. From the twenty site NAPS2 consortium, 219 affected subjects and 239 controls were later screened for the three common nonsynonymous single nucleotide polymorphisms (SNPs) seen in the coding region of the intracellular CASR tail in exon 7 which appeared to be the region of interest. These were A9865G (rs \# 1801725), R990G (rs \# 1042636), and Q1011E (rs \# 1801726).

DNA preparation and mutation analysis
Genomic DNA was extracted from whole blood as described\(^{[20]}\). PCR primers were designed for CASR gene exons 2, 3, 4, 5 and 7, which contains most of the commonly seen activating and inactivating mutations as well as the novel mutations found in Germany and India (Table 1). Exons 4 and 7 were lengthy and thus were divided into 2 and 4 fragments respectively.

PCR was performed in a total volume of 25 \(\mu\)L; 200 nmol of forward and reverse primer, 200 \(\mu\)mol of dNTP and 1 \(\times\) PCR Buffer II (ABI, CA) with 10 ng of DNA. Amplification settings were 95°C for 12 min \(\times\) 1 cycle, 95°C for 30 s, annealing temperature (Table 1) \(\times\) 20 s and 72°C \(\times\) 20 s for 35 cycles and 72°C for 2 min \(\times\) 1 cycle. Annealing temperatures and magnesium concentrations for different primers are shown in Table 1. PCR amplification products were purified with exonuclease 1 (NEB, Beverly, MA) and shrimp alkaline phosphatase (Roche Diagnostics, Indianapolis, IN) according to the manufacturer’s recommendations. Cycle sequencing was performed using the ABI Prism Big Dye Terminator Sequencing Kit v3.1 diluted 1:8 (ABI, Foster City, CA) using the appropriate PCR primers. Products from the sequencing reaction were purified by ethanol EDTA precipitation. Sequence products were run on
Table 1  Polymerase chain reaction primer pairs, magnesium concentration and annealing temperatures used for genetic analysis of the CASR gene

| Scanning region | Forward and reverse primer sequences | MgCl₂ (mmol/L) | Annealing temperature (°C) |
|-----------------|--------------------------------------|----------------|---------------------------|
| Exon 2          | 5'-ACCACCCACATTACACGT-3' 5'-GTCTTTCTCCACACACCACTC-3' | 2.5 2.5 | 55 55 |
| Exon 3          | 5'-AGACCCACAGAGCAGATTACAC-3' 5'-TAAACGTGAAAGTGTTTTGGG-3' | 2.5 2.5 | 58 58 |
| Exon 4a         | 5'-GTCTTTCTACCTTTCTCTTATC-3' 5'-ATACACCTTACCCAATGCTGTG-3' | 2 2 | 58 58 |
| Exon 4b         | 5'-CATATGCTGGACGCCCTATC-3' 5'-GCAGCCCAACCTTTGGTT-3' | 2 2 | 59 59 |
| Exon 5          | 5'-TGGGGCTTGACTACATTTCT-3' 5'-ATGTTTTCTGATGGCTATTGGG-3' | 1.5 1.5 | 57 57 |
| Exon 7a         | 5'-CACACAAATAACTCACTCTT-3' 5'-CAGAGGAAAACCAGCAGGAAC-3' | 2.0 2.0 | 61 61 |
| Exon 7b         | 5'-AAAACCCACAGGTCTTGGTG-3' 5'-ATGCCCTTACACCAATACC-3' | 1.0 1.0 | 53 53 |
| Exon 7c         | 5'-GTCTATCTTTCTACAGTGCTG-3' 5'-GTATATGGCTTCATTTGGGG-3' | 1.0 1.0 | 58 58 |
| Exon 7d         | 5'-CCCCGAAGGAGCAGAGCAG-3' 5'-ACACCTTCTACGCTTCC-3' | 1.0 1.0 | 58 58 |

