Abstract

Background  Heavy drinkers have a high incidence of chronic pancreatitis (CP), but the mechanism of alcohol-related CP is largely unknown. Exocrine pancreatic insufficiency exists in about 90% of the patients with cystic fibrosis (CF), which results from an abnormal cystic fibrosis transmembrane conductance regulator gene (CFTR).

Aim  To investigate in Japanese alcoholics the association between bicarbonate concentration in pure pancreatic juice and one of the polymorphisms of the CFTR gene, the (TG)m Tn tract length in intron 8.

Methods  Fifty-six patients under treatment for alcohol dependence were stimulated by intravenous injection of secretin during endoscopic retrograde cholangiopancreatography to provide pancreatic juice specimens. Individual maximum bicarbonate concentrations (MBC) were compared with (TG)m Tn tract polymorphisms identified by directly sequencing lymphocyte DNA.

Results  Among the 41 patients able to provide adequate pancreatic juice specimens, 15 had low MBC and 26 had normal MBC. The frequencies of the six haplotypes identified in these patients were 17.1% (TG)11T7/(TG)11T7, 46.3% (TG)11T7/(TG)12T7, 29.3% (TG)12T7/(TG)12T7, 2.4% (TG)10T9/(TG)11T7, 2.4% (TG)12T5/(TG)11T7, and 2.4% (TG)12T6/(TG)12T7. Among the 92.7% of patients who had the common (TG)m1T7/(TG)m2T7 haplotype, all of the 7 with homozygous (TG)11 alleles had normal MBC (p<0.05).

Conclusion  Alcoholics with homozygous (TG)11 alleles in intron 8 of the CFTR gene appear to be protected against decreased MBC, compared with those who have the (TG)11/(TG)12 and (TG)12/(TG)12 genotypes, suggesting a role for CFTR gene polymorphism in the progression of alcohol-related pancreatic disease.

Key words: cystic fibrosis, cystic fibrosis transmembrane conductance regulator gene, chronic pancreatitis, bicarbonate (HCO₃⁻), pancreatic juice

Introduction

Cystic fibrosis (CF) is rare in the Japanese population, having an incidence of only 3.1 per million live births during the period 1969–1980 (1), and since 1980 about 1 in 350,000 (2). The emphasis of CF in Western countries is higher; the incidence of CF in the white population is about 1 in 2,500 (3). Few studies in Japan have investigated the relationship between CF and its clinical complications. About 90% of CF patients have exocrine pancreatic insufficiency. Pancreatic disease is thought to result from a reduced volume of pancreatic secretion with low concentrations of bicarbonate (HCO₃⁻) (4). Without sufficient fluid and bicarbonate, digestive proenzymes are retained in pancreatic ducts and prematurely activated, ultimately leading to tissue destruction and fibrosis (5).

Heavy alcohol consumption also disturbs human pancreatic function. Heavy drinkers have a high incidence of acute or chronic pancreatitis (6). Between 38% and 94% of cases
of chronic pancreatitis (CP) in Western countries and more than half of all cases of CP in Japan are related to alcohol consumption (7, 8), but the etiology of CP has not yet been fully elucidated and there is no clear evidence pertaining to the mechanisms of alcoholic CP. Many heavy drinkers have no symptoms and no problems with the pancreas, while others have severe pancreatic disease. The difference between heavy drinkers with and without pancreatic injury is an important issue.

CF is caused by mutations in a 230-kb gene on chromosome 7, encoding a 1,480-amino acid polypeptide, the cystic fibrosis transmembrane regulator (CFTR), which functions as a chloride channel in epithelial membranes. More than 1,000 mutations in this gene have been described (9). The lengths of the dinucleotide (TG)m and mononucleotide Tn repeats, both located at the intron 8/exon 9 splice acceptor site of the CFTR gene, whose mutations cause CF, have been shown to influence the skipping of exon 9 in CFTR mRNA (10).

Understanding of the genetic basis, pathogenesis, and natural history of pancreatitis has grown tremendously in the past decade (11). Mutations in cationic trypsinogen (12–14), pancreatic secretory trypsinogen inhibitor (PSTI) (15–17), and CF genes (i.e., CFTR) have recently been associated with CP (18). One genetic polymorphism related to the CFTR gene is the (TG)m polythymidine (Tn) length in intron 8. Some studies of the association between CP and polymorphism of the Tn tract in intron 8 of the CFTR gene have demonstrated that the 5T allele has a stronger association with CP than the 7T and 9T alleles (19–21), whereas others have reported no association between CP and polymorphisms of the Tn tract (22–24).

