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Genetic Polymorphisms Influencing Xenobiotic Metabolism and Transport in Patients With Primary Biliary Cirrhosis

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Epidemiological data suggest that environmental factors may trigger autoimmunity in genetically susceptible individuals. In primary biliary cirrhosis (PBC), it has been postulated that halogenated xenobiotics can modify self-molecules, facilitating the breakdown of tolerance to mitochondrial antigens. The transport and metabolism of xenobiotics is highly dependent on key genetic polymorphisms that alter enzymatic phenotype. We analyzed genomic DNA from 169 patients with PBC and 225 geographically and sex-matched healthy subjects for polymorphisms of genes coding for cytochromes P450 (CYPs) 2D6 (CYP2D6*4, CYP2D6*3, CYP2D6*5, and CYP2D6*6) and 2E1 (c1/c2), multidrug resistance 1 (MDR1 C3435T) P-glycoprotein, and pregnane X receptor (PXR C-25385T, C8055T, and A7635G). We compared the genotype frequencies in patients and controls and also correlated polymorphisms with PBC severity. The distributions of the studied genotypes did not significantly differ between patients and controls. However, when clinical characteristics of patients with PBC were compared according to genotype, the CYP2E1 c2 allele was associated with signs of more severe disease. In conclusion, genetic polymorphisms of CYP 2D6 and 2E1, PXR, and MDR1 do not appear to play a role in the onset of PBC. (HEPATOLOGY 2005;41:55–63.)

The etiology of primary biliary cirrhosis (PBC) remains elusive, but recent data suggest that the breaking of tolerance to the highly conserved 2-oxoacid dehydrogenase complex lipoylated domains of the mitochondrial autoantigen could result from molecular mimicry initiated by an immune response directed toward xenobiotic structural analogues of lipoic acid.[1] For example, sera from patients with PBC present antimitochondrial antibodies (AMAs) recognizing a number of synthetic structures that mimic a xenobiotic-modified lipoyl hapten conjugated to a peptide from the E2 subunit of the pyruvate dehydrogenase complex.[2] In addition, rabbits immunized with a xenobiotic (6-bromohexanoate) bovine serum albumin conjugate produce immunoglobulin G autoantibodies that react not only with xenobiotic but also self-reactive AMAs.[3] Although many genetic factors conferring susceptibility to PBC have been suggested in population and family studies,[4,5] no definitive genetic association with the onset of the disease or its outcome has yet been found. The liver is the primary organ involved in the metabolism and disposition of foreign chemicals. In such an environment, chemicals and/or their reactive metabolites may modify cellular proteins to form neoantigens.[6] The recent findings of a possible role of molecular mimicry prompted us to determine whether polymorphisms in the genes involved in xenobiotic metabolism could contribute to the pathogenesis of

Abbreviations: PBC, primary biliary cirrhosis; CYP, cytochrome P450; MDR, multidrug resistance; PXR, pregnane X receptor; AMA, antimitochondrial antibody; SNP, single nucleotide polymorphism; PM, poor metabolizer; PGP, P-glycoprotein; PCR, polymerase chain reaction.

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Table 1. Characteristics of Patients With PBC at Time of Enrollment

		PBC		
	All Patients (n = 169)	Early Disease (n = 77)	Advanced Disease (n = 92)	P Value
Female sex (n)	153 (91%)	68 (88%)	85 (92%)	NS
Age at enrollment (yr)	62 ± 12	58 ± 12	66 ± 11	< .001
Disease duration (mo)	124 ± 72	90 ± 51	152 ± 75	< .001
AMA-positive (n)	139 (82%)	61 (79%)	78 (85%)	NS
Total bilirubin (mg/dL) (n.v. < 1.0)	1.6 ± 3.2	0.7 ± 0.35	2.4 ± 4.2	< .001
Albumin (g/dL) (n.v. >3.5)	4.2 ± 0.9	4.9 ± 0.7	3.7 ± 0.7	< .001
Prothrombin time (INR) (n.v. < 1.2)	1.04 ± .017	0.99 ± 0.08	1.08 ± 0.21	.001
With ascites (n)	22 (13%)	0	22 (24%)	< .001
Mayo score	5.7 ± 1.4	4.7 ± .07	6.5 ± 1.4	< .001

NOTE. Continuous variables are expressed as the mean ± SD.
Abbreviations: NS, not significant; INR, international normalized ratio; n.v., normal value.

PBC by genotyping a large population of patients with PBC and controls searching for a range of single nucleotide polymorphisms (SNPs). In particular, we decided to concentrate our efforts on mechanisms controlling the absorption of xenobiotics from the intestinal lumen, as well as their secretion into the bile (multidrug resistance 1 [MDR1]). Moreover, we were interested in analyzing the enzymes directly responsible for the metabolism of exogenous compounds (cytochromes P450 [CYPs]) or influencing the activity of the latter enzymes through regulation of their transcription (pregnane X receptor [PXR]).

Within the group of CYPs, CYP2D6 (debrisoquine/sparteine hydroxylase) is involved in the metabolism of approximately 20% of drugs.[7] Several coding genetic polymorphisms have been identified that are associated with significant reduction of drug metabolism rates *in vivo* and *in vitro*; these are known as "poor metabolizers" (PMs).[8] In particular, the PM phenotype is found in as many as 10% of Caucasian subjects[9] and is most commonly due to the presence of null alleles for single base pair mutations (CYP2D6*3, CYP2D6*4, and CYP2D6*6, among others) or deletion of the whole gene (allele CYP2D6*5).[7] As such, four alleles—CYP2D6*3, CYP2D6*4, CYP2D6*5, and CYP2D6*6—account for 93% to 97% of the PM phenotypes in Caucasians.[10]

CYP2E1 metabolizes several compounds, including ethanol, estrogenic metabolites, and halothane, and its activity is altered by nicotine.[11] These characteristics make this enzyme particularly interesting in PBC because of a striking female predominance.[12] Furthermore, PBC shares several characteristics (including the presence of serum AMAs) with halothane-induced hepatitis,[13] and is diagnosed more commonly among smokers.[14] A specific genetic polymorphism of CYP2E1 (*Rsa*I restriction polymorphisms alleles c1/c2) has been widely investigated[15] and is associated with reduced activity *per se*[16] or altered phenotype following interaction with specific compounds (*e.g.*, ethanol or isoniazid), despite similar baseline activity.[17,18]

