

## CYTOTOXIC T-LYMPHOCYTE ANTIGEN-4 A49G POLYMORPHISM IS ASSOCIATED WITH SUSCEPTIBILITY TO AND SEVERITY OF ALCOHOLIC LIVER DISEASE IN ITALIAN PATIENTS

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**Abstract—Aims:** To determine whether the functional A49G polymorphism of cytotoxic T-lymphocyte antigen-4 (CTLA-4), a T-cell surface molecule that modulates T-lymphocyte activation and influences the risk of developing alcohol-induced autoantibodies, plays a role in susceptibility to alcoholic liver disease (ALD) and influences disease severity in Italian alcohol abusers. **Methods:** One hundred and eighty-three patients with chronic ALD (61 cirrhosis), 115 end-stage HCV cirrhosis, 102 non-alcoholic fatty liver disease (NAFLD), 93 healthy subjects and 43 heavy drinkers without liver disease were studied. CTLA-4 gene polymorphism was analysed by restriction analysis. **Results:** The frequency of the CTLA-4 polymorphism was higher in patients with ALD than in patients with HCV chronic hepatitis and NAFLD, healthy subjects ( $P < 0.0001$ ), and heavy drinkers without liver disease ( $P = 0.02$ ). In patients with ALD, homozygosity for the CTLA-4 polymorphic allele (G/G genotype) was more represented in subjects with cirrhosis ( $P = 0.047$ ), and independently associated with the risk of cirrhosis (OR 3.5;  $P = 0.03$ ). **Conclusions:** The CTLA-4 polymorphic G allele, probably by interfering with the immune response, may confer susceptibility to ALD and, in homozygous state, to alcoholic cirrhosis.

### INTRODUCTION

Genetic susceptibility has been reported to play a key role in alcohol induced liver disease and to account for the relatively low proportion of heavy drinkers who develop alcoholic liver disease (ALD) (Day, 2000). However, although the potential role of polymorphisms of genes encoding for proteins involved in alcohol metabolism has been studied extensively, conflicting results have been obtained in Caucasian populations (Monzoni *et al.*, 2001).

Clinical and laboratory studies have provided evidence that altered immune response, including both lymphocyte-mediated reaction to autologous hepatocytes and circulating antibodies to self protein adducts with products of alcohol metabolism, is present in patients with ALD. It has been suggested that these immune mechanisms could be involved in the pathogenesis of ALD (Paronetto, 1993; Klassen *et al.*, 1995; Clot *et al.*, 1996; 1997; Israel, 1997). Thus, immunoregulatory genes are potential candidates in determining genetic susceptibility to ALD, and polymorphisms of cytokines, including TNF- $\alpha$  and IL-10, have been associated with alcoholic acute hepatitis and liver disease in populations of north European ancestry (Day, 2000).

Cytotoxic T-lymphocyte antigen-4 (CTLA-4) is a T-cell surface molecule that inhibits the immune response by interacting, in competition with the co-stimulatory molecule CD28, with the ligand B7 on antigen presenting cells

(Schwartz *et al.*, 2001), thus playing a significant role in the peripheral control of the immune response (Bluestone, 1997; Thompson and Allison, 1997; Doyle *et al.*, 2001). The functional importance of this molecule is emphasized by the demonstration that CTLA-4-deficient mice develop lethal self-reactive lymphoproliferative disease (Waterhouse *et al.*, 1995) and that blockade of CTLA-4-B7 interaction promotes disease onset and progression in experimental models of autoimmunity (Perrin *et al.*, 1996; Luhder *et al.*, 1998). The CTLA-4 gene, mapped on chromosome 2q33, includes a single base exchange polymorphism in exon 1 (G49A), resulting in a threonine for alanine substitution in the protein (Argawal *et al.*, 2000a). Individuals carrying the polymorphic G allele show significantly decreased expression of both cell-surface CTLA-4 in activated T-helper lymphocytes and of CTLA-4 mRNA in resting cells (Ligers *et al.*, 2001).

