Alcoholic Pancreatitis and Polymorphisms of the Variable Length Polythymidine Tract in the Cystic Fibrosis Gene

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Background: The observation that only a minority of alcoholics develops clinical pancreatic disease has led to a search for a predisposing factor to the disease. One possible predisposing factor is mutation of the cystic fibrosis transmembrane conductance regulator (CFTR) gene as cystic fibrosis leads to pancreatic injury. We have recently demonstrated that 15 common CFTR mutations are not found in patients with alcoholic pancreatitis. Another common polymorphism of the CFTR gene has recently been implicated in the pathogenesis of idiopathic chronic pancreatitis, the 5T variant of the variable length polythymidine tract in intron 8 (the normal genotypes are 7T and 9T). The 5T variant inhibits transcription of exon 9 resulting in a CFTR protein lacking chloride channel activity. The aim of this study was to determine whether the 5T variant is associated with alcoholic pancreatitis. Methods: Fifty-two patients with alcoholic pancreatitis were identified using standardized diagnostic criteria. Fifty alcoholics without pancreatitis were also studied as controls. Genomic DNA was extracted from peripheral blood leukocytes and the polythymidine tract of intron 8 was amplified by nested polymerase chain reaction using established primers. The polymerase chain reaction products were digested with Msel, separated by electrophoresis on 15% polyacrylamide gels and genotypes assigned by comparison with known positive controls. Results: The 5T allele was found in only two patients with alcoholic pancreatitis (3.0% of the index group; 95% confidence intervals 0-10%) and in seven alcoholic controls. Allele frequencies for 5T, 7T, and 9T in patients with alcoholic pancreatitis were 1.9%, 85.6%, and 12.5%, respectively. These did not differ from the allele frequencies in alcoholic controls (7%, 79%, and 14% for 5T, 7T, and 9T, respectively). Conclusion: The 5T allele was not associated with alcoholic pancreatitis. Individual susceptibility to this disease remains unexplained.

Key Words: Alcoholic Pancreatitis, CFTR Mutations, Individual Susceptibility.

PANCREATITIS is a serious complication of alcoholism. The pathogenesis of alcoholic pancreatitis remains unknown and its treatment is largely empirical. A fundamental observation is that only a minority of alcoholics develop clinical pancreatic disease indicating that some alcoholics are more susceptible to the pancreatotoxic effects of ethanol than others. Identification of factors mediating susceptibility to alcoholic pancreatitis may help understand the pathogenesis of this disease and may assist identification of those at increased risk. The basis for this individual susceptibility has not been defined despite numerous studies of diet and drinking habits, smoking, and other factors. A genetic basis for alcoholic pancreatitis has not been established but is suggested by familial clustering of the disease, reports of human leukocyte antigens associations, and the recently reported existence of a gene predisposing to hereditary pancreatitis.

One potential predisposing genetic factor is mutation of the cystic fibrosis (CFTR, cystic fibrosis transmembrane conductance regulator) gene. Cystic fibrosis (CF) is one of the most common genetic disorders in Caucasian populations and exocrine pancreatic insufficiency is a clinical hallmark of this disease. The prevalence of heterozygous CF mutations is approximately 5% which is similar to the prevalence of pancreatitis among alcoholics. There are many similarities in the pancreatic pathology of cystic fibrosis and alcoholic pancreatitis including parenchymal atrophy, interstitial fibrosis, and small duct injury. Also, increased protein concentration in pancreatic secretion has been documented in both patients with cystic fibrosis and alcoholics. Nonalcoholic pancreatitis is associated with CFTR mutations but to date, no association between alcoholic pancreatitis and common CFTR mutations has been found.

The recently described 5T variant of the CFTR gene is part of a noncoding region of intron 8 that lies 5 bp upstream from exon 9 and comprises either 5, 7, or 9 consecutive thymidines (5T, 7T, or 9T). The presence of the 5T variant results in decreased expression of the adjacent exon 9. It is described as a variant rather than a mutation as it is associated with, but not yet proven to cause, disease.