The MDR1 gene encodes for the P-glycoprotein (PGP), a molecule that controls the cellular trafficking of substrates such as bilirubin and cancer drugs. In particular, PGP plays a role in excreting toxic xenobiotics and metabolites into the intestinal lumen as well as urine and bile. Although multiple mutations have been identified in MDR1, the exon 26 C3435T SNP is of special interest because of its association with a lower PGP expression in the intestine.[19] It has also been suggested that the C3435T SNP might be associated with susceptibility to ulcerative colitis,[20] although the latter observation was not confirmed in another study.[21]

PXR is a nuclear receptor for steroid hormones and select xenobiotics whose activity regulates the expression of CYP3A4 and MDR1 in the liver and intestine. Importantly, there is an association of specific PXR SNPs with the CYP3A4 phenotype.[22] We report the prevalence of CYP2D6*4, CYP2E1 c1/c2, MDR1 C3435T, and PXR (C-25385T, C8055T, A7635G) polymorphisms in patients with PBC and controls and have identified a correlation between CYP2E1 c1/c2 genotype and disease severity.

Patients and Methods

Patients. A total of 169 Italian patients with PBC who attended the Liver Unit at San Paolo Hospital (Milan, Italy) were enrolled in the study (Table 1). The diagnosis of PBC was based on internationally accepted criteria,[23,24] and the AMA status of each patient was verified via indirect immunofluorescence. Of these 169 patients, 30 (18%) were AMA negative and 139 (82%) were AMA positive. All patients were negative for hepatitis B surface antigen and antibodies to hepatitis C virus and

denied alcohol abuse during the previous 12 months. The disease duration was calculated as the time between the date of the earliest recorded evidence of liver disease and the date of blood sampling. The latter evidence was determined through an extensive search of all laboratory data for alterations in cholestasis indicators (alkaline phosphatase > 1.5 normal values with or without altered γ-glutamyltransferase). All patients had undergone liver biopsy during the 12 months before blood sampling. Patients who did not have fibrosis according to liver histology (*i.e.*, Stages I-II according to Ludwig et al.[25]) were considered to have early-stage PBC. Patients with liver fibrosis or cirrhosis (*i.e.*, Stages III-IV according to Ludwig et al.[25]), patients who had a history of a major complication from cirrhosis (*e.g.*, ascites or gastrointestinal bleeding caused by portal hypertension), and patients who had undergone orthotopic liver transplantation for PBC were considered to have advanced-stage PBC. Advanced disease was found in 92 individuals (54.4%). Based on age, serum bilirubin and albumin, prothrombin time, and the presence of ascites, the Mayo score, the only validated prognostic index in PBC,[26] was calculated at the time of blood sampling or orthotopic liver transplantation. Two hundred twenty-five healthy subjects (blood donors) geographically and sex-matched with patients with PBC were used as a control population. Namely, 4 matched controls were obtained for every 3 patients. The study protocol followed the ethical guidelines of the most recent Declaration of Helsinki (Edinburgh, 2000); all patients provided written informed consent.

Genotyping. Whole blood samples were obtained from each patient and control and stored at −20°C before DNA extraction. DNA extraction was performed using a commercially available kit (Instagene Matrix; Bio-Rad Laboratories, Segrate, Italy). SNP genotypes were determined with polymerase chain reaction (PCR)/restriction fragment length polymorphism or the TaqMan SNP detection system (Applied Biosystems, Foster City, CA).

CYP2D6. CYP2D6*3 (2549A deletion) and CYP2D6*6 (1707T deletion) variants were analyzed using TaqMan-based methods. Amplification was performed using TaqMan universal master mix (Applied Biosystems) and 40X primers (forward 5′-CCTGACCCAGCTGGATGAG-3′, reverse 5′GCCAGGAAGGCCTAGT-3′ for CYP2D6*3 and forward 5′-GGGCCTGGGCAAGAAGTC-3′, reverse 5′-CGAAGGCGGCACAAAGG-3′ for CYP2D6*6) and allele-specific probes (VIC-ACTGAGCACAGGATGA-8NFQ, FAM-TAACTGAGCACGGATGA-8NFQ for CYP2D6*3 and VIC-CACCCACTGCTCCAG-8NFQ, FAM-TCACCCCTGCTCCAG-8NFQ for CYP2D6*6). PCR amplification included one cycle at 95°C for 10 minutes followed by 40 cycles of 92°C for 15 seconds and 65°C for 1 minute. Allelic discrimination was performed on the post-PCR product by the 7900HT Sequence Detection System (Applied Biosystems). The presence of homozygosity for the CYP2D6*5 allele (deletion of the whole gene) was determined as described previously.[27]

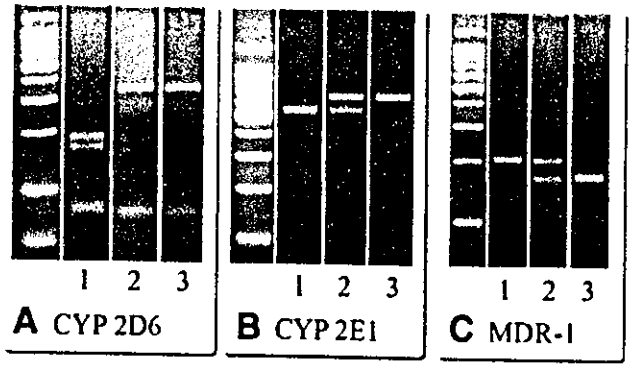

Fig. 1. Restriction fragment length polymorphism analysis of (A) CYP2D6 G1934A, (B) CYP2E1 c1/c2, and (C) MDR1 C3435T SNPs. PCR products of CYP2D6, CYP2E1, and MDR1 were digested with restriction enzymes *Bst*NI, *Rsa*I, and *Sau*3AI, respectively, and resolved via agarose gel electrophoresis. Restriction fragment patterns were scored as: (A) non-CYP2D6*4 homozygotes (77, 161, and 183 bp) in lane 1, heterozygous in lane 2, or CYP2D6*4 homozygous (77 and 344 bp) in lane 3; (B) CYP2E1 wild-type homozygous c1/c1 (360 and 50 bp) in lane 1, heterozygous c1/c2 in lane 2, or mutant-type homozygous c2/c2 (410 bp) in lane 3; and (C) MDR1 homozygous TT (197 bp) in lane 1, heterozygous CT in lane 2, or homozygous CC (158 and 39 bp) in lane 3. CYP, cytochrome P450; MDR, multidrug resistance.