CTLA-4 polymorphism has been associated with the susceptibility to organ-specific autoimmune diseases, including type 1 diabetes (Florez *et al.*, 2003), primary biliary cirrhosis (Argawal *et al.*, 2000b), and type 1 autoimmune hepatitis (Czaja and Donaldson, 2000). In northern European patients with ALD, the mutant CTLA-4 G allele, which was more represented than in controls, has been shown to increase the risk of developing autoantibodies towards cytochrome P4502E1 (CYP2E1). These autoantibodies have been involved in the pathogenesis of alcohol-induced liver injury through the induction of antibody-dependent cytotoxicity (Clot *et al.*, 1997; Vidali *et al.*, 2003). No data on the relationship between CTLA-4 and ALD in patients from Mediterranean area and in patients with other non-autoimmune liver diseases have been reported.

The aim of this study was to determine in a series of Italian subjects whether the A49G CTLA-4 polymorphism confers specific susceptibility to ALD and to assess a possible relationship between this polymorphism and the severity of liver damage.

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## PATIENTS AND METHODS

*Study population*

**ALD.** 183 consecutive unrelated adult patients of whom a DNA sample was available were analysed. 169 subjects (92%) were from northern Italy and 14 (8%) from southern Italy. 121 patients attended the outpatient service in Milan, 60 in Venice, and 12 in Turin. ALD was defined as the presence of clinical and/or biochemical signs of liver disease in patients with a daily alcohol consumption higher than 60 g/day in men and 40 g/day in women for more than 5 years. An accurate record of alcohol abuse was assessed as previously described (Fargion *et al.*, 2001). Patients with coexistent chronic HCV or HBV viral hepatitis, inherited and autoimmune liver diseases (autoimmune hepatitis, primary biliary cirrhosis, sclerosing cholangitis) were excluded.

The presence of cirrhosis was assessed by liver biopsy in 47 patients and by clinical evidence in 13 patients (evidence of chronic liver failure/signs of portal hypertension) (Fargion *et al.*, 1996). Biopsy was not performed in 42 patients because of normalization of all biochemical tests within 6 weeks after alcohol withdrawal in 17, and refusal in 25. Because of the lack of histology, these subjects were excluded from analyses in which patients were compared according to the presence or the absence of cirrhosis.

Serum ferritin, IgA and gamma globulin at presentation were available for all of the patients. Associated pathological conditions were recorded. Clinical and demographic characteristics of patients with ALD are shown in Table 1. Cirrhotic patients (60 cases) were older than those who were non-cirrhotic (81 cases); mean age was  $60 \pm 10.8$  versus  $50.4 \pm 10.7$  ( $P < 0.001$ ). No significant difference in sex distribution, degree and duration of alcohol consumption and liver enzymes was observed between patients with and without cirrhosis.

Of the 128 patients of whom liver biopsy was available, 47 had cirrhosis. Of the remaining 81, 70 showed steatosis with variable degree of fibrosis (perivenular fibrosis in 38, moderate in 32 cases), whereas minimal fibrosis was observed in 11 cases. Seven of the 11 patients without significant fibrosis had evidence of steatohepatitis.

**HCV related cirrhosis.** One hundred and fifteen subjects, submitted to liver transplantation in Milan between 1994 and

2000 because of HCV-related end-stage liver disease, were considered. Demographic characteristics of the patients are shown in Table 1.

**NAFLD.** One hundred and two patients with an ultrasonographic and clinical diagnosis of NAFLD (confirmed by liver biopsy in 87 cases) were considered. Other possible causes of liver disease were excluded. Demographic and clinical features of these patients are shown in Table 1.

**Healthy subjects.** Ninety-three healthy subjects of a similar ethnic and geographical origin, assessed until the third generation [83 (89%) from northern Italy and 10 (11%) from central and southern Italy], were considered. The group consisted of volunteers among hospital staff, medical students and acquired relatives of the patients. Sixty-two (67%) were men, 31 (33%) women; mean age was  $45 \pm 15$  years; none referred alcohol abuse.