Table 2  Participant characteristics

| Demographic | CP (n = 219) RAP (n = 115) Controls (n = 305) |
|-------------|------------------------------------------|
| Age, mean (SD) | 45.3 (18.1) 46.1 (16.2) 54.7 (14.5) |
| Race, % White | 91 91 94 |
| Sex (M/F) | 125/94 94/66 121/184 |
| Alcohol drinking pattern (%) | |
| Abstainers | 45 (22.5) 30 (27) 72 (25) |
| Light | 38 (18) 26 (23) 83 (28) |
| Moderate | 34 (17) 22 (20) 56 (19) |
| Heavy | 39 (19.5) 20 (18) 58 (20) |
| Very heavy | 44 (22) 13 (12) 23 (8) |

Statistical analysis

Genotype frequencies were assessed for Hardy-Weinberg equilibrium. The frequencies of genotypes among cases and controls were compared using Chi square test or the Fisher's exact test when appropriate. Odds ratio (OR) and 95% confidence intervals (95% CI) for genotypes were calculated using an autosomal dominant model. Therefore, caution must be exercised before this association is either accepted or rejected.

RESULTS

Subject demographics and alcohol drinking patterns are given in Table 2. The proportion of subjects reporting a moderate or heavy alcohol drinking pattern was similar between patients and controls. Of the 334 patients with pancreatitis, 219 (66%) had CP and 115 (34%) had RAP.

The initial study consisted of 115 patients (CP = 82 and RAP = 33) and 66 controls, of which 57 patients (CP = 47 and RAP = 10) and 21 controls carried the SPINK1 N34S high risk haplotype. Of the 58 patients without SPINK1 N34S, 35 were diagnosed with CP and 23 had RAP.

The genotype frequencies were found to be in Hardy-Weinberg equilibrium. The R990G polymorphism (AGG→GGG transition) in exon 7 of the CASR gene, the G allele was more common among CP patients (n = 35) than controls (n = 45), but only in subjects without SPINK1 N34S. In comparing CP patients (n = 47) and controls (n = 21) with SPINK1 N34S, there was a non-significant trend towards an increased occurrence of the G allele in patients (OR, 4.03; 95% CI, 0.48-190.8, P = 0.255). One limitation of this study was the small number of SPINK1 N34S subjects for comparison; Therefore, caution must be exercised before this association is either accepted or rejected.

From the 112 mutations reported previously, the following three mutations--E191E, Y440C and A746A (AGG→GGG transition) in exon 7 of the CASR gene, were each observed once in CP patients with SPINK1 N34S. Recently identified novel CASR mutations from Germany and India seen in exons 3 (P163R), 4 (L173P, F391F, I425S, D433H), 5 (V477A) and 7 (E870E, R896E) were not observed in either patients or controls. Two intronic polymorphisms 493-94 C>T and 493-134 T>C included in exon 4 amplicon occurred with similar frequency in CP patients without SPINK1 N34S, 72 had CP and 32 had RAP.

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no difference in A986S and Q1011E polymorphisms among RAP and CP patients, and controls.

To determine if the risk was modified with alcohol use we compared CASR R990G genotypes in subjects with moderate and heavy alcohol drinking pattern. CP was strongly associated with the CASR R990G in moderate and heavy alcohol drinkers, as is demonstrated in Table 4 (OR, 3.12; 95% CI, 1.14-9.13; \( P = 0.018 \)). No association was observed with this particular polymorphism in abstainers or in subjects with self-reported light or very heavy alcohol drinking patterns.

### DISCUSSION

In the past, CP was commonly attributed to heavy alcohol consumption. More recent studies, however, suggest there is also a strong genetic basis for this illness\[^{25}\]. Growing knowledge of complex gene-environment interactions has provided fundamental insight into the pathophysiological mechanisms that result in fibrotic destruction of the pancreas\[^{11,23-25}\]. Studies from Germany and India have recently identified 8 novel CASR mutations that were associated with SPINK1 N34S in idiopathic and tropical CP subjects. Our study did not detect these novel CASR mutations. However, we were able to demonstrate and verify that CASR R990G confers significant risk for developing CP especially when linked to moderate and heavy alcohol consumption.