The CYP2D6 G1934A SNP (CYP2D6*4) was investigated as described by Brown et al.[8] Briefly, using primer pairs 5′-GGTGTTCCTCGCGCGCTATG-3′ and 5′-CTCGGTCTCTCGCTCCGCAC-3′, DNA amplifications were performed with the PCR System 9700 (Applied Biosystems). PCR amplification consisted of an initial denaturation for 5 minutes at 94°C followed by 35 cycles of denaturation at 94°C for 1 minute, annealing at 60°C for 1 minute, and extension at 72°C for 1 minute. The terminal elongation was performed at 72°C for 7 minutes. PCR products were digested with restriction enzyme *Bst*NI (New England BioLab, Beverly, MA) overnight at 60°C, resolved in 3% agarose in Tris-acetate buffer, and visualized using ultraviolet ethidium bromide staining. Based on the pattern of the detected bands, restriction fragment patterns were scored as CYP2D6*4 homozygous (77 and 344 bp), non-CYP2D6*4 homozygous (77, 161, and 183 bp), or heterozygous (Fig. 1).

CYP2E1 c1/c2. CYP2E1 c1/c2 SNP was investigated as described by Choi et al.[28] Briefly, using primer pairs 5′-CCAGTCGAGTCTACATTGTCA-3′ and 5′-TTCATTCTGTCTTCTAACTGG-3′, DNA amplification was performed with an initial denaturation of 4 minutes at 94°C followed by 34 cycles at 94°C for 60

seconds, 60°C for 60 seconds, and 72°C for 60 seconds. The terminal elongation was performed at 72°C for 4 minutes. PCR products were digested with restriction enzyme *Rsa*I (Invitrogen, Carlsbad, CA) at 37°C for 3 hours, resolved on 3% agarose gels in Tris-acetate buffer, and visualized with ethidium bromide staining. Based on the size of detected bands, samples were identified as either wild-type homozygous c1/c1 (360 and 50 bp), mutant-type homozygous c2/c2 (410 bp), or heterozygous c1/c2 (see Fig. 1).

MDR1 C3435T. The MDR1 C3435T SNP was determined as described by Cascorbi et al.[29] Briefly, using primer pairs 5′-TGTTTTCAGCTGCTTGATGG-3′ and 5′-AAGGCATGTATGTTGGCCTC-3′, DNA amplification was performed with an initial denaturation of 2 minutes at 94°C followed by 35 cycles of 94°C for 30 seconds, 60°C for 30 seconds, and 72°C for 30 seconds. The terminal elongation was performed at 72°C for 7 minutes. PCR products were digested with restriction enzyme *Sau*3AI (New England BioLab) overnight at 37°C, resolved on 3.8% agarose gels in Tris-acetate buffer, and visualized with ethidium bromide staining. Based on the size of the detected bands, samples were identified as C/C homozygous (158 and 39 bp), T/T homozygous (197 bp), or heterozygous (Fig. 1).

PXR. Three PXR SNPs—C-25385 T, A7635G, and C8055T—were analyzed using a TaqMan-based method. Using the TaqMan universal master mix and 40× assay mix including primers and probes 5′-ACCACGATTGAGCAAACAGGTA-3′, 5′-ACCTGAAGACAACTGTGGTCATT-3′, VIC-TCCCAGGTTCTCTTTT-8NFQ, and FAM-TCCCAGGTTTTCTTTT-8NFQ (Assays-by-Design; Applied Biosystems), the PXR C-25385T SNP was determined. PCR amplification consisted of one cycle at 95°C for 10 minutes followed by 40 cycles of 92°C for 15 seconds and 65°C for 1 minute. Allelic discrimination was performed on the post-PCR product using the 7900HT Sequence Detection System (Applied Biosystems). Potentially, there were four clusters of points that corresponded to the three genotypes (CC, CT, TT) or to no amplification. Similarly, PXR A7635G SNP was analyzed using primers and probes 5′-CACAGTCATCCTCAGGGAAAGG-3′, 5′-CAGCCATCCCATAATCCAGAAGT-3′, VIC-CTCTTCCTCTCACCCCCA-8NFQ, and FAM-CTTCCTCTCGCCCCCA-8NFQ. PXR C8055T SNP was analyzed using primers and probes 5′-GGGATGATTAGATCTTGGTCAGCTT-3′, 5′-CTGGAAGCCACCTGTGGAT-3′, VIC-CCCCTCCATCCTGTTAC-8NFQ′, and FAM-CCCCTCCATTCTGTTAC-8NFQ′.

Statistical Analysis. Fisher exact tests were used for the analysis of categorical variables. In the case of continuous variables, the Mann-Whitney *U* test was used to compare two groups, and the Kruskal-Wallis nonparametric one-way ANOVA was used to compare more than two groups. In the presence of statistically significant differences in categorical variables, odds ratios and 95% confidence intervals were calculated, and *P* values were corrected for age at enrollment and duration of disease. Statistical comparisons were made using Stata Statistical Software (Stata Corp., College Station, TX) or SAS (SAS Institute Inc., Cary, NC). All of the analyses were two-sided, and *P* values of less than .05 after correction were considered statistically significant.

Results

CYP2D6. The CYP2D6*4 allele frequencies in patients with PBC and controls are shown in Table 2. The obtained genotype frequencies corresponded to the Hardy-Weinberg equilibrium in our healthy Caucasian sample. Among 169 patients with PBC, 3 (2%) were found to be homozygous for the mutant allele (*4/*4), 118 (70%) were homozygous for the wild-type allele (N/N), and 48 (28%) were heterozygous (*4/N). No significant differences were observed between patients and controls in the frequencies of CYP2D6 genotypes. When the clinical characteristics of patients with PBC were analyzed according to the presence of the CYP2D6*4 allele (Table 3), no significant differences in genotype distributions were observed for age at enrollment, disease duration, or AMA status. Patients carrying the *4 allele presented signs of a slightly more advanced disease as indicated by higher prevalence of ascites (22% vs. 9%, $P = .031$ after correction for age and disease duration); higher but nonsignificant total bilirubin levels (2.2 ± 4.3 vs. 1.4 ± 2.7; *P* value not significant) were also observed. Moreover, the presence of the *4 allele was found to be associated with ascites (odds ratio, 2.76; 95% CI, 1.1-6.92). Data obtained from a preliminary study of CYP2D6*3 and CYP2D6*6 showed low allele frequencies in both patients (n = 90; 5/180 for CYP2D6*3 and 1/180 for CYP2D6*6) and controls (n = 90; 0/180 for CYP2D6*3 and 6/180 for CYP2D6*6). A similar preliminary study showed that homozygous CYP2D6*5 was not found in 80 patients with PBC and 80 controls. Based on these findings, further genotyping for these variants was not performed.