**Heavy drinkers without liver disease.** Forty-three subjects who reported alcohol abuse (>60 g/day for men, >40 g/day for women, for more than 5 years), assessed as previously described (Fargion *et al.*, 2001) with liver enzymes below the upper normal range, no clinical or biochemical sign of liver failure and no ultrasonographic sign of liver damage or portal hypertension were considered. Forty-one were men, two (4%) women, mean age  $44 \pm 12$  years, mean alcohol intake  $142 \pm 102$  g/day.

Informed consent was obtained from each patient included in the study.

*CTLA-4 gene polymorphism*

A 328-bp fragment of the first exon of the CTLA-4 gene was amplified using the primers 5'-CCACGGCTTCCTTTCTCGTA-3' (sense) and 5'-AGTCTCACTCACTCACCTTTGCAG-3' (antisense) (obtained from Genenco, Italy). After amplification, the 328-bp amplicon was digested with Bbv I for 2 h at 37°C. Three different CTLA-4 genotypes (GG, GA and AA) were identified by electrophoresis on 4% agarose gel and ethidium bromide staining. Control samples of predetermined genotype, as well as negative controls, were included in each batch.

*Statistical analysis*

Results are expressed as means  $\pm$  standard deviation and considered significant when  $P < 0.05$  (two-tailed). Mean values

Table 1. Demographic and clinical characteristics of 183 Italian patients with alcoholic liver disease (ALD), 115 patients submitted to liver transplantation because of HCV-related liver disease and 102 patients with non-alcoholic fatty liver disease (NAFLD)

	ALD ( $n = 183$ )	HCV ( $n = 115$ )	NAFLD ( $n = 102$ )
Sex [M/F (%)]	160/23 (87/13)	78/37 (68/32)	80/22 (80/22)
Age [years (range)]	$54.4 \pm 12$ (22–79)	$51.6 \pm 8$	$49.7 \pm 10$
Alcohol (g/day)	$166 \pm 96$	–	$9 \pm 8$
Duration of abuse (years)	$29.5 \pm 10$	–	0
Cirrhosis (%)	60/141 (43)	115 (100)	2/87 (2)
Bridging fibrosis (%)	32/141 (23)	–	17/87 (20)
ALT (IU/ml)	$68 \pm 56$	–	$53 \pm 45$
AST	$60 \pm 41$	–	$34 \pm 43$
GGT (IU/ml)	$197 \pm 250$	–	$62 \pm 76$
Ferritin (ng/ml)	$602 \pm 702$	–	$471 \pm 320$
$\gamma$ -globulin (g/dl)	$1.56 \pm 0.5$	–	–
IgA (mg/dl)	$430 \pm 270$	–	–

were compared by Student's *t*-test. Frequencies were compared by Fisher's exact test. Logistic regression analysis was performed to assess the relationship between CTLA-4 genotype, ALD and liver cirrhosis; the presence of ALD and liver cirrhosis were set as dependent variables.

## RESULTS

The prevalence of subjects positive for the exon 1 CTLA-4 polymorphism in patients with ALD and controls is shown in Table 2. The prevalence of subjects positive for the CTLA-4 polymorphism was significantly higher in patients with ALD than in healthy subjects ( $P = 0.0002$ ), in heavy drinkers without liver disease ( $P = 0.02$ ) and in patients with NAFLD ( $P = 0.005$ ). The prevalence of the carriers of the CTLA-4 polymorphism was higher ( $P = 0.07$ ) in patients with ALD than in those submitted to liver transplantation because of end-stage HCV cirrhosis; the difference was significant (62 vs. 50%;  $P = 0.047$ ) when only patients with alcoholic cirrhosis were included in the analysis.

