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• *BRIEF REPORTS* •

CD14 promoter polymorphism in Chinese alcoholic patients with cirrhosis of liver and acute pancreatitis

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Abstract

AIM: To investigate the relationship between genetic polymorphism of the CD14 promoter and the occurrence of alcoholic cirrhosis and alcoholic pancreatitis, and to challenge the conclusion made earlier that the patients with acute alcoholic pancreatitis and patients with alcoholic cirrhosis of liver are two different subpopulations.

METHODS: Using the polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method, we determined the polymorphism of CD14 gene and aldehyde dehydrogenase gene 2 (ALDH 2) in 335 alcoholic patients with different organ complications i.e., cirrhosis of liver (n = 100), acute pancreatitis (n = 100), esophageal cancer (n = 82) and avascular necrosis of hip joint (AVN) (n = 53) and 194 non-alcoholic controls in a Chinese group.

RESULTS: The results showed that the carriage of T allele was not different among alcoholic patients with cirrhosis of liver, alcoholic patients with other complication and non-alcoholic controls. On the other hand, the carriage of the C allele was significantly more prevalent for alcoholic pancreatitis than for esophageal cancer (0.79 *vs* 0.60, P<0.001), alcoholic AVN (0.79 *vs* 0.65, P<0.025) and non-alcoholic controls (0.79 *vs* 0.68, P<0.025). Furthermore, when only subjects with ALDH2 1-1 genotype were examined, the C allele frequency was significantly more prevalent for alcoholic pancreatitis than for alcoholic liver cirrhosis (0.82 *vs* 0.69, P<0.025), esophageal cancer (0.82 *vs* 0.61, P<0.01), alcoholic AVN (0.82 *vs* 0.64, P<0.01) and non-alcoholic controls (0.82 *vs* 0.69, P<0.05).

CONCLUSION: The C allele may be associated with some mechanism, which is important in the pathogenesis of alcoholic pancreatitis, and that alcoholic patients with acute pancreatitis and cirrhosis of liver are probably two different subpopulations.

Key words: CD14 promoter; Polymorphism; Alcoholic patients

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INTRODUCTION

It is well known that alcoholism causes numerous physical complications[1]. However, it is still a mystery why certain organ-specific complications occur only in some alcoholics. Twin concordance studies suggest a contribution by genetic factors. In a large study, consisting of 16 000 male twin pairs, the concordance rate of alcoholic cirrhosis was significantly greater among monozygous (14.6%) than dizygous (5.4%) twins[2]. Later, Reed *et al*., also confirmed the findings in 1996[3]. Based on the above findings, many studies were designed to search for candidate genes that contribute to the susceptibility to alcoholic liver disease (ALD)[4-8]. As we know, the alcohol metabolizing enzymes alcohol dehydrogenase (ADH), aldehyde dehydrogenase (ALDH) and cytochrome P4502E1 are polymorphic at the ADH2, ADH3, ALDH2 loci and the 5'-fling region of the P4502E1[9,10]. Many studies have investigated the differences in these alcohol-metabolizing enzymes to explain susceptibility to alcoholism and to alcohol-induced liver disease[9-12]. The studies have been inconclusive in the linkage of the susceptibility to ALD and polymorphism of alcohol-metabolizing enzymes[13-16].

More recently, accumulating clinical and experimental data indicate that inflammatory responses are involved in the pathogenesis of ALD. Elevated serum endotoxin levels are found in alcoholic patients and in alcohol-treated experimental animals[17,18], as a consequence of intestinal wall leakage, bacterial overgrowth, or reduced phagocytic clearance[19]. Circulating endotoxin is bound mainly to the lipopolysacchride binding protein. This complex has a high affinity for the CD14 receptor, which is expressed on monocytes and macrophages[20]. Binding of the lipopolysaccharide–lipopolysaccharide binding protein complex to CD14 initiates transcription and release of several proinflammatory cytokines, such as tumor necrosis factor-α and interleukin-1β[21].