CYP2E1 c1/c2. The CYP2E1 genotype and allele frequencies among patients with PBC and controls are shown in Table 2. The obtained genotype frequencies corresponded to the Hardy-Weinberg equilibrium in our healthy Caucasian sample. 159 (94%) of 169 patients with PBC were found to be homozygous c1/c1 and 10 (6%) heterozygous c1/c2. No significant differences were observed between patients and controls in the frequencies of CYP2E1 genotypes.

Table 2. Distribution of CYP2D6, CYP2E1, and MDR1 Genotypes and Alleles in Patients With PBC and Controls

		PBC		
	Controls	Total	Early Disease	Advanced Disease
CYP2D6				
N/N	164/225 (73%)	118/169 (70%)	57/77 (74%)	61/92 (66%)
*4/N	57/225 (25%)	48/169 (28%)	19/77 (25%)	29/92 (32%)
*4/*4	4/225 (2%)	3/169 (2%)	1/77 (1%)	2/92 (2%)
*4 allele frequency	0.144	0.159	0.136	0.179
CYP2E1				
C1/C1	212/223 (95%)	159/169 (94%)	76/77 (99%)*	83/92 (90%)*
C1/C2	9/223 (4%)	10/169 (5%)	1/77 (1%)	9/92 (10%)
C2/C2	2/223 (1%)	0	0	0
C2 allele frequency	0.029	0.033	0.006	0.049
MDR1				
3435 C/C	60/225 (27%)	47/169 (28%)	23/77 (30%)	24/92 (26%)
3435 C/T	110/225 (49%)	88/169 (52%)	39/77 (51%)	49/92 (53%)
3435 T/T	55/225 (24%)	34/169 (20%)	15/77 (19%)	19/92 (21%)
T allele frequency	0.488	0.461	0.448	0.472

*P = 0.043 after correction for age and disease duration; odds ratio 11.39 (95% CI 1.09-119.47) for c1/c2 genotype determining advanced disease.

When patients with PBC were analyzed according to disease stage, the frequency of the c1/c2 genotype was found to be significantly higher in advanced-stage PBC compared with early-stage PBC ($P = .043$ after correction for age and disease duration). Moreover, the presence of the c2 allele was found to be associated with advanced disease (odds ratio, 11.39; 95% CI, 1.09-119.47). When the clinical characteristics of patients with PBC were analyzed according to the presence of the c2 allele (Table 4), patients with this allele presented signs of more advanced disease as indicated by higher total bilirubin levels (3.6 ± 5.3 vs. 1.5 ± 3.0; $P = .054$), lower serum albumin levels (3.6 ± 0.9 vs. 4.3 ± 0.8; $P = .036$ after correction for age and disease duration), and higher Mayo score values (6.9 ± 1.9 vs. 5.6 ± 1.4; $P = .015$ after correction for age and disease duration).

MDR1 C3435T. The MDR1 C3435T genotypes and allele frequencies among patients with PBC and controls are shown in Table 2. The obtained genotype frequencies corresponded to the Hardy-Weinberg equilibrium in our healthy Caucasian sample. Forty-seven (28%) of 169 patients with PBC were found to be homozygous C/C, 34 (20%) were homozygous T/T, and 88 (52%) were heterozygous C/T. No significant differences were observed between patients and controls in the frequencies of MDR1 genotypes. No significant differences in genotype distributions were observed between patients with early and advanced disease (T allele frequency 0.442 in early disease vs. 0.473 in advanced disease) nor in the clinical characteristics across the three genotypes (data not shown).

PXR. The PXR1 C-25385T genotypes and allele frequencies among patients with PBC and controls are shown in Table 5. The obtained genotype frequencies corresponded to the Hardy-Weinberg equilibrium in our healthy Caucasian sample. Seventy-two (43%) of 167 patients with PBC were found to be homozygous C/C, 24

Table 3. Clinical Features of Patients With PBC and the Presence of the CYP2D6*4 Allele

	N/N (n = 118)	*4/N + *4/*4 (n = 51)	P Value (Corrected)
Female sex (n)	106 (90%)	47 (92%)	NS
Age at enrollment (yr)	62 ± 13	62 ± 11	NS
Disease duration (mo)	122 ± 71	127 ± 76	NS
AMA-positive (n)	99 (84%)	40 (78%)	NS
Total bilirubin (mg/dL) (n.v. < 1.0)	1.4 ± 2.7	2.2 ± 4.3	.194
Albumin (g/dL) (n.v. >3.5)	4.3 ± 0.9	4.2 ± 0.9	NS
Prothrombin time (INR) (n.v. < 1.2)	1.04 ± 0.18	1.03 ± 0.14	NS
Ascites (n)	11 (9%)	11 (22%)	.031 (.031)*
Mayo score	5.6 ± 1.4	5.9 ± 1.5	NS

NOTE. Continuous variables are expressed as the mean ± SD.
Only P values below .2 for the comparison between patient groups are reported before and after correction for age and disease duration.
*Odds ratio 2.76 (95% CI, 1.1-6.92) for *4 allele determining the presence of ascites.
Abbreviations: NS, not significant; INR, international normalized ratio; n.v., normal value.

Table 4. Clinical Features of Patients With PBC and the Presence of the CYP2E1 c2 Allele

	c1/c1 (n = 159)	c1/c2 (n = 10)	P Value (Corrected)
Female sex (n)	143 (90%)	10 (100%)	NS
Age at enrollment (yr)	62 ± 12	64 ± 13	NS
Disease duration (mo)	122 ± 69	155 ± 112	NS
AMA-positive (n)	131 (82%)	8 (80%)	NS
Total bilirubin (mg/dL) (n.v. < 1.0)	1.5 ± 3.0	3.6 ± 5.3	.054
Albumin (g/dL) (n.v. > 3.5)	4.3 ± 0.9	3.6 ± 0.9	.011 (.036)
Prothrombin time (INR) (n.v. < 1.2)	1.03 ± 0.16	1.13 ± 0.20	.109
Ascites (n)	19 (12%)	3 (30%)	.125
Mayo score	5.6 ± 1.4	6.9 ± 1.9	.011 (.015)

NOTE. Continuous variables are expressed as the mean ± SD. Only *P* values below .2 for the comparison between patient groups are reported before and after correction for age and disease duration.