Variations in the (TG)m and Tn polymorphic repeats at the 3’ end of intron 8 of the CFTR gene are associated with the alternative splicing of exon 9, which results in a nonfunctional CFTR protein (25–28). Because the CFTR gene protein is connected to the chloride ion channel, this mutation may affect ion balance with a negative charge, leading to the change in bicarbonate (HCO₃⁻) concentration in internal organs. Studies by Choi and colleagues (29) show that among CF-associated mutants, those that exhibit pancreatic insufficiency do not support bicarbonate transport, and even those with sufficient pancreatic activity have reduced bicarbonate transport. CFTR regulates both bicarbonate secretion and bicarbonate salvage in secretory epithelia (30).

Although several investigators have examined the association between CP and polymorphisms of Tn tract length in intron 8 of the CFTR gene (19–24), no one has investigated the relationships between bicarbonate concentration in pure pancreatic juice and these polymorphisms. The present study was designed to examine these possible associations in Japanese alcoholics.

Methods

Subjects

Among male patients admitted to the National Alcoholism Center, Kurihama Hospital, Japan, from February to December 2002 for treatment of alcohol dependence, our subjects included those who were interested in their pancreatic conditions or in learning the reason for experiencing acute pancreatitis (with or without chronic pancreatitis, as previously diagnosed by their physicians). All potential subjects gave informed consent to undergo endoscopic retrograde cholangiopancreatography (ERCP) and to use their lymphocyte DNA for analysis of their genotypes. After the exclusion of patients with major medical history such as surgery or malignancy, we enrolled 56 patients, all of whom agreed to receive secretin injections during ERCP to obtain their pancreatic juices.

We obtained an adequate volume of pure pancreatic juice from 41 patients. More than half of the remaining 15 showed moderate to marked changes on pancreatography (marked, 4; moderate, 5; normal to mild, 6). We could not obtain adequate pure pancreatic juice from 6 subjects with normal to mild changes because of their abnormalities, including 1 who showed no connection between the accessory and main pancreatic duct, 2 who had curious loops in the main pancreatic duct, and 3 who showed long common channels (anomalous pancreatobiliary ductal union). None of the enrolled patients needed treatment for acute pancreatitis after ERCP examination.

Patient history

Background information was obtained from the patients themselves and from their partners, when available. All subjects were surveyed by face-to-face interviews, conducted by one of the authors, regarding the duration of habitual drinking and the types of alcoholic beverages they most frequently consumed. Daily ethanol consumption was calculated using a standard conversion for alcoholic beverages in which beer is considered to be 5% ethanol (v/v); wine, 12%; sake, 15%; shochu, 25%; gin, 37%; brandy, 40%; and whiskey, 43%.

Bicarbonate concentration in pancreatic juice

All patients were examined by ERCP 3 weeks after stopping the use of alcohol. To obtain pure pancreatic juices following intravenous injection of secretin (100 U; Eisai Co., Tokyo, Japan), we used a clean, uncontaminated tube inserted through the ampulla of Vater during the ERCP examination and limited collection to a 20-minute period to avoid patient discomfort. We analyzed each 5-minute sample for bicarbonate concentration, using detectors of GASTAT-3 (Tachno Medica, Yokohama, Japan), and identified the maximum bicarbonate (HCO₃⁻) concentration (MBC) for each patient.

CFTR gene analysis

The 3’ site of intron 8 of the CFTR gene, which includes
the (TG)m Tn tract, was amplified by polymerase chain reaction (PCR), using 100 ng of lymphocyte DNA (DNA Extractor WB Kit; Wako Chemicals, Tokyo, Japan) and 5 pmol of two primers (sense, 5'-ATGGGCCATGTGCTTTTCAAAC-3'; antisense, 5'-CTGAAGAAGAGGCTGTCATCACC-3'). The 200-base pair (bp) PCR products were purified in a microcolumn and sequenced using the Dye Terminator Cycle Sequencing Ready Reaction Kit with an ABI Prism 310 Genetic Analyzer automatic sequencer (Perkin Elmer Applied Biosystems, Wellesley, MA, USA).

Statistical analysis

All data were expressed as mean values±standard deviation (SD). The Mann-Whitney U test was used to analyze differences between groups. The association of risk between polymorphisms of (TG)m Tn in the CFTR gene and MBC in pure pancreatic juice was analyzed by the Fisher exact test. p<0.05 was regarded as statistically significant.

Results

The polymorphisms of the (TG)m Tn tract in intron 8 of the CFTR genes from our 41 subjects were sequenced into six haplotypes. Seven subjects had (TG)11T7/(TG)11T7 (17.1%); nineteen, (TG)11T7/(TG)12T7 (46.3%), twelve, (TG)12T7/(TG)12T7 (29.3%); one, (TG)10T9/(TG)11T7 (2.4%); one, (TG)12T5/(TG)11T7 (2.4%); and one, (TG)12T6/(TG)12T7 (2.4%) (Fig. 1).