A polymorphic C/T form at position-159 in the promoter region of the human CD14 gene was recently detected. The polymorphism is within the Spl transcription factor binding site, known to affect CD14 expression. The T variants of the -159 polymorphism promote CD14 gene transcription and cause higher expression of CD14 on monocytes, which seems to lead to an enhanced inflammatory response[22,23]. The T allele has a suggested association with myocardial infarction, which also is known to have an inflammatory component[23,24]. Later, Jarvelainen *et al*., investigated the allele frequency of the CD14 promoter genotype in a Finnish group and found that the T allele was associated with advanced ALD. The T allele confers increased risk of alcoholic liver damage. The TT homozygotes are at a high risk to develop cirrhosis[25]. On the other hand, Koppelman *et al*., reported the importance of the C allele influencing the expression and severity of the atopic phenotype in a Dutch group[26]. The CD14 genotype does not appear to represent a susceptibility gene for the development of atopy, yet it appears to produce a more severe atopic phenotype. Considering the ethnic variations, we conducted a study in a Chinese group to investigate the relationship between genetic polymorphism of the CD14 promoter and the occurrence of alcoholic cirrhosis and alcoholic pancreatitis, two common necro-inflammatory complications in alcoholic patients. Because the preliminary data revealed that the C allele was higher in alcoholic pancreatitis patients, we also included patients with non-alcoholic pancreatitis for comparison. By investigating the polymorphism of CD 14 gene, we also want to challenge the conclusion we made before that the Chinese alcoholic patients with cirrhosis of liver and acute pancreatitis are two different subpopulation[27].

MATERIALS AND METHODS

Blood samples were obtained from 335 alcoholic and 194 non-alcoholic patients at the Tri-Service General Hospital in Taipei, from September 2002 to March 2004. Of the 335 alcoholic patients, 100 were diagnosed with cirrhosis of liver, 100 with acute pancreatitis, 82 with esophageal cancer, and 53 with avascular necrosis of the hip joint (AVN), with all instances of the respective diseases deemed to be alcohol-induced. Of the 194 non-alcoholic controls, 77 were diagnosed with gallstone pancreatitis and 117 were non-alcoholic controls that had been admitted to hospital during the same observation period (clinical diagnoses for the controls included peptic ulcer, inguinal hernia, acute appendicitis, bone fracture and acute gastroenteritis). All of the alcoholic patients had consumed in excess of 60 g alcohol per day, on average, for at least 6 years. None of the patients in the non-alcoholic subgroup had a history of alcoholism and consumption of alcoholic beverages was infrequent for these individuals. The 100 patients in the alcoholic cirrhosis subgroup were all negative for serum antinuclear and anti-mitochondrial antibodies, and negative for antibodies to the hepatitis C virus and its RNA (determined using a second-generation test kit, Abbott Laboratories, Chicago, IL, USA) and PCR, respectively. The hepatitis B surface antigen and HBV DNA were negative for all 100 of these patients. All the cirrhotic patients presented with typical sonographic signs suggestive of cirrhosis, and all had endoscopically proven esophageal varices. The distributions of these cirrhotic patients according to Child-Pugh score (A/B/C) were 4/70/26. Liver biopsy was not performed for most cases because of decompensated hepatic function or massive ascites. None of the patients in the alcoholic cirrhosis subgroup had a history of acute pancreatitis. The acute pancreatitis patients presented with typical symptoms and signs, with elevations of serum amylase and lipase at least three-fold normal levels. Abdominal sonography, computerized tomography (CT), and endoscopic retrograde cholangiopancreatography were used for patient evaluation. For the alcoholic pancreatitis subgroup (n = 100), risk factors for the pancreatitis, other than alcoholism, were carefully assessed before being ruled out. Of these 100 patients, 40 had experienced two or more episodes, with mild but persistent elevation for serum transaminases noted among 12 during a 6-mo follow-up period after discharge, indicating the possibility of a coexisting alcohol-induced liver disease. The serum albumin, total bilirubin, prothrombin time and peripheral platelet count for these 12 acute alcoholic pancreatitis patients were within normal limits. No evidence of esophageal varices or congestive gastropathy was noted for any of the 100 patients in this subgroup. All the patients in the alcoholic AVN subgroup had undergone total hip replacement, with diagnosis proven from pathology; for 11 of these, hip-joint involvement was bilateral. None of the 53 patients in alcoholic AVN subgroup had a history of acute pancreatitis. Eleven patients were diagnosed with alcoholic liver disease because of persistently abnormal transaminase levels; however, there was no clinical evidence of cirrhosis. The pathology diagnosis of the patients in esophageal cancer subgroup revealed that 72 of the 82 were squamous cell carcinoma and the other 10 were adenocarcinoma. The 82 patients showed no clinical evidence of liver cirrhosis on abdominal sonography, gastroscopy, and biochemical studies. However, seven of them had mildly elevated serum transaminase levels. None of them had any history of acute pancreatitis. Alcohol consumption histories were obtained using a standard questionnaire. The physician on duty ensured that the informed consent was obtained and the questionnaire was reliably completed by interviewing both the patient and a member of the family, usually the spouse or mother.

