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GENETICS AND CELL BIOLOGY

Interleukin-10 Gene Polymorphism is Associated with Alcoholism but not with Alcoholic Liver Disease

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Abstract — Aims: To determine whether the functional polymorphism −592C>A of the interleukin (IL)-10 gene (*IL10*) influences the development of alcoholic liver disease or alcoholism in alcoholic Spanish subjects. **Methods:** The −592C>A *IL10* polymorphism was analyzed by the polymerase chain reaction and digestion with restriction enzymes in 257 male alcoholics [161 without alcoholic liver disease and 96 with alcoholic liver cirrhosis (ALC)] and 100 male healthy controls. **Results:** We found no association between the −592C>A *IL10* polymorphism and ALC. Meta-analysis combining this result and data from previous studies failed also to show any significant association between this polymorphism and alcoholic liver disease. However, the frequency of allele A carriers (CA and AA genotypes) was significantly higher in alcoholic patients (defined as patients with abuse or dependence of alcohol) than in healthy controls. **Conclusion:** The −592C>A *IL10* polymorphism is not related to the risk of ALC. Nevertheless, our study shows that alcoholism is associated with an excess of allele A carriers in alcoholic patients.

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INTRODUCTION

At present, there is strong evidence that genetic factors could play an important role in the susceptibility to alcoholic liver cirrhosis (ALC) (Reed *et al*., 1996; Stickel and Osterreicher, 2006). An increased production of inflammatory cytokines, such as interleukin-1 (IL-1) and tumor necrosis factor-α (TNF-α), has been involved in the pathogenesis of liver injury in chronic alcoholism (McClain *et al*., 2004; Crews *et al*., 2006; Laso *et al*., 2007b, 2007a). Some authors, based on results from animal models, have also suggested that elevated circulating levels of TNF-α may lead to progressive neuroinflammation and degeneration of brain, which could be involved in the development of alcohol dependence (Lin *et al*., 1998; Crews *et al*., 2006; Qin *et al*., 2007). In line with these findings, certain polymorphisms in genes encoding IL-1 and TNF-α have been associated either with alcoholic liver disease or with alcoholism (Takamatsu *et al*., 2000; Pastor *et al*., 2005a, 2005b).

Decreased levels of IL-10, a cytokine that inhibits the secretion of TNF-α (Wang *et al*., 1994), have been shown in patients with ALC (McClain and Cohen, 1989; Le Moine *et al*., 1995; McClain *et al*., 2004; Crews *et al*., 2006). Moreover, IL-10 knockout mice are more sensitive to develop alcoholic liver damage (Hill *et al*., 2002; Zhong *et al*., 2006).

In recent years, the relationship between alcoholic liver disease and polymorphisms at positions −1082 (G>A, rs1800896) (Bathgate *et al*., 2000; Grove *et al*., 2000; Ladero *et al*., 2002; Richardet *et al*., 2006), −819 (C>T, rs1800871) (Ladero *et al*., 2002) and −592 (C>A, rs1800896, also called by others −597 or −627) (Grove *et al*., 2000; Ladero *et al*., 2002; Martins *et al*., 2005; Richardet *et al*., 2006) in the promoter region of the IL-10 gene (*IL10*) has been analyzed, although a clear role for these genetic variants is not established. Two common haplotypes, called ATA and GCC (Lim *et al*., 1998; Edwards-Smith *et al*., 1999), are defined by these three polymorphisms. Secretion of IL-10 in ATA haplotype is lower than that in GCC haplotype (Lim *et al*., 1998; Edwards-Smith *et al*., 1999; Hoffmann *et al*., 2001; Zhang *et al*., 2007). Due to the strong linkage disequilibrium showed by these polymorphisms, the possession of the −592A allele could fully determine the presence of the common ATA haplotype (Lin *et al*., 2003).

In the present study, we analyze if there is a relationship between the −592C>A polymorphism of *IL10* and either ALC or alcoholism in chronic alcoholic Spanish men. Additionally, we perform a meta-analysis of both our data and data from previous studies in which this polymorphism has been analyzed in patients with alcoholic liver disease.