The overall average MBC for the 41 subjects was 101.2±32.65 mEq/l. We divided the patients into two groups on the basis of their MBCs. A concentration over 100 mEq/l was considered normal (n=26), while a concentration of 100 mEq/l or below was considered low (n=15). The volume of pure pancreatic juice obtained in 20 minutes was greater for patients in the normal MBC group than for those in the low MBC group (32.5±7.58 ml vs. 20.3±9.05 ml, p<0.001), but the maximum amylase levels did not differ significantly between the two groups (61,000±14,900 IU/l vs., 62,000±14,500 IU/l, respectively). The profiles of the 41 patients enrolled in this study are presented by MBC group in Table 1. Although the normal MBC and low MBC groups did not differ significantly in age, duration of habitual drinking, or daily ethanol consumption, subjects in the low MBC group tended to be older and to have habitually consumed more...
ethanol per day for a longer period than the normal MBC group.

In the Tn tract, the frequencies of the T5, T6, T7, and T9 alleles were 1.2%, 1.2%, 96.3%, and 1.2%, respectively. The genotype frequencies were 2.4%, 2.4%, 92.7%, and 2.4% for T5/T7, T6/T7, T7/T7, and T7/T9. In the (TG)m tract, the TG10, TG11, and TG12 alleles were present in 1.2%, 41.5%, and 57.3% of subjects, respectively, while the
frequencies of the TG10/TG11, TG11/TG11, TG11/TG12, and TG12/TG12 genotypes were 2.4%, 17.1%, 46.3%, and 34.1%, respectively.

T7/T7 in intron 8 of the CFTR gene was the most common genotype among our subjects, occurring in all but 3 of 41 (92.7%). Within the group of patients who had the T7/T7 genotype, none who also had the (TG)11/(TG)11 genotype fell into the low MBC category, i.e., all seven had normal MBC. Fisher exact test analysis of the results for the 38 alcoholic patients with the T7/T7 genotype demonstrated protection of the bicarbonate concentration in those who also had homozygous (TG)11 alleles, compared with the results for their counterparts with the (TG)11/(TG)12 and (TG)12/(TG)12 genotypes (p<0.05). Three subjects did not possess the T7/T7 genotype; the one in the low MBC group had the (TG)10T9/(TG)11T7 haplotype, and the two in the normal MBC group who had the (TG)12T6/(TG)12T7 and (TG)12T5/(TG)12T7 haplotypes.

Computed tomography (CT) scans of these 41 patients detected calcification in the pancreas of 3 in the low MBC group (20.0%) and 4 in the normal MBC group (15.4%). Investigation of the relationship between MBC and ERCP grade revealed moderate to marked ERCP change in 5 subjects in the low MBC group (33.3%) and in 8 of those in the normal MBC group (30.8%).

We had originally divided our subjects into low or normal MBC groups on the basis of the average MBC for all 41 subjects (approximately 100 mEq/l). However, other researchers had used 125 mEq/l for pancreatic function (31), so we then divided our subjects at a threshold of 125 mEq/l, showing 12 with normal MBC and 29 with low MBC. Among the 12 patients with normal MBC, 4 (33.3%) had TG11T7/TG11T7, 6 (50.0%) had TG11T7/TG12T7, and 2 (16.7%) had TG12T7/TG12T7. Among the 29 patients with low MBC (≤125 mEq/l), 3 (10.3%) had TG11T7/TG11T7, 13 (44.8%) had TG11T7/TG12T7, 10 (34.5%) had TG12T7/TG12T7, and 1 each (3.4% each) had TG10T9/TG11T7, TG12T5/TG12T7, and TG12T6/TG12T7. In subjects with the T7/T7 genotype and normal MBC, we also found that TG11/TG11 subjects were predominant over those with TG11/TG12 and TG12/TG12 (odds ratio 3.8, 95% C.I. 0.70–21.0).

**Discussion**

Among our 41 patients, 96.3% had the T7 allele. In fact, only 3 patients did not have the T7/T7 genotype. This distribution is similar to that reported by Kimura et al (19), in which all 47 healthy Japanese had the homozygous T7/T7 genotype. The frequencies of the T5, T7, and T9 alleles were 0.043, 0.894, and 0.064, respectively, in the Japanese patients with CP in that study. The Tn tract polymorphism in intron 8 of the CFTR gene reportedly has three variants: T5, T7, and T9, of which T7 and T9 generate a predominantly normal transcript, whereas the T5 is associated with an anomaly (10, 32, 33). Investigating the variation in the Tn repeat, Andrieux et al (34) found a more incorrectly spliced transcript associated with the T5 allele (38.4% aberrant transcript among those with the T5/T7 genotype, 3.5% in those with T7/T7, and 0.6% in those with T9/T7). In contrast, only 1 (2.4%) of our 41 subjects had the T5/T7 genotype, and only 1 had the T9/T7 genotype. Evaluation of the significance of the possible role of Tn tract polymorphism in developing alcohol-related CP was difficult, because the vast majority of our subjects had the T7/T7 genotype. Moreover, our sample included a patient with the T6 allele, a finding not reported previously. These issues suggest that additional studies of larger samples are warranted.