DNA isolation and CD14 genotyping

DNA was extracted from WBC pellets, obtained after lysis of the red cells with ammonium bicarbonate. The genotyping of a polymorphism in the promoter region of the CD14 receptor characterized by a C to T transition at -159 was accomplished by restriction fragment length polymorphism analyses. The genomic DNA was amplified using the following primer pair: forward, 5' GTGCCAACAGATGA-GGTTCAC 3'. And reverse, 5' GCCTCTGACAGTTTAT-GTAATC 3'. The PCR was carried out in a final volume of 50 μL, containing 1 mmol/L $MgCl_2$, 0.2 mmol/L of each deoxynucleotide triphosphate, 0.2 μmol/L of each primer, and 1 U Taq DNA polymerase. After an initial denaturation of 5 min at 94 ¡æ, the samples were subjected to 35 cycles at 94 ¡æfor 30 s, 60 ¡æfor 30 s, and 72 ¡æfor

30 s, with a final extension of 7 min at 72 ¡æ The 497-bp product was restricted with Eca 471 (Ava II; Fermentas, Amherst, NY) overnight at 37 ¡æ The unrestricted 497-bp product represents the C allele, where a T allele was cut into 144- and 353-bp fragments. The three genotypes were scored after running on a 2.5 % agarose gel. A T/T homozygote was included in PCR as a positive control of digestion.

ALDH2 genotyping

ALDH2 genotyping was determined by our previously published method, using PCR-directed mutagenesis[14].

Statistical analysis

χ^2 analysis was performed to compare the allele frequencies between groups.

RESULTS

The patient's age, sex, and alcoholism history are listed in Table 1. The CD14 genotype and allele frequencies for alcoholic and non-alcoholic patients are presented in Table 2. Carriage of C allele was significantly more prevalent in alcoholic pancreatitis than in esophageal carcinoma ($P<0.001$), alcoholic AVN ($P<0.025$), non-alcoholic pancreatitis ($P<0.001$) and non-alcoholic controls ($P<0.025$). When the subjects with genotype CC compared with genotype TT, the CC genotype was more prevalent in alcoholic pancreatitis than in esophageal cancer ($P<0.005$), non-alcoholic pancreatitis ($P<0.005$), and non-alcoholic controls ($P<0.025$).

We also compared subjects with genotype CC *vs* CT and TT (T- *vs* T+). The CC genotype was more prevalent in alcoholic pancreatitis than in alcoholic esophageal cancer ($P<0.001$), alcoholic AVN ($P = 0.005$), non-alcoholic pancreatitis ($P<0.001$) and non-alcoholic controls ($P<0.002$). The CC genotype was more prevalent in alcoholic liver cirrhosis than in alcoholic esophageal cancer ($P<0.05$) (Table 2).