Three common nonsynonymous SNPs are located in the region coding the intracellular tail of CASR\[^{26}\] and play an important role in cellular signal transduction that alters serum ionized calcium level\[^{27,28}\]. Previously, it was reported that individuals carrying the 990 variant G allele may experience very mild decrease in serum ionized calcium levels from 4.92 mg/dL to 4.84 mg/dL\[^{26}\]. Although serum ionized calcium levels

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Table 3  Genotype analysis of CASR R990G polymorphism in patients and controls

|                | Patients | Controls | \( P \) | OR (95% CI) |
|----------------|----------|----------|---------|-------------|
| CP patients vs controls without SPINK1 N34S (%) |          |          |         |             |
| AA            | 140 (82) | 255 (90) |         |             |
| AG            | 31 (18)  | 28 (10)  |         |             |
| GG            | 1 (1)    | 1 (0.5)  |         |             |
| AA vs AG/GG   |          |          | 0.015   | 2.01 (1.12-3.59) |
| RAP patients vs controls without SPINK1 N34S (%) |          |          |         |             |
| AA            | 93 (89)  | 255 (90) |         |             |
| AG            | 10 (9)   | 28 (10)  |         |             |
| GG            | 2 (2)    | 1 (0.5)  |         |             |
| AA vs AG/GG   |          |          | 0.712   | 1.28 (0.67-2.47) |
| SPINK1 N34S positive CP patients vs controls (%) |          |          |         |             |
| AA            | 39 (83)  | 20 (95)  |         |             |
| AG            | 8 (17)   | 1 (5)    |         |             |
| GG            | 0        | 0        |         |             |
| AA vs AG/GG   |          |          | 0.255   | 4.1 (0.48-35.14) |

CP: Chronic pancreatitis; RAP: Recurrent acute pancreatitis. SPINK1: Serine protease Kazal type 1 gene. \(^1\)Fisher exact test.

Table 4  CASR genotype comparison for R990G polymorphism in CP patients and controls with similar alcohol drinking patterns

|                | Patients | Controls | \( P \) | OR (95% CI) |
|----------------|----------|----------|---------|-------------|
| A/L Alcohol CP patients vs controls (%) |          |          |         |             |
| AA            | 69 (83)  | 136 (88) |         |             |
| AG            | 13 (16)  | 18 (11)  |         |             |
| GG            | 1 (1)    | 1 (1)    |         |             |
| AA vs AG/GG   |          |          | 0.332   | 1.45 (0.69-3.07) |
| M/H Alcohol CP patients vs controls (%) |          |          |         |             |
| AA            | 59 (81)  | 106 (95) |         |             |
| AG            | 14 (19)  | 8 (7)    |         |             |
| GG            | 0        | 0        |         |             |
| AA vs AG/GG   |          |          | 0.018   | 3.12 (1.14-9.13) |
| VH Alcohol CP patients vs controls (%) |          |          |         |             |
| AA            | 37 (84)  | 21 (91)  |         |             |
| AG            | 7 (16)   | 2 (9)    |         |             |
| GG            | 0        | 0        |         |             |
| AA vs AG/GG   |          |          | 0.708   | 1.99 (0.38-0.45) |

A: Abstainer; L: Light; M: Moderate; H: Heavy; VH: Very heavy. Alcohol categories: Abstainers: No alcohol use or < 20 drinks in lifetime; Light drinkers: < 3 drinks/week; Moderate drinkers: 4-7 drinks/week for females, 4-14 drinks/week for males; Heavy drinkers: 8-34 drinks/week for females, 15-34 drinks/week for males; Very heavy drinkers: > 35 drinks/week. \(^1\)Fisher’s exact test.
alter the cytosolic calcium ion concentrations in acinar cells in a concentration-dependent manner, and may alter the risk of acute pancreatitis\cite{29,30}, the CASR R990G allele associated with increased risk of CP should slightly reduce the risk of acute pancreatitis. Furthermore, the magnitude of change in serum calcium levels due to CASR R990G alone is small, and it is difficult to imagine that this small change would, by itself, significantly alter the risk of acute pancreatitis. Indeed, our data suggests that CASR R990G is associated with CP rather than RAP. Our speculation is that CASR R990G might induce direct changes in the acinar and ductal cells that increase the risk for CP. However, the mechanism remains unknown.