Our results suggest that among alcoholic subjects with the T7/T7 genotype, the (TG)11/(TG)11 genotype offers protection against decreased bicarbonate concentrations in pancreatic juice, compared with the (TG)11/(TG)12 and (TG)12/(TG)12 genotypes. Cuppens and colleagues (35) reported that on a T7 background, comparison with the (TG)10 allele showed that (TG)11 and (TG)12 had 2.8-fold and 6-fold higher rates of skipping exon 9. Manson and Huxley (36) reported that among transgenic mice carrying a yeast artificial chromosome with the intact human CFTR gene, about 50% of those with (TG)10 and T7 at the splice acceptor lacked exon 9 in most tissues, whereas among those with (TG)12/T7, the rate of skipping was about 90%. In that light, our results strongly suggest that the (TG)11 allele associated with a lower rate of exon 9 skipping is more likely to be associated with the production of functional protein than is the (TG)12 allele. The presence of exon 9 might deter a decrease in the pancreatic bicarbonate concentration even after long-term excessive alcohol consumption. Recently, Naruse et al (37) suggested that a higher proportion of the (TG)12 allele may provide the genetic background for elevated sweat chloride concentration in Japanese patients, and reported that the (TG)12/T2 genotype was present in 29% of patients with alcoholic pancreatitis compared with the presence of (TG)11/11 in only 10%.

As previously suggested, it is important to use pure pancreatic juice rather than duodenal juice in the assessment of pancreatic function (31, 38). When setting the normal limit of MBC at 125 mEq/l (mean value–1.5 SD) in a pancreas function test to detect CP, Wada et al (31) found sensitivity, specificity, and overall efficiency of 86%, 100%, and 94%, respectively. They found the MBC fractions 10 minutes after the bolus intravenous injection of 100 IU of secretin. The mean value of MBC in our sample (101 mEq/l) was lower than their normal limit. Although most of the patients in this study showed little morphological change in the pancreatic duct or in symptoms of CP, long-term heavy alcohol consumption may have caused lowered pancreatic bicarbonate secretion. We observed the tendency toward low pancreatic bicarbonate concentration in alcoholics who reported greater daily alcohol consumption and longer duration of habitual drinking, and in older alcoholics, compared to their respective counterparts. Because these differences were not significant, we could not rule out the association between TG polymorphism and hereditary or idiopathic pancreatitis. The
investigation of larger samples will be needed to show true significant differences.

This study did not demonstrate a significant relationship between MBC and ERCP grade. There have been several reports about the lack of relationship between the secretin test and ERCP grade. Lankisch et al (39) suggested that the use of ERCP may result in overdiagnosis of the disease, as much as duct changes that may only reflect scars after severe acute pancreatitis or old age do not necessarily constitute a sign of chronic pancreatitis. They reported that the secretin-pancreozymin test can be used to diagnose chronic pancreatitis with more reliability. They also reported that pancreatic calcification does not indicate severe exocrine pancreatic insufficiency or the necessity of pancreatic enzyme substitution (40).

Several other studies have shown that in CP the morphological changes of the main pancreatic duct disturb exocrine pancreatic function, reducing the secretion of pancreatic juice (41–43). It is difficult to collect adequate specimens of pancreatic juice for evaluation in patients with advanced CP. More than half of the 15 patients in this study who could not provide adequate pancreatic juice after secretin injection had moderate to marked changes on ERCP evaluation.

CFTR polymorphism has been reported to have a relationship with several other diseases, such as bronchiectasis (34), oligozoospermia (44), and rhinosinusitis (45), as well as chronic pancreatitis (46). Further investigation is needed to clarify the association between human diseases and CFTR gene polymorphisms, and to provide much more information on the Japanese population.

In conclusion, this study revealed some of the genetic background of alcohol-related pancreatic diseases, including an association between bicarbonate concentration in pure pancreatic juice and polymorphism of the (TG)m Tn tract in the 3’ lesion of intron 8 of the CFTR gene. People with the (TG)11/(TG)11 genotype appear to be protected against decrease in pancreatic bicarbonate concentration, even after long-term alcohol consumption, compared with those who have (TG)11/(TG)12 or (TG)12/(TG)12. Because our sample included too few patients lacking the T7 allele, we could not fully examine the relationship between Tn polymorphisms and pancreatic bicarbonate concentration; it remains an issue to be addressed in future studies.

References

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