For further clarification of the role of C allele in the development of different alcohol-related complications, we checked the allele frequency of CD14 only in subjects with the ALDH2 1-1 genotype (Table 3). The C allele frequency was significantly more prevalent in alcoholic pancreatitis than in alcoholic liver cirrhosis ($P<0.025$), esophageal cancer ($P<0.01$), alcoholic AVN ($P<0.01$), non-alcoholic pancreatitis ($P<0.001$) and non-alcoholic controls ($P<0.05$). In a subgroup of subjects with genotype ALDH2 1-1, the genotype CC *vs*

Table 1 Age, sex, and alcohol consumption in the different groups

Groups (*n*)	Alcohol consumption			
	Age (yr)	Sex (M/F)	Daily (g)	Duration (yr)
Alcoholic cirrhosis (100)	50.5±12.9	97/3	189±119	25.8±11.6
Alcoholic pancreatitis (100)	40.2±11.7	95/5	143±93	15.8±8.8
Alcoholic esophageal Ca (82)	64.4±12.7	82/0	200±175	35.8±13.8
Alcoholic AVN (53)	44.7±9.7	53/0	197±139	19.6±8.2
Non-alcoholic pancreatitis (77)	53.2±17.5	38/39	-	-
Non-alcoholic controls (117)	60.6±18.3	75/32	-	-

Table 2 CD14 polymorphism

Groups (*n*)	CC	CT	TT	*C	*T
Alcoholic pancreatitis (100)	59[c,d,f,h,2,3]	39	2	0.79[a,b]	0.21
Alcoholic cirrhosis (100)	46[e]	49	5	0.71[1]	0.29
Alcoholic esophageal Ca. (82)	25	49	8	0.60	0.40
Alcoholic AVN (53)	18	33	2	0.65	0.35
Non-alcoholic pancreatitis (77)	25	43	9	0.60	0.40
Non-alcoholic controls (117)	49	62	6	0.68	0.32

C allele frequency, [a]$P<0.025$ *vs* alcoholic AVN and non-alcoholic controls; [b]$P<0.001$ *vs* esophageal carcinoma and non-alcoholic pancreatitis; [1]$P = 0.055$ *vs* esophageal carcinoma. Genotype CC *vs* TT, [c]$P<0.005$ *vs* esophageal cancer and non-alcoholic pancreatitis; [d]$P<0.001$ *vs* alcoholic AVN. Genotype CC *vs* CT and TT (T- *vs* T+), [f]$P<0.001$ *vs* alcoholic esophageal cancer; [h]$P<0.001$ *vs* non-alcoholic pancreatitis; [2]$P = 0.005$ *vs* alcoholic AVN; [3]$P = 0.017$ *vs* non-alcoholic controls. Genotype CC *vs* CT and TT (T- *vs* T+), [e]$P<0.05$ *vs* alcoholic esophageal cancer.

Table 3 CD14 polymorphism in ALDH2 1-1 homozygotes

Group (*n*)	CC	CT	TT	*C	*T
Alcoholic pancreatitis (65)	41[e,g,i,k]	24	0	0.82[a,b,c,d]	0.18
Alcoholic cirrhosis (77)	33	40	4	0.69	0.31
Alcoholic esophageal Ca (23)	8	12	3	0.61	0.39
Alcoholic AVN (42)	14	26	2	0.64	0.36
Non-alcoholic pancreatitis (40)	13	21	6	0.59	0.41
Non-alcoholic controls (54)	22	30	2	0.69	0.31

C allele frequency, [a]$P<0.025$ *vs* alcoholic cirrhosis; [b]$P<0.001$ *vs* non-alcoholic pancreatitis; [c]$P<0.05$ *vs* non-alcoholic controls; [d]$P<0.01$ *vs* esophageal cancer and alcoholic AVN. Genotype CC *vs* CT and TT, [e]$P<0.05$ *vs* alcoholic cirrhosis and alcoholic esophageal cancer; [g]$P<0.025$ *vs* alcoholic AVN; [i]$P<0.005$ *vs* non-alcoholic pancreatitis; [k]$P<0.05$ *vs* non-alcoholic controls.