PATIENT AND METHODS

Subjects

We studied 257 male alcoholic patients referred to the Alcoholism Unit of the University Hospital of Salamanca (Salamanca, Spain). The patients were aged between 24 and 80 years (52.15 ± 12.56 years, mean ± SE) and had consumed >120 g of ethanol daily for at least 10 years. Of them, 161 were alcoholics without alcoholic liver disease (48.45 ± 11.42 years, AWLD group), whereas 96 patients were diagnosed as having ALC (58.26 ± 12.00 years, ALC group). All patients met criteria for alcohol abuse or dependence according to the DSM-IV criteria and they were diagnosed by a psychiatrist specialized in treatment of alcoholism. Ninety-seven patients had alcohol abuse (41 with ALC and 56 in the AWLD group) and 160 patients had alcohol dependence (55 and 105, respectively) (American Psychiatric Association, 2000).

Histopathological examination of the liver of 87 cirrhotic patients in which liver biopsy was performed revealed micronodular cirrhosis. This test was not performed in the remaining nine patients due to coagulation abnormalities. In these cases, the diagnosis of liver cirrhosis was established on the basis of the presence of the physical stigmata of chronic liver disease (i.e. cutaneous signs, hepatosplenomegaly, gynecomastia,

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testicular atrophy), a history of ascites, variceal bleeding or hepatic encephalopathy, as well as on gastroscopic and/or ultrasonographic findings (Erlinger and Benhamou, 1999).

AWLD patients had no clinical evidence of liver disease, normal liver blood tests [with the exception of an isolated rise in gamma-glutamyltransferase as an alcohol intake marker (Wu *et al*., 1976)] and normal liver ultrasonography. Alcoholic patients presenting abnormalities in liver blood tests (bilirrubin, transaminsases or alkaline phosphatase), in which liver biopsy was not clinically indicated, were excluded from this study. In this group of patients performing a liver biopsy was not considered ethically justifiable, and therefore it was impossible to classify these individuals as suffering from ALC or not having liver damage.

Hepatitis B surface antigen and antibodies to hepatitis C virus were negative in all the alcoholic patients; other causes of chronic liver disease, such as hemochromatosis or autoimmune liver disease, were excluded using standard tests.

One hundred sex-matched healthy volunteers were included in the study (41.04 ± 12.56 years). All of them consumed <10 g of ethanol per day and none had a history of alcohol abuse or alcohol dependence and neither did their first or second degree relatives.

All patients and control subjects were from Castilla and León (northwestern Spain) as were their parents and grandparents and were all from Caucasian origin. All subjects gave informed consent to participate, and the study was approved by the Ethics Committee of the University Hospital of Salamanca.

Genetic analyses

Genomic DNA was extracted from nucleated peripheral blood cells using standard proteinase K digestion, phenol–chloroform extraction and ethanol precipitation and was stored at −20°C.

IL10 polymorphism was detected using the following primers: 5′-CCTAGGTCACAGTGACGTGG-3′ and 5′-GGTGAGCACTACCTGACTAGC-3′. DNA samples were amplified in a 50 μl reaction volume that contained 45 μl of polymerase chain reaction (PCR) Supermix (Life Promega Technologies, Eggenstein, Germany), 4 μl of the above-described primers at 0.1 mg/ml and 500 ng of the DNA sample. PCR conditions consisted of a denaturation step at 94°C for 5 min and then 35 cycles of denaturation at 94°C for 30 s, annealing at 52°C for 30 s and extension at 72°C for 30 s, with a final extension step at 72°C for 10 min. The PCR amplification yielded a fragment of 412 bp electrophoresed on 2.5% agarose gels with ethidium bromide staining. After its digestion overnight at 37°C with *AfaI*, the fragments obtained (of 412, 236 and 176 bp) were separated on 2.5% agarose gel electrophoresis, stained with ethidium bromide and visualized with ultraviolet light. If the nucleotide −592 was C, the PCR fragment remained uncut (412 bp), but if the nucleotide −592 was A, we obtained two PCR fragments (236 and 176 bp).