The hypothesis for this study was that the 5T variant of CFTR is a predisposing factor for alcoholic pancreatitis. It was postulated that in the presence of alcoholism, heterozygotes for the 5T variant (who do not usually manifest
pancreatic injury) produce sufficiently abnormal pancreatic juice to result in pancreatitis. To test this hypothesis, a simplified method for analysis of the polythymidine tract was devised and used to test 52 patients with alcoholic pancreatitis and 50 alcoholics without clinically evident pancreatitis. The findings were also compared to those previously reported for the general population.\textsuperscript{17}

METHODS

Subjects

Alcoholics with Pancreatitis. A total of 52 Caucasian patients with alcoholic pancreatitis were identified from clinical records of the Prince of Wales Hospital, Sydney and the Queen Elizabeth Hospital, Adelaide. These individuals had an alcohol intake exceeding 80 g/day for at least 5 years before the onset of pancreatitis. Pancreatitis was diagnosed by the presence of at least one of the following criteria: (1) attacks of abdominal pain, tenderness, and serum lipase or amylase levels at least three times more than the upper limit of normal; (2) chronic abdominal pain with pancreatic calcification on abdominal x-ray or computed tomography; (3) abdominal pain and endoscopic pancreatography changes of moderate or severe pancreatitis according to published criteria; or (4) histopathological changes of pancreatitis in tissue obtained at operation in patients with a clinical history of abdominal pain suggestive of pancreatitis. Excluded from the study were patients with other known associations of pancreatitis (gallstones, hypercalcaemia, or hypertriglyceridaemia). None of the patients had a personal or family history of CF.

Alcoholics Without Pancreatitis. Fifty consecutive consenting patients with no clinical history of pancreatic disease were selected from an alcoholism treatment program (Langton Clinic, South Eastern Sydney Area Health Service).

Extraction and Amplification of DNA

Genomic DNA was isolated from peripheral blood leukocytes according to standard methods.\textsuperscript{19} Nested polymerase chain reactions (PCR) were carried out in a reaction mix of 25 μl containing 100 ng of genomic DNA (or 1 μl of first-round product), 1.5 mmol/liter of MgCl₂, 2 μl of nucleotide triphosphates, 1 U AmpliTaq Gold polymerase (Perkin Elmer, Branchburg, NJ), and 5 μmol/liter of each primer. The first round primers were 9⅔ and 9⅓,\textsuperscript{20} and the second round primers were 9⅔D9 and 9⅔R2.\textsuperscript{17} Both reactions commenced with incubation at 94°C for 5 min followed by 25 cycles of PCR comprising denaturation at 95°C for 30 sec, annealing at 54°C for 30 sec, and extension at 74°C for 40 sec.

The final PCR products were digested with MseI (5 units; New England Biolabs, MA) which recognizes AATT as the cut site in two positions, generating three digestion products (Fig. 1a). These were separated by electrophoresis on 15% polyacrylamide mini-gels under nondenaturing conditions at 125 V for 90 min (Fig. 1b). Gels were stained with ethidium bromide and visualized under UV light using the BioRad GelDoc imaging system (BioRad, Hercules, CA). Genotypes were assigned by comparison of the smallest of the three products with DNA size markers and bands derived from known standards (generous gift of T. Casals, Hospital Duran i Reynals, Barcelona, Spain) (Fig. 1b).

Statistical Analysis

Allele frequencies in the two study groups and those reported in the general population\textsuperscript{17} were compared using the χ² test (StatView 4.5i, Abacus Concepts Inc., CA). Odds ratios and confidence intervals were calculated as described by Morris and Gardner.\textsuperscript{21}

Ethics Approval

Ethics committee approval for this study was obtained from the South Eastern Sydney Area Health Service, Sydney, and the Queen Elizabeth Hospital, Adelaide. Written informed consent was obtained from all subjects.

RESULTS

The 5T variant was found in only two alcoholic pancreatitis subjects (3.9% of the index group; 95% confidence intervals 0 to 10.0%) compared with seven alcoholic control subjects. The odds ratio for pancreatitis among those with the 5T variant was 0.26 (95% confidence interval, 0.05–1.3), which was not statistically significantly different from 1, indicating that the 5T variant is not associated with alcoholic pancreatitis. Allele frequencies for 5T, 7T, and 9T were similar in alcoholics with and without pancreatitis and were comparable to those reported in the general population\textsuperscript{17} (Table 1).