Table 4 CD14 polymorphism in alcoholic patients with and without pancreatitis

Group (*n*)	CC	CT	TT	*C	*T
Alcoholics without pancreatitis (235)	89[b,c]	131	15	0.66[a]	0.34
Alcoholics with pancreatitis (100)	59	39	2	0.79	0.21
Alcoholics with genotype ALDH2 1-1					
Alcoholics without pancreatitis (142)	59[g,i]	78	9	0.66[e]	0.34
Alcoholics with pancreatitis (65)	41	24	0	0.82	0.18

C allele frequency, [a]$P<0.005$ *vs* alcoholics with pancreatitis. Genotype CC *vs* TT, [b]$P<0.001$ *vs* alcoholics with pancreatitis. [c]$P<0.05$ *vs* alcoholics with pancreatitis. Genotype CC *vs* CT and TT (T- *vs* T+), C allele frequency, [e]$P<0.005$ *vs* alcoholics with pancreatitis. Genotype CC *vs* TT, [g]$P<0.05$ *vs* alcoholics with pancreatitis. Genotype CC *vs* CT and TT (T- *vs* T+), [i]$P<0.005$ *vs* alcoholics with pancreatitis.

CT and TT (T- *vs* T+) was also more prevalent for the alcoholic pancreatitis than for the alcoholic liver cirrhosis ($P<0.05$), alcoholic esophageal cancer ($P<0.05$), alcoholic AVN ($P<0.01$), non-alcoholic pancreatitis ($P<0.005$) and non-alcoholic controls ($P<0.05$). We did not check CC *vs* TT, and TT *vs* CC and CT in subjects with ALDH2 1-1 genotype because there were no TT homozygous patients in alcoholic pancreatitis group. We also compared alcoholic patients with and without pancreatitis in Table 4. The frequency of C allele was more prevalent for the alcoholic patients with pancreatitis than for the alcoholic patients without pancreatitis ($P<0.005$). Genotype CC was more prevalent for alcoholic patients with pancreatitis than for patients without pancreatitis ($P<0.05$). The genotype CC *vs* CT and TT (T- *vs* T+) was also more prevalent in alcoholic patients with pancreatitis than patients without pancreatitis ($P<0.001$). In subjects with genotype ALDH2 1-1, the C allele frequency was significantly higher in alcoholic patients with pancreatitis than without pancreatitis ($P<0.005$). The CC genotype was more prevalent for alcoholic patients with pancreatitis than patients without pancreatitis ($P<0.05$). And, the genotype CC *vs* CT and TT (T- *vs* T+) was more prevalent in alcoholic patients with pancreatitis than patients without pancreatitis ($P<0.005$).

DISCUSSION

The genotype distribution of the CD14 promoter polymorphism was unique in that the TT genotype was less prevalent in the Chinese than in the Finnish group (4.8% in Chinese alcoholics *vs* 19.7% in Finnish alcoholics and 7.7% in Chinese non-alcoholics *vs* 16.4% in Finnish non-alcoholics)[25]. In this study, we investigated the genotype distribution of CD14 in alcoholic patients with necro-inflammatory complications, pancreatitis and liver cirrhosis and other complications i.e., esophageal cancer and avascular necrosis of hip joint. We did not confirm the results in Chinese alcoholic patients that the T allele confers increased risk of alcoholic liver damage, reported by Jarvelainen *et al*., in the Finnish group[25]. This indicates that this polymorphism is probably not, at least in the Chinese population, an important factor determining the severity of liver disease in alcoholic patients. But we found that the C allele frequency was significantly higher in alcoholic patients with pancreatitis than alcoholic patients with other complications.