Abbreviations: NS, not significant; INR, international normalized ratio; n.v., normal value.

(14%) were homozygous T/T, and 71 (43%) were heterozygous C/T. No significant differences in genotype distribution were observed between patients and controls nor between patients with early and advanced disease. Similarly, no significant differences were found in the clinical features of patients with different genotypes (data not shown).

The PXR A7635G genotypes and allele frequencies among patients with PBC and controls are shown in Table 5. Twenty-eight (28%) of 100 patients with PBC were found to be homozygous A/A, 19 (19%) were homozygous G/G, and 53 (53%) were heterozygous A/G. This genotype distribution was similar to what was observed among controls. Patients with PBC presented similar clinical characteristics across the three genotypes (data not shown).

The PXR C8055T genotypes and allele frequencies among patients with PBC and controls are shown in Table 5. No significant differences were observed between patients and controls in the frequencies of C8055T genotypes nor between patients with early and advanced disease. When the clinical characteristics of patients with PBC were analyzed according to their PXR genotypes, similar features were encountered in all groups (data not shown).

Discussion

We investigated several key SNPs of CYP2D6, CYP2E1, MDR1, and PXR in patients with PBC and geographically and sex-matched controls to determine if particular alleles contribute to a link between xenobiotics and PBC. We did not identify an association between such alleles and PBC. In addition, we correlated the genotypes of the investigated genes with histological stages and other clinical and biochemical features of patients in a cross-sectional fashion and identified a significant association of the frequency of CYP2E1 c2 allele with PBC severity. Our series, one of the largest series of PBC cases ever genotyped for candidate genes, included an unusually high frequency of AMA-negative patients, but in all cases the diagnosis was verified as described herein. Moreover, data from AMA-negative patients were similar to the AMA-positive group. Indeed, our comparisons (between patients and controls as well as among affected individuals) should therefore be regarded as statistically powerful.

Table 5. Distribution of PXR Genotypes and Alleles In Patients With PBC and Controls

		PBC		
	Controls	Total	Early Disease	Advanced Disease
−24385 C/C	76/225 (34%)	72/167 (43%)	36/77 (47%)	36/90 (40%)
−24385 C/T	119/225 (53%)	71/167 (43%)	31/77 (40%)	40/90 (44%)
−24385 T/T	30/225 (13%)	24/167 (14%)	10/77 (13%)	14/90 (16%)
−24385 T allele frequency	0.397	0.356	0.331	0.378
7635 A/A	32/102 (31%)	28/100 (28%)	12/42 (29%)	16/58 (28%)
7635 A/G	49/102 (49%)	53/100 (53%)	25/42 (59%)	28/58 (48%)
7635 G/G	20/102 (20%)	19/100 (19%)	5/42 (12%)	14/58 (24%)
7635 G allele frequency	0.436	0.455	0.417	0.483
8055 C/C	66/102 (65%)	63/99 (63%)	29/43 (68%)	34/56 (61%)
8055 C/T	29/102 (28%)	34/99 (34%)	13/43 (30%)	21/56 (37%)
8055 T/T	7/102 (7%)	2/99 (2%)	1/43 (2%)	1/56 (2%)
8055 T allele frequency	0.211	0.192	0.174	0.205

PBC has a wide spectrum of disease progression—some patients remain asymptomatic for decades after diagnosis, while others present a rapidly progressing disease leading to orthotopic liver transplantation or death. Although a number of genetic factors have been proposed to explain such differences, results obtained thus far have proven to be weak or limited to specific geographical areas.[4,5,30,31]

Four CYP2D6 alleles (CYP2D6*3, CYP2D6*4, CYP2D6*5, and CYP2D6*6) are known to account for 93% to 97% of PM cases,[7] with CYP2D6*4 alone accounting for approximately 75% of these[10,32]; this is the most widely studied allele in association studies. Brown et al.[8] demonstrated a link between the CYP2D6*4 allele and ankylosing spondylitis and postulated that the poor metabolism of xenobiotics by a defective CYP2D6 polymorphism might explain this association. Others have suggested that a PM state might increase susceptibility to Parkinson's disease because of impaired detoxification of neurotoxins.[33] It is interesting to note that patients with CYP2D6*4 had slight signs of more severe clinical features as indicated by the prevalence of ascites, though differences in other variables (*e.g.*, total bilirubin) did not reach statistical significance. The low or null allelic frequencies observed for CYP2D6*3, CYP2D6*5, and CYP2D6*6 in a preliminary study performed on both patients and controls did not provide sufficient statistical power and did not warrant further investigation. It is important to note that the observed allelic frequencies are similar to previous reports for all the CYP2D6 polymorphisms studied herein.[34]

CYP2E1 is involved in the metabolism of drugs, chemicals (including ethanol), and carcinogens, and its c1/c2 polymorphism has been shown to influence enzyme activity *per se*[16] or when induced by specific agents.[17,18] Interestingly, Tsutsumi et al.[35] demonstrated that the c2 allele is associated with the development of alcoholic liver disease, likely through the reduced observed activity.[16] Conversely, subjects with the c1/c1 genotype had higher CYP2E1 activity induced by isoniazid, suggesting an association between such a genotype and susceptibility to antituberculosis drug-induced hepatitis.[18] We can assume that the c1/c2 SNP is involved in determining CYP2E1 activity possibly induced by specific agents, thus influencing the clinical manifestations of several diseases.[18,35–37] Many CYP2E1 substrates have been identified, including halothane, isoniazid, acetone, acetonitrile, estrogen metabolites, and ethanol. Halothane is of great interest in the association of xenobiotic metabolism and susceptibility to PBC, because the sera of patients with halothane-induced hepatitis have autoantibodies to the pyruvate dehydrogenase complex similar to PBC sera.[13] Moreover, previous data have also indicated that both oxidative and reductive metabolism of halothane can lead to active xenobiotics *in vivo*.[2] On the other hand, the influence of low doses of nicotine on the expression of liver CYP2E1 in animal models[38] is also interesting considering that smoking is a risk factor for PBC.[14]