DISCUSSION

This study was designed to test the hypothesis that the 5T variant of the CFTR gene predisposes alcoholics to pan-
creatic disease. No association between the 5T variant of the CFTR gene and alcoholic pancreatitis was found.

There has been considerable recent interest in the role of the CFTR gene mutations in pancreatic disease in the absence of overt cystic fibrosis. A number of individuals presenting with idiopathic pancreatitis with minimal or no respiratory tract disease have been reported with homozygous CFTR mutations. These cases have been described as a “forme fruste” of CF. Others have reported an association between heterozygous CFTR mutations and idiopathic pancreatitis. Three studies have examined selected CFTR mutations in alcoholic pancreatitis but no increase in the prevalence of mutations has been found.

The 5T variant may predispose toward pancreatitis in subjects who drink ethanol but at moderate levels not usually associated with pancreatitis. Indeed, previous studies included a number of these individuals. Further study of the 5T variant in subjects with unexplained pancreatitis associated with moderate ethanol consumption using appropriate controls is needed to address this question.

The In8pT tract was of interest as a predisposing factor to alcoholic pancreatitis because the 5T variant is common in the general community. Indeed, the prevalence of this variant in the general community, 5%, is strikingly similar to the reported prevalence of alcoholic disease in alcoholics. The 5T variant inhibits expression of the adjacent exon 9 by about 90% in homozygotes and 50% in heterozygotes. Exon 9 encodes part of the nucleotide binding fold 1. CFTR molecules without exon 9 do not bind ATP and consequently lack chloride channel activity. The 5T mutation is regarded as a mild mutation as even homozygotes retain 10% of CFTR transcripts with normal exon 9 and normal chloride channel activity. In general, the mild CFTR mutations are associated with pancreatic sufficiency (i.e., with “functioning” pancreatic acinar cells) whereas the severe CFTR mutations are associated with loss of acinar cell function and therefore, pancreatic insufficiency. The presence of “functioning” acinar cells that produce digestive enzymes may increase the risk of pancreatitis (due to autodigestive injury) more than “nonfunctioning” acinar cells seen with severe CFTR mutations.

The In8pT variant was initially defined by sequencing analysis but this method is unsuitable for analysis of large numbers of subjects. Two subsequent groups have progressively simplified this analysis but the methods remained cumbersome. Chillon et al.17 amplified exon 9 by PCR and used XmnI digestion to differentiate In8pT alleles, but long sequencing gels were required to distinguish the resulting large DNA fragments that differed in length by only two base pairs. Another recent approach involved amplification of the region using allele-specific PCR primers. Each sample requires separate PCR analysis for each of the three alleles plus a control gene. The three alleles and the PCR primers required to selectively amplify each of these alleles differ only by two thymidine base pairs. Thus, the PCR conditions to selectively amplify the correct allele are fastidious, so that false-positive or false-negative reactions can occur. These false-positive or false-negative reactions may be difficult to recognize, as a genotype may be assigned unless PCR is negative for all three alleles or positive for all three alleles. The presence of a control gene in each reaction only provides a partial solution to this problem as the control gene amplification is not as sensitive to minor day-to-day variations in PCR conditions as the three allele specific reactions. During the course of this study, a simplified method for the analysis of the In8pT tract was developed and this method may prove suitable for study of this region of the CFTR gene in other disease states. The method permits identification of the 5T, 7T, and 9T alleles using a single PCR reaction without fastidious techniques or radio-isotopes, thereby facilitating analysis of large numbers of samples.

It remains possible that an untested minor CFTR mutation or polymorphism predisposes an alcoholic to pancreatitis. More than 750 CFTR gene mutations have been identified. However, as the proportion of alcoholics who develop pancreatitis is 5%, only a mutation with approximately this prevalence in alcoholics could account for the susceptibility to pancreatitis. In view of the very low frequency of these minor mutations in the population, such a mutation could account, at best, for only the occasional case of “alcoholic” pancreatitis. Nevertheless, the only certain way to exclude a role for CFTR mutations in alcoholic pancreatitis would be to fully sequence the gene in a large cohort and also in an appropriate control group. This major undertaking does not appear justified on the basis of existing evidence.

In summary, this study has demonstrated that polymorphisms of the variable length polythymidine tract of CFTR are not associated with alcoholic pancreatitis. Individual susceptibility to this disease, therefore, remains unexplained.

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REFERENCES