

Microsatellite scan identifies new candidate genes for susceptibility to alcoholic chronic pancreatitis in Japanese patients

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Abstract. Alcohol abuse is one of the most common risk factor for chronic pancreatitis, but the underlying pathophysiological mechanisms remain unclear. The aim of this study was to identify genes that contribute to susceptibility or resistance for alcoholic chronic pancreatitis by screening the whole genome. Sixty-five patients with alcoholic chronic pancreatitis (63 men and 2 women, mean age 55.2 years) and 99 healthy Japanese controls were enrolled in this study. This was an association study using 400 polymorphic microsatellite markers with an average spacing of 10.8 cM distributed throughout the whole genome. This search revealed 10 candidate susceptibility regions and 5 candidate resistant regions throughout the genome. No specific microsatellite markers were detected in association with previously reported susceptibility genes for chronic pancreatitis, such as *PRSSI*, *PRSS2*, *CTRC*, *SPINK1*, *CFTR*, *ALDH2*, and *CYP2E1*. Among the statistically significant markers, *D15S1007* on chromosome 15q14 showed strong evidence for disease susceptibility (70.8% vs. 35.1%, $P_c = 0.0001$). Within 500 kb of *D15S1007*, several genes were candidate genes for susceptibility, including *FMN1*, *DKFZP686C2281*, *LOC440268*, *RYR3*, and *AVEN*. This study identified 10 candidate susceptibility and 5 candidate resistant regions that may contain genes involved in ACP pathogenesis.

Keywords: Chronic pancreatitis, microsatellite, D15S1007, RYR3

1. Introduction

Chronic pancreatitis is clinically characterized by severe abdominal pain, exocrine and endocrine dysfunction, and pancreatic calcification [1]. Alcohol abuse remains one of the highest-impact risk factors associated with chronic pancreatitis [2,3]. However, alcohol abuse might not be sufficient alone pancreatitis, as there is a limited prevalence of chronic pancreatitis in alcoholics. Accordingly, the pathophysiological

mechanisms responsible for alcoholic chronic pancreatitis (ACP) remain unknown [4]. It seems likely that ACP is a multifactorial disease, with both genetic and environmental factors contributing to pathogenesis.

Several studies have reported a genetic predisposition to chronic pancreatitis. Mutations that appear to affect disease susceptibility have been identified in isoforms of trypsinogen (*PRSS1*, *PRSS2*, *CTRC*) [5–9], in the pancreatic secretory trypsin inhibitor (*SPINK1*) gene [10–13], and in the cystic fibrosis transmembrane conductance regulator (*CFTR*) gene [14,15]. Alcohol may be a cofactor in the development of alcoholic pancreatitis in susceptible humans [4], and the alcohol metabolizing enzymes *ADH2* [16], *ADH3* [17], *ALDH2* [18,19], *CYP2E1* [20], and the *HLA* (Human

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Leukocyte Antigen) may also impact the pathogenesis of the disease [21,22]. However, mutations in these genes are not solely responsible for ACP. Accordingly, it is still unclear whether these mutations directly affect the clinical course of ACP.

Genome-wide analysis using microsatellite markers could identify genetic causative or contributing factors to ACP. Microsatellites, also called short tandem repeat polymorphisms, are tandem arrays of short stretches of non-coding nucleotide sequences that are usually repeated between 15 and 30 times [23]. The advantages of using microsatellites are high heterozygosity, ubiquity throughout the genome, and easy genotyping by PCR. Association analysis using microsatellite markers is a powerful yet cost-efficient method for mapping candidate susceptibility genes in multifactorial genetic diseases [24,25]. In this study, we performed an association analysis using 400 microsatellite markers spaced an average of 10.8 cm apart to search for additional genes in the whole genome that influence the development of ACP.

2. Materials and methods

2.1. Subjects

Sixty-five patients with ACP, 63 men and 2 women with a mean age of 55.2 years, and 99 healthy Japanese controls, 50 men and 49 women, were enrolled in this study. ACP was defined as a daily intake of at least 80 g of ethanol for more than 10 years. We obtained alcohol history of patients from not only patients but near relatives, as far as possible, in order to identify this more accurately. All these patients had intermittent pain attacks characteristic for CP. They showed calcification of the pancreas by ultrasonography or computed tomography and marked irregular dilatation of the main pancreatic duct by endoscopic retrograde cholangiopancreatography (ERCP). ERCP results were assessed using the Cambridge classification [26]. Most patients also showed impaired exocrine function by secretin test or bentiromide test. Diagnosis of ACP was based on the Japan Pancreas Society Diagnostic Criteria for Chronic Pancreatitis published on May 9, 1995 [27].