The frequency of the c1/c2 genotype in patients with advanced stage PBC was significantly higher than that of early-stage PBC. Accordingly, among other variables, the Mayo score values in patients carrying the c2 allele were significantly higher than those observed in patients with the c1/c1 genotype. A possible confounding effect of age (one of the factors in the calculation of such prognostic index) or disease duration was considered in this comparison, and we also note that these variables were not significantly different in patients with different genotypes. Our results indicate that genetically determined alterations of the metabolism of xenobiotics possibly mediated by other compounds might play a role in determining disease severity. Interestingly, in one of our previous studies, AMA from patients with PBC often reacted with a higher titer against the xenobiotically modified peptide than with the native lipoyl domain; that is, altered lipoic acid actually increased antibody binding.[2] Considering such data and the results described herein, we hypothesize that higher CYP2E1 activity induced by the presence of the c2 allele may make patients with PBC produce more active xenobiotics, including drugs, resulting in an enhanced T-cell reactivity to organic modified autoepitopes. Although our findings on the CYP2E1 c2 allele were limited to a small subgroup of patients, this polymorphism in PBC should be further assessed as a prognostic marker. Our data therefore support the previously suggested hypothesis, derived from the weak association between PBC and human leukocyte antigen haplotypes,[39] that susceptibility and progression are most likely caused by a combination of several factors, including more than one genetic determinant (*e.g.*, abnormalities in sex chromosomes[40]) together with specific genomic variations.

The MDR1 gene is responsible for the production of PGP, which is highly expressed in intestinal epithelial cells, and its genetic polymorphisms play a major role in determining local defense against bacteria and xenobiotics.[41] Individuals carrying the homozygous MDR1 3435TT genotype had on average a two-fold lower intestinal level of PGP expression compared with the CC genotype, resulting in decreased transport of PGP substrates into the gut lumen and higher absorption from the gastrointestinal tract.[20] We have postulated that xenobiotic modification of lipoic acid occurs on microbial proteins.[42] In particular, we reported that sera from patients with PBC react in a highly directed and specific fashion against proteins from the ubiquitous xenobiotic-metabolizing

bacterium *Novosphingobium aromaticivorans*.[43] In the data presented herein, homozygous TT genotype was found in 20% of patients with PBC, and no significant differences were observed between patients and controls in the frequencies of MDR1 genotypes and alleles (T allele frequency 0.461 in patients with PBC vs. 0.488 in controls). In the future, studies designed to investigate MDR1 genetic polymorphisms linked to alterations in PGP expression or SNPs of other genes related to gastrointestinal protection against bacteria and xenobiotics should be addressed. Based on a similar hypothesis, we note that Pauli-Magnus et al.[44] recently investigated the genetic polymorphisms of two different adenosine triphosphate–dependent binding cassettes (ABC B11 and B6, or MDR3) and reported a lack of correlation between polymorphisms of such genes and susceptibility to PBC.

PXR is a nuclear hormone receptor that acts as a xenobiotic sensor to transcriptionally regulate many important genes such as CYP3A4 and MDR1. Although there is the extent of CYP3A4 phenotypic variation, few allelic polymorphisms with altered activity have been reported.[45] Functional genetic variations in PXR, on the other hand, influence the expression of CYP3A4.[22] Among PXR SNPs, C-25385T was associated with CYP3A4 inducibility phenotype in the liver, while A7635G and C8055T were involved in intestinal CYP3A induction. In particular, 3A4 is the most abundant CYP detectable in the liver (18.4% of the total CYP activity) and that an increased CYP3A4 induction in the liver is associated with the PXR -25385CC genotype.[46] We postulate that higher liver CYP3A4 activity in patients with PBC directly caused by the PXR -25385 CC genotype may alter the metabolism of xenobiotics, thus leading to the induction of autoimmunity through the enhanced production of xenobiotic structural analogues of lipoic acid. Our data, however, showed that the frequency of the latter genotype among patients with PBC was not significantly different compared with matched controls (43% vs. 33%; $P = .06$). Similarly, no differences were observed in the prevalence of A7635G and C8055T genotypes. Because PBC presents a striking female predominance,[23] it is interesting to note that progesterone is one of the substrates of CYP3A4.[47]

In summary, we concentrated on genes for which sound evidence has suggested characteristics constituting a possible link with PBC. Second, we note that our design allowed to investigate the crucial steps of xenobiotic transport and metabolism. Third, we chose variations within such genes that were demonstrated to be "coding" for phenotypic differences, although in the case of CYP2E1 such differences can be mediated by other compounds. Our findings herein, along with our previous data, point toward a "multi-hit" pathogenesis of PBC, with different genetic factors leading to onset and severity of disease.

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Aldehyde Dehydrogenase 2 and β_3-Adrenergic Receptor Gene Polymorphisms: Their Association With Elevated Liver Enzymes and Metabolic Syndrome

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Recent studies indicate that some patients with nonalcoholic fatty liver have ongoing liver injury that may progress from steatosis to steatohepatitis or fibrosis. The present study was designed to clarify the clinical features of liver dysfunction observed in the course of workplace physical check-ups in relation to multiple risk factor syndrome including obesity, hyperlipidemia, hypertension, and impaired glucose tolerance, and to clarify the involvement of aldehyde dehydrogenase 2 (ALDH2) and β_3-adrenergic receptor (β3-AR) gene polymorphisms in elevation of liver enzymes. One hundred forty-eight male workers 35 years of age were enrolled. They were requested to answer questionnaires about drinking and smoking habits, and underwent urinalysis, physical and peripheral blood examinations, blood chemistry, electrocardiogram and chest x-rays. The genotypes of ALDH2 and β3-AR were analyzed by polymerase chain reaction–restriction fragment length polymorphism (PCR-RFLP). The subjects were divided into active ALDH2 or inactive ALDH2 groups. They were also divided into 2 groups according to the β3-AR genotype. The relationships between ALDH2 and β3-AR gene polymorphism and the results of the physical examination including liver function tests were analyzed. The subjects were also divided according to the number of components of metabolic syndrome. The prevalence of elevated alanine aminotransferase (ALT) level increased with the accumulation of components of metabolic syndrome. Active ALDH2 was associated with elevated ALT level to a greater degree than β3-AR polymorphism. Among those with normal body mass index (BMI), the genotypes of ALDH2 and β3-AR were strongly associated with elevated ALT level. Logistic regression analysis revealed that BMI, triglyceride level, and ALDH2 genotype were associated with ALT elevation. In conclusion, evaluating the genotype of ALDH2 and β3-AR may assist in predicting and preventing the development of fatty liver which may be related to multiple risk factor syndrome.