Statistical analysis

The Student's t and χ^2 tests were used for the comparison of quantitative and qualitative variables, respectively. The deviation of genotype frequencies from the Hardy–Weinberg equilibrium in healthy subjects was assessed by the χ^2 test. Carrier and genotypic frequencies among groups were compared by means of the χ^2 test and Fisher's exact test when necessary (expected values below 5). The odds ratio (OR) and 95% confidence intervals (95% CI) were also calculated. A P-value <0.05 was regarded as significant. All these analyses were performed using the software program SPSS 12.0 (Chicago, IL, USA).

Meta-analysis

All studies published before November 2007 were identified by searching the PubMed database, using the following entries as a search criterion: *polymorphism, interleukin-10* and *alcoholism, alcohol* or *alcoholic liver disease*. Case-control studies that analyzed the distribution of the −592C>A *IL10* polymorphism in alcoholic liver disease were eligible for inclusion. We considered as cases patients with heavy alcohol intake (>80 g of ethanol/day for at least 5 years) with histological diagnosis of ALC or alcoholic hepatitis, as well as patients with ALC diagnosed by clinical, ultrasonography and gastroscopic findings. Controls were defined as individuals with heavy alcohol intake but without alcoholic liver disease or with simple steatosis diagnosed by liver biopsy. Allelic and genotypic frequency were extracted or calculated from published data. Bibliographic search and data extraction were done independently by two authors (M. M. and F. J. L.), and consensus was achieved for all data. As previous data (Grove *et al*., 2000) and our own results suggested that the effect of the A allele was dominant, the main meta-analysis studied the association between the possession of the A allele and the risk of alcoholic liver disease; the recessive contrast of the allele A was also examined. We also performed an exploratory meta-analysis analyzing the association between the possession of the A allele and the risk of an excessive alcohol intake. The OR and its 95% CI were estimated for each study. Cochran's Q-statistic was used to assess heterogeneity: a significant Q-statistic ($P < 0.10$) indicated heterogeneity across studies, and therefore the random effect model (DerSimonian and Laird) was used for meta-analysis; otherwise, fixed effect model (Mantel–Haenszel) was used. A sensitivity analysis was performed examining the effect of excluding specific studies (Cochran, 1954; Davey Smith and Egger, 1997). The meta-analysis was carried out using the computer software package RevMan 4.2.8 (RevMan Analyses, 2003).

RESULTS

Analysis of the −592C>A IL10 polymorphism

The distribution of −592C>A *IL10* genotypes in control subjects were similar to that previously published for Caucasians (Grove *et al*., 2000; Ladero *et al*., 2002) and did not significantly differ from those expected from Hardy–Weinberg equilibrium ($\chi^2 = 1.508$; $P = 0.219$).

Genotypic and allelic distribution of the −592C>A *IL10* polymorphism are summarized in Table 1. We did not observe any statistical difference when we compared the groups of patients with the presence or absence of alcoholic liver disease. However, the frequency of allele A carriers was significantly higher in alcoholic patients than in healthy subjects (52.1 and 40.0%, respectively; $P = 0.039$). The possession of the A allele (CA and AA genotypes) conferred an OR of 1.25 (95% CI 1.02–1.54; $P = 0.039$) for the presence of alcoholism.

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Table 1. Distribution of the −592C>A IL-10 gene polymorphism in alcoholic patients and healthy subjects

Subjects	Genotypic frequency (%)			Allelic carriers (%)	
	CC	CA	AA	CC + CA	CA + AA
Total ($n = 357$)	183 (51.3%)	144 (40.3%)	30 (8.4%)	327 (91.6%)	174 (48.7%)
Alcoholics ($n = 257$)	123 (47.9%)	112 (43.6%)	22 (8.6%)	235 (91.4%)	134 (52.1%)*
AWLD group ($n = 161$)	77 (47.8%)	68 (48.2%)	16 (9.9%)	145 (90.1%)	84 (52.2%)
ALC group ($n = 96$)	46 (47.9%)	44 (45.8%)	6 (6.3%)	90 (93.8%)	50 (52.1%)
Healthy subjects ($n = 100$)	60 (60.0%)	32 (32.0%)	8 (8.0%)	92 (92.0%)	40 (40.0%)*

*Odds ratio (95% confidence interval) for the possession of one or two A alleles in alcoholics versus healthy subjects: 1.25 (1.02–1.54); $P = 0.039$.