All participants gave informed consent for genetic analysis, and the Ethics Committee of the Shinshu University School of Medicine approved this study.

2.2. Preparation of genomic DNA

Genomic DNA from patients and controls was isolated by phenol extraction of sodium dodecyl sulfated and proteinase K-treated cells as described previously [28].

2.3. Microsatellite typing

The genome scan was carried out using 400 microsatellite polymorphisms (ABI Linkage Mapping Set v.2.5 - MD10; Applied Biosystems, Foster City, CA) with an average heterozygosity of 79% and an average intermarker distance of 9.4 ± 2.9 cM (mean \pm SD). The entire marker set consisted of 28 panels, each containing markers pooled according to size and fluorescent tag (6-FAM, VIC, NED). The markers were amplified by polymerase chain reaction (PCR) according to the manufacturer's protocols in 10 μ l reactions containing 40 ng of genomic DNA. After PCR, the pooled panels were analyzed using an ABI 3130 DNA Analyzer. Semiautomated genotyping was performed using GeneMapper v 3.5 (Applied Biosystems).

2.4. Statistical analysis

Frequencies of alleles in each microsatellite marker were estimated by direct counting. The significance of the difference in the distribution of alleles between patients with ACP and healthy control subjects was determined by a χ^2 -test. A Fisher's exact probability test was used for fewer than six samples. *P* values were corrected by multiplication with the number of different alleles observed at each locus (*P_c*). The odds ratio (OR) was calculated using Woolf's formula, and Haldan's modification of the formula was used in sets containing zero. The Hardy-Weinberg proportion (HWP) for multiple alleles was obtained by the Markov chain method within the GENEPOP software package.

3. Results

3.1. ACP susceptibility or resistance loci

Four hundred microsatellite markers were used in a genome-wide linkage search in ACP patients with age- and sex-matched controls to identify genetic intervals that contained ACP susceptibility or resistance loci. This search revealed 10 candidate susceptibility regions throughout the genome, including *D2S125*,

Table 1
Statistically significant alleles associated with susceptibility and resistance to ACP

	Chromosome	Significant marker	Allele	CP	Control	OR	χ^2	P	Pc
				(n = 65) %	(n = 99) %				
Susceptibility	2q37.3	D2S125	87	21.5	5.3	4.58	9.6	0.0019	0.0252
	4p16.1	D4S2935	87	87.7	67	3.36	8.87	0.0029	0.0232
	6p24.1	D6S308	336	35.4	17	2.63	7	0.0082	0.0408
	10p14	D10S547	250	0	19.1	0.03	14.04	0.0002	0.002
	10q23.33	D10S185	215	80	55.3	3.15	10.35	0.0013	0.0156
	10q26.2	D10S217	100	58.5	35.1	2.6	8.48	0.0036	0.0395
	15q14	D15S1007	92	70.8	35.1	4.48	19.55	1E-05	0.0001
	15q26.2	D15S130	285	13.8	0	31.78	13.8	0.0002	0.0031
	18q22.2	D18S61	210	0.09	0	20.65	9.02	0.0027	0.0348
	18q23	D18S462	298	0.11	0	24.23	10.59	0.0011	0.0114
Resistance	1q42	D1S213	107	24.6	50	0.33	10.35	0.0013	0.0194
	2p22	D2S367	318	1.5	16	0.12	8.83	0.003	0.0445
	4q32	D4S413	284	67.7	88.3	0.29	10.15	0.0014	0.0202
	7p15	D7S516	318	40	62.8	0.4	8	0.0047	0.0327
	12q24.3	D12S1723	206	29.2	55.3	0.34	10.58	0.0011	0.0091

Abbreviations: ACP, alcoholic chronic pancreatitis; OR, odds ratio; Pc, corrected P.

D4S2935, *D6S308*, *D10S547*, *D10S185*, *D10S217*, *D15S1007*, *D15S130*, *D18S61*, and *D18S462* (Table 1). We also found 5 additional regions that contained genes that might confer resistance to ACP (Table 1). No specific markers were detected for genes previously reported to be associated with chronic pancreatitis, such as *PRSS*, *SPINK1*, *CFTR*, *ALDH2*, and *CYP2E1*.