Interestingly, while 55%-80% of pancreatitis cases may be attributed to alcohol abuse, less than 5% of heavy alcohol users develop pancreatitis\cite{31}. Alcohol abuse may not be the sole risk for the development of CP\cite{32}; rather alcoholic CP is likely the result of an interaction of several co-factors\cite{33}. It has been demonstrated that chronic alcohol consumption accelerates fibrosis in response to cerulein-induced CP in rats\cite{34}. Alcohol metabolites in pancreatic acinar cells induce persistent cytosolic Ca\textsuperscript{2+} signals in a concentration-dependent manner and depolarize mitochondria.

The discovery and characterization of a genetic cause of hereditary pancreatitis generated renewed interest in a possible genetic predisposition to alcoholic CP\cite{35}. Several CP-related gene mutations have been described previously with CFTR, PRSS1, SPINK1 and others\cite{36}. Our study also demonstrates the association of CASR R990G with CP, especially with moderate and heavy alcohol consumption. The presence of CASR R990G alone doubled the risk of developing CP, while in those individuals reporting moderate and heavy alcohol consumption, the risk was increased by 3-fold. Our hypothesis for testing CASR R990G in subjects with moderate and heavy drinking patterns is that this group represented a “threshold” alcohol pancreatitis risk group in which the addition of another risk factor would increase the overall risk of developing CP. The risk of CP in subjects with CASR R990G but with minimal or no alcohol consumption would be lower, while very heavy drinkers would be at high risk, regardless of the CASR genotype. Our experimental findings support this hypothesis.

The novel CASR gene mutations that were identified in German and Indian populations appeared to be closely associated with the SPINK1 N34S haplotype. We did not detect these, or other novel CASR mutations, and our study was not powered to demonstrate an interaction between SPINK1 N34S and CASR R990G. On the other hand, it was not clear whether or not the German and Indian studies tested for an effect of alcohol in a “threshold” dose range. However, both studies suggest that the overall effect of CASR polymorphisms are relatively small, and become clinically significant in the presence of additional risk factors in an additive or multiplicative way. This is consistent with current concepts that CP is a complex syndrome.

The present study confirmed the association of CASR genetic variants with CP. Our genotyping results in a US population were different from those reported from Germany and India. CASR R990G significantly increased the risk of developing CP and this effect was enhanced in subjects who consumed alcohol in a moderate to heavy dose range. Certain polymorphisms in the CASR gene may be considered risk factors for the development of CP, especially within the context of alcohol consumption. The relationship with SPINK1 mutations warrants further study.

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COMMENTS

Background

Chronic pancreatitis is a highly morbid, complex disease whose development depends on the combination of genetic and environmental factors. Elucidating the genetic links to this illness is critical in diagnosis, treatment and risk assessment.

Research frontiers

This study adds another gene to the growing number of genetic and other factors that confer increased risk of chronic pancreatitis. As new factors continue to be identified and confirmed, the emphasis will turn to integrating these risks, using systems approaches, as described in reference #1.

Innovations and breakthroughs

This study is one of the first to consider the complexity of gene-environment and gene-gene interactive paradigms by evaluating alcohol consumption and serine protease inhibitor Kazal 1 type (SPINK1) N34S variants with calcium sensing receptor (CASR) polymorphisms. The confirmation of CASR genetic variants as risk factors for chronic pancreatitis strengthens the importance of dysfunctional calcium regulating genes in the etiology of pancreatitis.

Application

With the inclusion of associated CASR polymorphisms in comprehensive evaluation of selected patients, we may improve the accuracy of overall pancreatitis risk prediction and may be able to provide a target for preventive approaches and possible treatment options.

Peer review

Our peer reviewers noted this brief manuscript to be well-developed and well-written. They felt that the abstract was clear and the hypothesis being tested and methodology were sound and well presented.

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