The frequency distribution of the A49G CTLA-4 polymorphism in patients with ALD and controls is shown in Table 3. The observed genotype frequencies were in Hardy-Weinberg equilibrium. The frequency distribution of CTLA-4 polymorphism was significantly higher in patients with ALD than in healthy controls ( $P = 0.0005$ ). The odds ratio (OR) for ALD adjusted for age, sex and geographical origin was 2.5 (95% confidence interval (CI) 1.3–4.6,  $P = 0.005$ ) for subjects

Table 2. Prevalence of subjects carrying the polymorphic 49G CTLA-4 allele in patients with alcoholic liver disease (ALD), control patients and healthy subjects

	<i>n</i>	CTLA-4 genotype		<i>P</i>
		G/G and G/A (%)	A/A (%)	
ALD	183	113 (62)	70 (38)	Ref.
HCV-related cirrhosis	115	58 (50)	57 (50)	0.07
NAFLD	102	41 (40)	61 (60)	0.005
Healthy subjects	93	35 (41)	58 (62)	0.0002
Heavy drinkers without ALD	43	18 (42)	25 (58)	0.025

A, wild-type allele; G, polymorphic allele; NAFLD, non-alcoholic fatty liver disease; Ref., reference.

Table 3. Frequency distribution of the A49G CTLA-4 polymorphism in patients with alcoholic liver disease (ALD) and healthy controls

	<i>n</i>	A/A <i>n</i> (%)	G/A <i>n</i> (%)	G/G <i>n</i> (%)
Healthy subjects	93	58 (62)	30 (32)	5 (6)
ALD	183	70 (38)	87 (48)	26 (14)
OR (95% CI)		1.0 (Ref.)	2.5 (1.3–4.6)	4.6 (1.5–14.2)

A, wild-type allele; G, polymorphic allele; CI, confidence interval  $P = 0.0005$  for the frequency distribution of the CTLA-4 polymorphism between patients and controls; OR, odds ratio for ALD (adjusted for age, sex and geographical origin); Ref., reference.

heterozygous for the CTLA-4 polymorphism and 4.6 (95% CI 1.5–14.2,  $P = 0.008$ ) for homozygous subjects.

No significant difference in age, prevalence of female sex, daily alcohol consumption, duration of abuse, and biochemical and immunological markers associated with alcohol abuse was observed between patients with and those without the CTLA-4 polymorphism. The prevalence of clinically manifest immune-mediated disorders (three patients had lymphocytic thyroiditis, two psoriasis) did not differ between patients with and without the CTLA-4 polymorphism.

The frequency distribution of the CTLA-4 polymorphism in patients with ALD subdivided according to the presence of liver cirrhosis is shown in Table 4. The prevalence of the GG genotype was significantly higher in patients with cirrhosis compared to those without (13/60, 22% vs. 7/81, 8%;  $P = 0.047$ ). At logistic regression analysis, which considered age, sex, the entity of alcohol intake and CTLA-4 genotype as independent variables, the CTLA-4 GG genotype (homozygosity for the polymorphic G allele) was independently associated with the presence of cirrhosis (OR 3.5; 95% CI 1.1–11,  $P = 0.03$ ). The presence of cirrhosis was significantly associated with age (OR 1.08; 95% CI 1.01–1.1,  $P < 0.0001$ ).

## DISCUSSION

In the present paper we studied the prevalence of the immunoregulatory CTLA-4 gene polymorphism in a series of Italian patients with chronic liver diseases of different aetiology to define whether this polymorphism confers susceptibility to ALD in a population at low risk for autoimmune liver diseases (Day, 2000) and to evaluate the relationship between the presence of this polymorphism and the severity of liver damage.

We were addressed to this study by the following. (1) The assumption that ALD results from a complex interaction between exogenous and genetic factors (Day, 2000; Monzoni *et al.*, 2001) (2) Several reports of a derangement of immune response in patients with ALD (Paronetto, 1993; Israel, 1997). (3) Data suggesting that a negative function polymorphism of the CTLA-4 gene, a T-cell surface molecule which inhibits immunological response (Schwartz *et al.*, 2001), may predispose to ALD (Vidali *et al.*, 2003). Our results indicate that the prevalence of the CTLA-4 polymorphism is significantly higher in patients with ALD than in those with non-autoimmune chronic liver disease and in controls, and that homozygosity for the polymorphic allele may predispose to alcoholic cirrhosis.