3.2. Candidate genes associated with *D15S1007* on chromosome 15q14

Among these susceptibility and resistance markers, marker *D15S1007* on chromosome 15q14 showed strong evidence of linkage (70.8% vs. 35.1%, OR = 4.48, $P_c = 0.0001$) (Fig. 1). We used the National Center for Biotechnology Information Map Viewer National Library of Medicine, National Institute of Health (<http://www.ncbi.nlm.nih.gov/mapview/>) to search for genes associated with this marker. Several candidate genes within 500 kb of *D15S1007* were identified: *FMN1*, *DKFZP686C2281*, *LOC440268*, *RYR3*, and *AVEN* (Table 2).

4. Discussion

Genome-wide studies have been used to identify genes associated with diseases such as rheumatoid arthritis, autoimmune thyroid disease, multiple sclerosis, and systemic lupus erythematosus, as well as with common diseases such as hypertension and type 1 diabetes. Microsatellite analysis is an effective tool for

this type of study, and we recently used it to identify genes associated with autoimmune hepatitis [25]. The present study is the first to search for candidate genes associated with ACP pathogenesis throughout the whole genome, although the number of enrolled subjects was relatively small for overcoming a type I error. This analysis revealed 10 candidate ACP susceptibility regions and 5 candidate ACP resistance regions throughout the genome. These regions were not all located near the genes associated with chronic pancreatitis susceptibility, namely *PRSS* [5–8], *CTRC* [9], *SPINK1* [10–13], *CFTR* [14,15], *ALDH2* [18,19], and *CYP2E1* [20]. These results suggested that new, previously unidentified genes responsible for the pathogenesis of ACP exist with proximity to the alleles of corresponding genes.

Among these alleles, strong evidence for an association with ACP was detected in marker *D15S1007* on chromosome 15q14. In the neighboring region of this marker, we noted several interesting candidate genes that might be linked to ACP susceptibility or influence the pathogenesis of ACP: *FMN1*, *DKFZP686C2281*, *LOC440268*, *RYR3*, and *AVEN*.

Among these candidate genes, *RYR3* (ryanodine receptor 3) is supposed to be associated with ACP from a functional viewpoint. The ryanodine receptor (RYR) consists of 3 subtypes, RYR1, RYR2, and RYR3 (Table 3). RYR1 and RYR2 were first isolated from mammalian skeletal muscle and cardiac muscle cytoplasmic reticulum as a calcium release channel in the cytoplasmic reticulum and as a component of the calcium channel supplying calcium to cardiac muscle. RYR3 was found in various mammalian cell types: neurons,

Table 2
Candidate genes within 500 kb of D15S1007

Marker	Position (kbp)	Gene symbol	Description
	30853.6–31147.3	FMN1	FMN1 and Name: formin 1
	31229.2–31274.1	DKFZP686C2281	similar to Formin-1 isoforms I/II/III
	31330.0–31316.4	LOC440268	hypothetical LOC440268
	31390.4–31945.6	RYR3	ryanodine receptor 3
D15S1007	31525.1		
	31945.7–32118.6	AVEN	apoptosis, caspase activation inhibitor

Table 3
Ryanodine receptor genes

Gene symbol	Location	Mutation
RYR 1	cytoplasmic reticulum (skeletal muscle)	malignant hyperthermia central core disease
RYR 2	cytoplasmic reticulum (cardiac muscle)	minicore myopathy with external ophthalmoplegia stress-induced polymorphic ventricular tachycardia arrhythmogenic right ventricular dysplasia
RYR 3	intracellular ion channels	

Abbreviations: RYR, ryanodine receptor.