NONALCOHOLIC fatty liver disease (NAFLD) is the most common form of liver disease in various countries. It is estimated to affect 10% to 24% of the general population.[1] NAFLD has been thought to be a relatively benign disease compared with chronic liver diseases caused by hepatitis B virus (HBV) or hepatitis C virus (HCV). However, a recent study indicated that some patients with nonalcoholic fatty liver have ongoing liver injury that may progress from steatosis to steatohepatitis or fibrosis.[2]

NAFLD is more common among patients with obesity, type 2 diabetes mellitus, and dyslipidemia.[2,3] Although the cause of NAFLD is not known, recent studies showed that most patients with NAFLD have hyperinsulinemia and insulin resistance regardless of the severity of liver inflammation.[4,5] These findings indicate the importance of insulin resistance as a causal role in the development of NAFLD.

Recently we noted that some younger people at one workplace had elevated liver enzyme levels in their routine medical examinations.[6,7] Among those aged 20 to 29 years, an abnormal liver function test was the only frequent finding. Since the majority of these workers were nonhabitual drinkers with no history of habitual drug use, showing negative for HBV, HCV, and markers of autoimmune hepatitis, it appears that they had NAFLD.[8]

Several reports indicate that polymorphism of β_3-adrenergic receptor (β3-AR) is related to insulin resistance or visceral fat obesity.[9-15] Therefore, we were interested in understanding the relationship between β3-AR polymorphism and alanine aminotransferase (ALT) level elevation in the development of fatty liver. Moreover, since chronic alcohol intake and acetaldehyde have been reported to be closely related with insulin resistance[16-18] and 40% of the Japanese population have a polymorphism of the aldehyde dehydrogenase 2 (ALDH2) gene, which plays a major role in the metabolism of ethanol in the liver, ALDH2 polymorphism may be another candidate for elevated liver enzymes. Additionally, we observed that inactive ALDH2 worsens glycemic control in patients with type 2 diabetes mellitus who drink low to moderate amounts of alcohol.[19]

Thus, the present study was designed to investigate the association between the clinical features of workers with elevated activity of liver enzymes and the involvement of the ALDH2 and β3-AR genes in liver dysfunction among young workers.

MATERIALS AND METHODS

Study Subjects

The subjects were 148 employees working at a car sales company in Japan. Since workers are required by law in Japan to have a routine health examination including blood chemistry at 35 years of age, we selected subjects who were 35 years of age who visited the medical clinic in the company between September 1, 1998, and July 31, 1999. Although about 400 workers at the company were 35 years of age during the period of investigation, approximately 50% of the workers

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preferred to go to a hospital for a complete medical examination, while the remaining workers visited the clinic at the company. The 148 workers who visited the clinic at the company during the period in question and who agreed to participate took part in the present study.

The industrial physician explained the purpose of the study to each worker. Each subject was asked to provide information on past medical history of illnesses and to fill out questionnaires regarding drinking and smoking habits, diet, health conditions, physical activity, sleeping time, and current drug use. The subjects underwent a physical examination, conventional laboratory tests including urinalysis, peripheral blood examination (red and white blood cell counts, hemoglobin), clinical chemistry (blood sugar level, total cholesterol, triglyceride, high-density lipoprotein [HDL]-cholesterol, creatinine, aspartate aminotransferase [AST], ALT, γ-glutamyl transpeptidase [γ-GTP]), electrocardiogram, and chest x-rays.

No subject had any health complaints, and no subject was undergoing therapy for hypertension, dyslipidemia, or diabetes mellitus. All subjects agreed to undergo genetic testing of their ALDH2 and β3-AR genes. All subjects signed informed consent forms to participate in this study. The ethics committee of the Tokai University School of Medicine, as well as the health care committee of the company approved the study protocol.

Genotype Analysis of the ALDH2 and β3-AR Genes

Genomic DNA was prepared from leukocytes using QIAamp DNA Blood kit (QIAGEN, Tokyo, Japan), and the genotypes of the ALDH2 and β3-AR genes of all subjects were analyzed. The genotype of the ALDH2 gene was examined by polymerase chain reaction–restriction fragment length polymorphism (PCR-RFLP), using the procedure described by Yokoyama et al with the restriction enzyme *Mbo*II.[20] Subjects with the ALDH2*1/*1 genotype were classified as having active ALDH2, and subjects with the ALDH2*1/*2 or ALDH2*2/*2 genotype were classified as having inactive ALDH2, as the ALDH2*1/*2 genotype shows nearly null activity compared to that of the ALDH2*1/*1 genotype.[20]

The β3-AR gene was analyzed for the Trp64Arg polymorphism by PCR-RFLP using the procedure described by Widen et al[14] with the restriction enzyme *Bst*OI. In most of the statistical analyses, we combined the Arg/Arg and Trp/Arg genotypes because the frequency of the Arg/Arg genotype was low.

Alcohol Drinking and Smoking Habits

The subjects filled out questionnaires about alcohol drinking and smoking habit. To avoid underreporting the amount of alcohol or tobacco and obtain accurate data, we explained to the subjects that the questionnaires would be used only for research purposes.

Regarding drinking habit, the questionnaire asked about the number of drinking days per week and daily alcohol intake. This was classified into 4 groups: none; less than 30 g of alcohol per day; 30 g per day; and more than 30 g per day. The amount of alcohol intake was calculated using the following standards: one 350-mL can of beer, one 120-mL glass of table wine, and one 90-mL cup of Japanese sake each contains 10 g of alcohol.[1]

Questions on smoking included the number of cigarettes per day and smoking history.

Evaluation of Liver Dysfunction

The serum levels of three liver enzymes, ie, AST, ALT, and γ-GTP, were used to determine liver dysfunction. A subject was considered to have liver dysfunction if AST was greater than 40 IU/L, ALT greater than 40 IU/L, and/or γ-GTP greater than 60 IU/L.

Table 1. Characteristics of the 148 Male Subjects Who Underwent Routine Physical Examination

	Mean ± SD
Age (yr)	35.2 ± 0.4
Height (cm)	171.3 ± 6.1
Body weight (kg)	69.0 ± 11.1
Body mass index (kg/m²)	23.5 ± 3.4
Systolic blood pressure (mm Hg)	127.9 ± 13.2
Diastolic blood pressure (mm Hg)	81.1 ± 9.3
AST (IU/L)	23.4 ± 13.4
ALT (IU/L)	28.0 ± 21.4
γ-GTP (IU/L)	30.6 ± 25.5
Fasting plasma glucose (mg/dL)	91.3 ± 8.9
Creatinine (mg/dL)	0.9 ± 0.1
Total cholesterol (mg/dL)	207.7 ± 39.0
HDL-cholesterol (mg/dL)	59.1 ± 11.5
Triglyceride (mg/dL)	118.7 ± 80.7
No. of subjects (%)	
Obesity*	51 (34.5%)
Hypertension†	39 (26.4%)
Dyslipidemia‡	71 (48.0%)
Impaired glucose tolerance§	6 (4.1%)

*If BMI was >24.2, which is 10% higher than the normal BMI.