ALC, alcoholics with liver cirrhosis; AWLD, alcoholics without liver disease.

Table 2. Characteristics of the studies considered in the meta-analysis of the −592C>A polymorphism of the IL-10 gene in alcoholic liver disease

First author, year	Selection criteria of cases and controls and genotype distribution
Grove *et al*., 2000	Patients with alcohol intake > 80 g/day for >10 years: Cases: patients with liver histology compatible with ALD of greater severity than simple steatosis, or clinical evidence of hepatic decompensation ($N = 287$; CC = 143, CA = 125, AA = 19) Controls: patients without clinical or biochemical evidence of ALD or with liver histology showing either normal liver or steatosis with no evidence of steatohepatitis or fibrosis ($N = 107$; CC = 68, CA = 125, AA = 3) Healthy subjects: $N = 227$; CC = 152, CA = 70, AA = 5
Ladero *et al*., 2002	Cases: patients with ALC diagnosed either by histology or by unequivocal clinical, ultrasonographic or endoscopic findings and with alcohol consume > 80 g/day for >10 years with ($N = 143$; CC = 89, CA = 43, AA = 11). Controls: healthy subjects ($N = 293$; CC = 170, CA = 103, AA = 20)
Richardet *et al*., 2006	Cases: patients with alcohol intake >80 g/day for at least one year hospitalized with histological evidence of alcoholic hepatitis ($N = 134$; CC = 77, CA = 51, AA = 6) Controls: healthy subjects with daily alcohol intake <20 g ($N = 145$; CC = 82, CA = 54, AA = 9)
Martins *et al*., 2005	Patients with alcohol intake >80 g/day for >5 years Cases: patients with liver histology compatible with ALD of greater severity than simple steatosis, or clinical evidence of hepatic decompensation ($N = 100$; CC = 57, CA = 34, AA = 8) Controls: patients without clinical or biochemical evidence of ALD ($N = 76$; CC = 38, CA = 30, AA = 8)

N = subjects in each group; CC, CA and AA = subjects with CC, CA and AA genotype, respectively.
ALC, alcoholic liver cirrhosis; ALD, alcoholic liver disease.

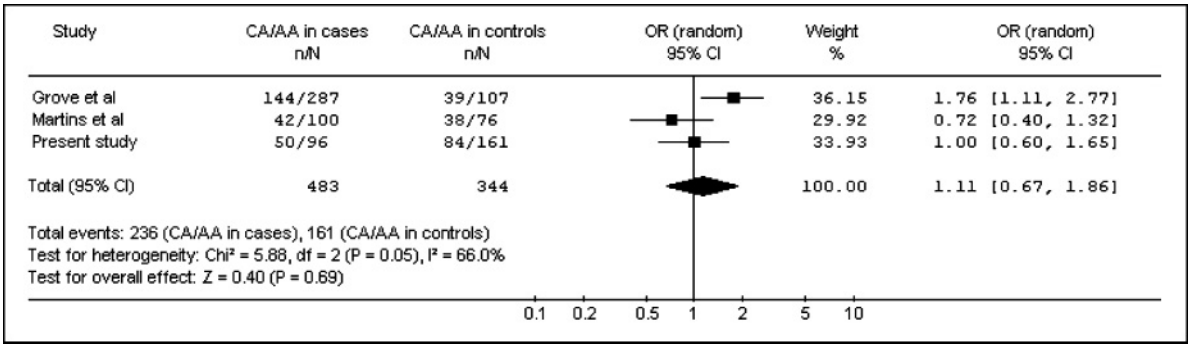

Fig. 1. Meta-analysis of whether the possession of the A allele of the −592C>A *IL10* polymorphism is related to the risk of alcoholic liver disease. Each study is shown by an OR estimate with the corresponding 95% CI.