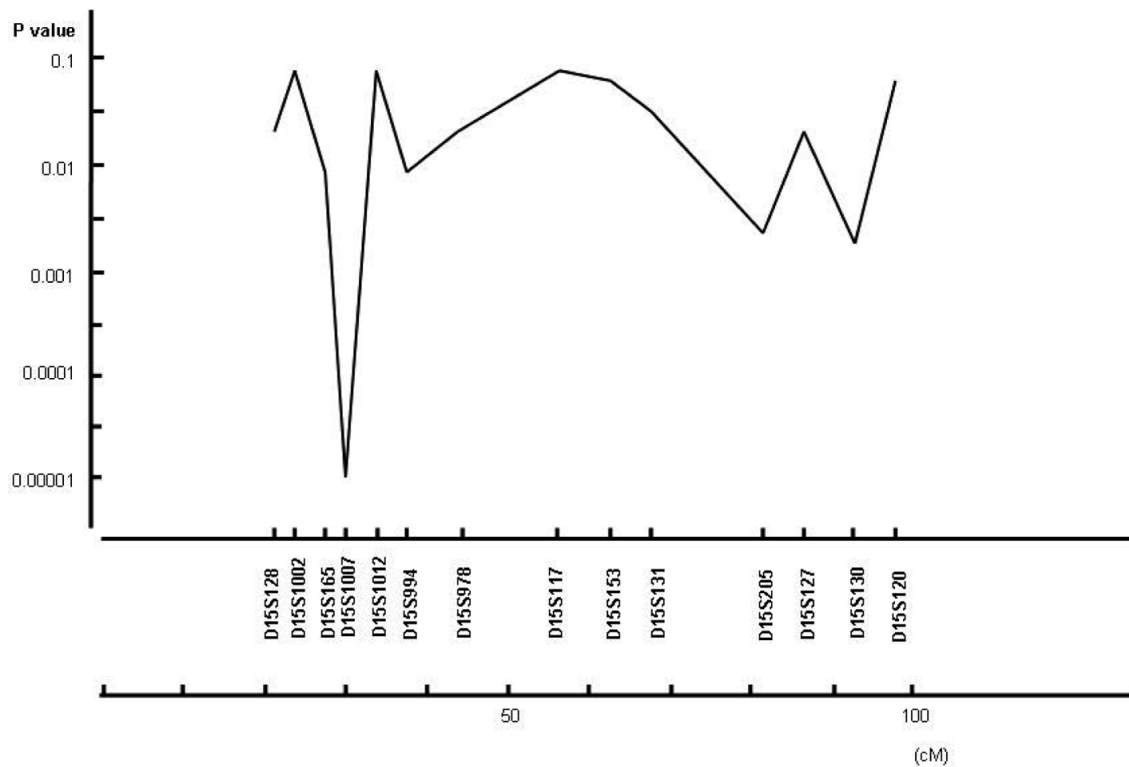


Fig. 1. ACP susceptibility gene mapping by association analysis on chromosome 15.

smooth muscle cells, skeletal muscle cells, and lymphocytes. In human pancreatic tissue, it has been uncertain whether RYR3 was expressed correctly. Recent report has demonstrated weaker and less constant expression of RYR1 or RYR3 than RYR2 in islet cells [29]. In contrast RYR1 and RYR2, functional properties of

RYR3 are less clear. To elucidate the biological roles of RYR3, some experiments have been performed using genetically engineered mutant mice lacking RYR3 [30–32]. Their analyses exhibited that the loss of RYR3 resulted in no gross abnormalities and normal phenotype. However, they pointed out biological impor-

tance of RYR3, which was basal locomotor activity, a physiological role in excitation-contraction coupling of neonatal skeletal muscles, and an enhancement of long-term potentiation induction through the RYR3-mediated Ca^{2+} release in hippocampal cell. RYR isoforms are involved in various aspects of neuronal activity and plasticity as well as in the function of non-excitable cells mediated a calcium release event [33]. Together RYR3 might be the intracellular calcium ion release channel responsible for the release of Ca^{2+} from intracellular stores following transduction of many different extracellular stimuli in pancreatic tissue. The RYR3 channel is diffusely distributed in the basolateral region of the pancreatic acinar cell, and induces Ca^{2+} release which in turn mediates early zymogen activation in pancreatic acinar cells. Ca^{2+} release by RYR3 may regulate zymogen activation under both physiological and pathological conditions [34], which induces pancreatitis in an animal model. Furthermore, bile acid can induce Ca^{2+} release through RYR3 [35]. Accordingly, alcohol abuse in conjunction with some RYR3 polymorphisms may trigger Ca^{2+} release and zymogen activation, which in turn are closely related to the pathogenesis of ACP. Analyzing the SNPs of RYR3 will provide further insights into this possibility. Further association studies by other groups will confirm whether RYR3 gene is really responsible for disease susceptibility to ACP.

In conclusion, this study identified 10 candidate ACP susceptibility and 5 candidate ACP resistance regions in the genome. Further study of these regions might help identify genes that play a role in the pathogenesis of ACP.

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