Table 4. Frequency distribution of the A49G CTLA-4 polymorphism in patients with alcoholic liver disease (ALD) with or without liver cirrhosis

	<i>n</i>	A/A <i>n</i> (%)	G/A <i>n</i> (%)	G/G <i>n</i> (%)
Cirrhosis				
Present	60	23 (38)	24 (40)	13* (22)
Absent	81	37 (46)	37 (46)	7* (8)
OR (95% CI)		1.0 (Ref.)	1.3 (0.6–3)	3.5 (1.1–11)

A, wild-type allele; G, polymorphic allele; CI, confidence interval; OR, odds ratio for cirrhosis (adjusted for age, sex, and alcohol intake),  $P = 0.047$ .

The allele distribution in the control group was similar to that found in other populations, including those of northern European ancestry (Argawal *et al.*, 2000b), and did not differ between individuals from northern and southern Italy, both in patients and in controls, indicating a uniform distribution of this polymorphism in different geographical areas. In the present study we observed a significantly higher prevalence of subjects carrying the mutated CTLA-4 allele [both in the heterozygous (OR 2.5) and homozygous (OR 4.6) form] in patients with ALD compared to healthy subjects, and this relationship was independent of age, sex and geographical origin.

We were able to recruit only 43 subjects with comparable alcohol abuse to that of patients with ALD and no liver disease. At least two reasons account for the small number of subjects included in this group: (1) the difficulty of finding completely 'normal' subjects (without ultrasonographic steatosis and alteration of liver enzymes) with such heavy and long lasting alcohol abuse; and (2) the impossibility of excluding ALD in the absence of liver biopsy. However, the prevalence of the CTLA-4 polymorphism in this group of subjects was not different from that of healthy controls and was significantly lower than that of patients with ALD, suggesting that the CTLA-4 polymorphism does not increase the susceptibility to alcohol abuse, but to alcohol-induced liver damage. We also analysed patients with non-autoimmune chronic liver disease, including HCV-related cirrhosis and NAFLD. The prevalence of the CTLA-4 polymorphism was higher in patients with ALD than in the other patient groups, suggesting a specific effect of this polymorphism on alcohol-induced liver disease and cirrhosis. In patients with HCV-related cirrhosis the difference was less evident, but we cannot exclude that some of these subjects were alcohol abusers in the years before transplantation. In fact, we have preliminary data of an increased prevalence of the CTLA-4 polymorphism in patients with cirrhosis due to co-existent HCV chronic infection and alcohol abuse (not shown).

We then analysed the relationship between the presence of the polymorphism and the severity of ALD. The GG genotype, homozygosity for the polymorphic allele, was over-represented in patients with liver cirrhosis compared to those without histological evidence of cirrhosis, the prevalence of this genotype being 22% in cirrhotic patients and 8% in those who were non-cirrhotic (6% in controls). At multivariate analysis, which considered age, sex, alcohol intake and the CTLA-4 genotype, the GG genotype was independently associated with cirrhosis (OR 3.5) suggesting that, when inherited in homozygous state, the polymorphism confers susceptibility to developing severe ALD. This is the first demonstration that the CTLA-4 polymorphism is not only involved in the breaking of immune tolerance in patients with ALD (Vidali *et al.*, 2003), but is also associated with the stage of the disease, indicating that autoimmunity may play an important role in the pathogenesis and progression of ALD.

In conclusion, the higher prevalence in patients with ALD compared to normal and pathological controls and the increasing prevalence with the severity of the liver damage suggest that the functional exon 1 CTLA-4 polymorphism confers susceptibility to ALD and may be a risk factor for its progression. These data indicate that the immunoregulatory CTLA-4 gene is involved in the pathogenesis of ALD, as

previously reported for autoimmune liver diseases. The simultaneous analysis of different genes involved in alcohol metabolism and immune response could provide the identification of the genetic background underlying ALD.

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