Meta-analysis of the −592C>A IL10 polymorphism

We identified four articles that met the search criteria, summarized in Table 2. The studies from Grove *et al*. (2000) and Martins *et al*. (2005) analyzed the termed '−627A' allele, and the one from Ladero *et al*. (2002) analyzed the '−597A' allele, both corresponding to the allele at position −592 bp from the transcription start site, according to the published sequence (Eskdale *et al*., 1997). However, as the works of Ladero *et al*. (2002) and Richardet *et al*. (2006) did not provide data about alcoholic patients without liver damage, we could not include that studies in our meta-analysis. Therefore, the meta-analysis was performed with the data from Grove *et al*. (2000), Martins *et al*. (2005) and our own data; no statistical differences were observed in the distribution of A allele carriers between alcoholics with or without liver disease (Fig. 1). The sensitivity analysis did not alter the pattern of results. In addition, we analyzed the effect of the A allele under a recessive model (comparing the AA genotype with the CA and CC genotypes combined), and we did not find any statistical relationship with the presence of alcoholic liver disease (data not shown).

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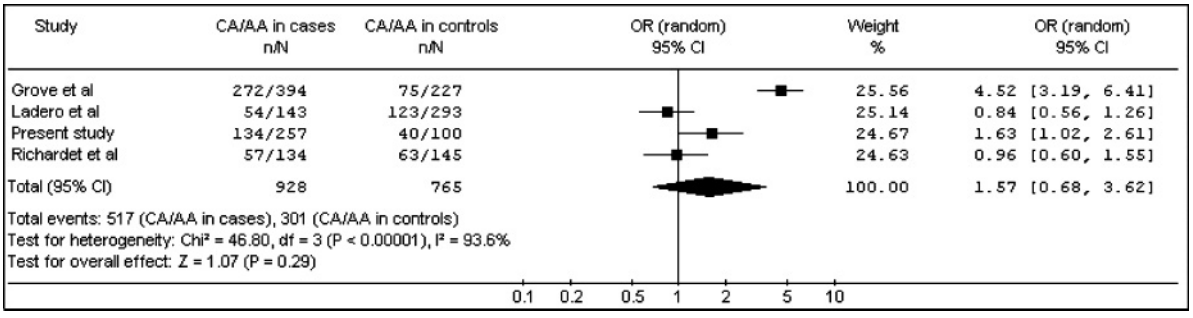

Fig. 2. Meta-analysis of whether the possession of the A allele of the −592C>A *IL10* polymorphism is related to the risk of a higher alcohol intake (>80 g/day for at least 1 year). Each study is shown by an OR estimate with the corresponding 95% CI.

Regarding the relationship between −592C>A *IL10* polymorphism and excessive alcohol intake, we performed a meta-analysis extracting data from the work of Grove *et al*. (2000), Ladero *et al*. (2002), Richardet *et al*. (2006) and our own work, in order to compare patients with an alcohol intake >80 g/day for at least 1 year with healthy subjects with an alcohol intake <20 g/day. This meta-analysis did not show a significant relationship (Fig. 2).

DISCUSSION

It is well established that only a minority of alcoholic patients have ALC; furthermore, alcoholic patients may or may not develop alcohol dependence or alcohol abuse. In an attempt to explain these findings, genetic studies have been conducted and previous reports have shown that polymorphic alleles of genes encoding inflammatory cytokines such as IL-1 and TNF-α could change the susceptibility of chronic alcoholic patients to develop ALC (Takamatsu *et al*., 2000; Pastor *et al*., 2005a). Additionally, a strong association between advanced alcoholic liver disease and the possession of the A allele in the promoter region of the IL-10 cytokine gene has been reported (Grove *et al*., 2000). These results, together with the data showing a reduced IL-10 secretion in patients with the −592A allele of this gene (Crawley *et al*., 1999; Hoffmann *et al*., 2001) as well as the pathophysiologic role of IL-10 in alcoholic liver injury (Hill *et al*., 2002; Zhong *et al*., 2006), raised the hypothesis that this allelic variant could be involved in the development of this disease.