The significant role of the T allele in CD14 promoter region was suggested by two findings: (1) it has been found to confer increased CD14 expression on monocytes[22,23] and (2) an increased risk for myocardial infarction has been observed among individuals who are CD14-promoter TT homozygotes[23,24]. The T allele seems to favor the inflammatory process. On the other hand, Ito *et al*., reported that no association between CD14 genotypes and serum CD14 levels was observed in the Japanese population[28]. Risley *et al*., suggested no significant differences in serum CD14 concentration between Caucasian healthy carriers and noncarriers of the T allele[29]. Furthermore, Fernandez-Real *et al*., also found that there was no difference in CD14 levels among healthy Spanish people with different CD14 genotypes[30]. However, the T/T homozygotes have higher levels of circulating CD14 in a subset of diabetic patients[27]. The CD14 genotype was associated with insulin sensitivity in both Caucasian healthy subjects and type 2 diabetic patients[29]. Taking these together, whether CD14 polymorphism was associated with circulating CD14 levels remains controversial. More functional studies are needed to study the effect of CD14/-159 on promoter activity.

Previous publications discuss the relationship between diseases and the CD14 genotypes and have revealed conflicting results. Hubacek *et al*., found the T allele and TT genotype elevated in Czech patients with myocardial infarction ($n = 178$) if compared with the control subjects, suggesting the -159 C/T nucleotide polymorphism to be a genetic risk factor[23]. In a large study which included 1 793 patients, conducted in Germany by Koch *et al*., data indicated that the -159 C/T polymorphism is not related to coronary artery disease or myocardial infarction[31]. In a Dutch population, Koppelman *et al*., found homozygotes for the C allele had a higher number of positive skin tests and higher total serum IgE levels and subsequently, more allergic symptoms including rhinitis and hay fever, compared with subjects with CT and TT alleles[26]. In our study, we found that the C allele frequency was significantly higher in alcoholic patients with pancreatitis than alcoholic patients with other complications. This difference persisted between alcoholic patients with pancreatitis and alcoholic patients with other complications when compared with CC to TT or T- to T+. This suggested that the C allele may be associated with some mechanism which is important and specific in the pathogenesis of alcoholic pancreatitis.

Previously, we reported that most Chinese alcoholic patients with liver cirrhosis never had an episode of acute alcohol-induced pancreatitis despite the fact that they are older and their daily alcohol consumption is larger than that of pancreatitis patients. Further analysis revealed that the ADH2*1 allele frequency is significantly different between Chinese alcoholic patients with cirrhosis and pancreatitis.

We also suggest that alcoholic patients with these two different complications are two different subpopulations[27]. In this study, we also want to investigate whether the CD14 allele frequency is different between alcoholic patients with liver cirrhosis and alcoholic pancreatitis. Initially when comparing the C allele frequency of CD14 in alcoholic patients with pancreatitis and liver cirrhosis, no significant difference was found in these two groups. As the alcohol-specific complications are considered to be influenced by multiple genes and also because the ALDH2 gene is one of the most important alcohol-metabolizing genes, we evaluated the relationship between CD14 genotypes and different complications only in alcoholic patients with genotype ALDH 2 1-1, the most common genotype in the Chinese alcoholic patients. The result showed that the C allele frequency is significantly different between alcoholic patients with cirrhosis of the liver and alcohol-induced pancreatitis. This further indicated that the C allele is associated with the development of alcoholic pancreatitis and alcoholic patients with cirrhosis and pancreatitis are of two different subpopulations.

Unlike alcoholic pancreatitis patients, those with gallstone pancreatitis share the similar frequency of C allele with general population. This indicates that the increased C allele frequency is specific for alcoholic, but not all types of pancreatitis.

The pathogenesis of alcoholic pancreatitis is not clear. There are several plausible explanations for a possible role of CD14 in alcoholic pancreatitis. CD14 is a multifunctional receptor and may play a role in different biological and pathophysiological processes such as: apoptosis, sepsis and inflammatory disease[32-35]. CD14 on monocytes and polymorphonuclear cells functions as a receptor for lipopolysaccharides, thereby inducing mediator and cytokine release. Thus, CD14 may be involved in a proinflammatory pathway through the release of cytokines.

The reason why alcoholic patients develop different organ complications is still unknown. It seems highly probable that the development of specific complications in alcoholic patients is determined by multiple genes, with most of these still not well understood.

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