As shown in Table 1 and Fig. 1, however, our results and the meta-analysis performed indicate that ALC is not associated with the distribution of −592C>A IL-10 gene polymorphism. In addition to this, the studies from Ladero *et al*. (2002) and Richardet *et al*. (2006), not included in our meta-analysis, also showed a similar distribution of the −592A allele in patients with advanced alcoholic liver disease with respect to controls. Interestingly Spanish alcoholics patients (defined as patients with alcohol abuse or dependence) showed a significantly higher frequency of the A allele in comparison to control subjects (Table 1).

Concerning genetic association studies, a number of potential biases may explain the disagreement between our results and those from Martins *et al*. (2005) compared with the study from Grove *et al*. (2000). The importance of genetic and clinical heterogeneity as well as population stratification as possible biases in association studies has been described in detail elsewhere (Hoffmann *et al*., 2001; Colhoun *et al*., 2003). Our study also raises another potential methodological problem concerning genetic studies in alcoholic liver disease: the possibility of alcoholism acting as a confounding factor. One of the inclusion criteria in our study was the presence of alcohol use disorders (alcohol abuse or dependence), which allows us to control the possible influence of these disorders in the development of alcoholic liver damage (Wodak *et al*., 1983; Grant and Harford, 1988; Barrio *et al*., 2004) and to investigate the relationship of *IL10* with alcoholism by itself. In contrast, the article from Grove *et al*. (2000) included 'heavy drinkers' (Table 2) as cases and controls, without considering the diagnosis of alcohol abuse or dependence. Therefore, the higher frequency of the A allele in patients with ALD found in this study may have been due to a higher frequency of patients with alcohol abuse or dependence in the group of patients with ALD.

An important issue in studies regarding alcoholic liver disease is the adequate selection of alcoholics who have not developed liver disease, given that performing a liver biopsy in patients with normal liver blood tests may be unethical. A previous biopsy study in asymptomatic alcoholics with normal blood tests showed that only a small percentage (4 of 85) of them had features of alcoholic hepatitis or cirrhosis (Bruguera *et al*., 1977). In view of both these data and ethical concerns, our study design seems reasonable and is similar as in other published articles (Grove *et al*., 2000; Martins *et al*., 2005). Another potential limitation of our work is the small number of studies that could be included in our meta-analysis. Nonetheless, this statistical method represents an evidence-based systematic approach and is the most accurate way of combining previous results (Egger and Smith, 1997).

Regarding the association found in our study between the −592A allele of IL-10 gene and alcoholism defined as alcohol abuse or dependence, we must admit that its functional role is uncertain. Considering both the lower secretion of IL-10 in patients with the A allele (Crawley *et al*., 1999; Hoffmann *et al*., 2001) and the inhibitory effects of IL-10 on TNF-α secretion (Wang *et al*., 1994), also shown in rodent microglia (Sawada *et al*., 1999; Kremlev and Palmer, 2005), our results support the notion that increased brain levels of TNF-α could reduce glutamate transport, inducing a hyperglutamatergic state that activates limbic dopamine and promotes neurotoxicity and alcohol consumption (Crews *et al*., 2006). In line with this, recent work conducted by our group has shown the association of IL-1 gene polymorphisms and alcoholism

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(Pastor *et al*., 2005b). Overall, these findings show a genetic association between alcoholism and genes that encode proteins involved in the immune response, although the exact relationship of the −592C>A polymorphism with alcoholism must be further investigated, given the possibility of a type I error and the lack of association found in the meta-analysis performed to analyze the relationship between the A allele and the risk of excessive alcohol intake. We must also consider that the studies integrated in this meta-analysis lack both homogeneous criteria for 'excessive alcohol intake' and alcohol use disorder diagnosis, which makes it more difficult to reach a conclusion about the association between alcoholism and this polymorphism.

In summary, the present study shows that alcoholism, but not ALC, is associated with the −592C>A polymorphism of the IL-10 gene. Moreover, due to the possibility that alcoholism may be a confounding factor for alcoholic liver disease in genetic association studies, its presence should be controlled in further candidate gene studies regarding this disease.

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