†If systolic blood pressure was >140mm Hg or diastolic pressure was >90 mm Hg.

‡If total cholesterol level was >220 mg/dL or triglyceride level was >150 mg/dL.

§If fasting plasma glucose level was >110 mg/dL.

Evaluation of the Components of Metabolic Syndrome

We evaluated the subjects for obesity, hypertension, dyslipidemia, and impaired glucose tolerance as the risk factors for multiple risk factor syndrome, and the upper and lower limits for the risk factors are shown in parentheses. We categorized the subjects into the following 3 groups according to body mass index (BMI): lean (BMI $<$ 19.8; lower than 10% of normal BMI, 22), normal (19.8 $\leq$ BMI $<$ 24.2), and obese (24.2 $<$ BMI; higher than 10% of normal BMI, 22). Hypertension (systolic blood pressure $\geq$ 140 mm Hg and/or diastolic blood pressure $\geq$ 90 mm Hg), dyslipidemia (total cholesterol level $\geq$ 220 mg/dL and/or triglyceride level $\geq$ 150 mg/dL), and impaired glucose tolerance (fasting plasma glucose level $\geq$ 110 mg/dL) were also measured.

Statistical Analyses

Categorical variables were assessed by the chi-square test or Fisher's exact test. Quantitative values are expressed as the mean ± SD. The significance of differences between groups was analyzed by the Student's unpaired *t* test, Mann-Whitney *U* test, 2-way analysis of variance (ANOVA), and post hoc tests. Logistic regression analysis was performed to predict factors that are associated with an elevated ALT level, and the factors examined were ALDH2 genotype, β3-AR genotype, and laboratory and physical data. A level of $P < .05$ was considered significant. All analyses were performed with the computer program StatView 5.0 (SAS Institute, Cary, NC), except for the logistic regression analysis, which was performed with SPSS 10.0 (SPSS Inc, Tokyo, Japan).

RESULTS

Characteristics of the 148 Male Subjects

The physical and clinical chemistry data of the 148 male subjects are summarized in Table 1. None of the subjects had

Table 2. Comparison of Clinical Data of Subjects Who Did or Did Not Have Liver Dysfunction

	Subjects With Liver Dysfunction	Subjects Without Liver Dysfunction	*P* Value*
No. of subjects	28	120	
BMI (kg/m^2)†	26.4 ± 3.5†	22.8 ± 3.0	<.0001
Systolic blood pressure (mm Hg)	135.0 ± 11.1	126.3 ± 13.1	.0003
Diastolic blood pressure (mm Hg)	87.2 ± 7.9	79.7 ± 9.0	<.0001
Fasting plasma glucose (mg/dL)	92.5 ± 8.8	91.0 ± 8.9	.578
Total cholesterol (mg/dL)	230.0 ± 42.1	202.5 ± 36.5	.0030
Triglyceride (mg/dL)	199.8 ± 113.6	99.8 ± 56.7	<.0001
	No. of subjects (%)		
Obesity‡	20 (71.4%)	31 (25.8%)	<.0001
Hypertension§	16 (57.1%)	23 (19.2%)	<.0001
Dyslipidemia¶	23 (82.1%)	48 (40.0%)	<.0001
Impaired glucose tolerance‖	2 (7.1%)	4 (3.3%)	.357

*P values were determined by Mann-Whitney U test (upper table) or chi-square test and Fischer's exact test (lower table).
†Values are mean ± SD
‡If BMI was >24.2, which is 10% higher than the normal BMI.
§If systolic blood pressure was >140 mm Hg or diastolic pressure was >90 mm Hg.
¶If total cholesterol level was >220 mg/dL or triglyceride level was >150 mg/dL.
‖If fasting plasma glucose level was >110 mg/dL.

chronic liver disease caused by HBV or HCV infection. Of the 148 subjects, 28 had liver dysfunction according to the criteria described earlier. They included 8 subjects with elevated AST level (5.4%), 23 with elevated ALT level (15.5%), and 14 with elevated γ-GTP level (9.5%). The subjects with elevated liver enzymes underwent ultrasonography to confirm the fatty infiltration of the liver. Excluding other liver diseases such as autoimmune and drug-induced, and known metabolic disorders by further serological tests, and based on the questionnaire responses regarding drinking habit (<30 g of alcohol per day), the majority of subjects were diagnosed as having nonalcoholic fatty liver. The percentages of subjects who had obesity, hypertension, dyslipidemia, or impaired glucose tolerance were 34.5%, 26.4%, 48.0%, and 4.1%, respectively. There was 1 patient with abnormal liver enzymes who did not have any of the 4 components of metabolic syndrome, ie, hypertension, dyslipidemia, glucose intolerance, and obesity.

Comparison of Clinical Data Between Subjects With or Without Liver Dysfunction

Twenty-eight subjects (18.9%) had liver dysfunction. BMI, blood pressure, total cholesterol level, and triglyceride level of the subjects with liver dysfunction were significantly higher than the respective values among the subjects without liver dysfunction (Table 2). The proportion of subjects with obesity, hypertension, and dyslipidemia was significantly high in the liver dysfunction group (Table 2).

Alcohol Drinking and Smoking Habits

One hundred thirty-four subjects filled out completely the questionnaire regarding drinking habits, and the same number of subjects answered completely the questions regarding smoking (Table 3). There were no significant differences in the level of alcohol consumption per week (P = .2028), amount of daily alcohol intake (P = .7420), and smoking (never; former; <20 cigarettes per day; >20 cigarettes per day) (P = .2500), between those who did and those who did not have liver dysfunction.

Comparison of Liver Function Among the Four Groups Classified According to the Number of Components of Metabolic Syndrome

Forty-nine (33.1%) of the 148 subjects had no component, 49 (33.1%) had one component, 35 (23.6%) had 2 components, 12 (8.1%) had 3 components, and 3 (2.0%) had 4 components of metabolic syndrome (Table 4). Ninety-nine subjects (66.9%) had 1 or more components of metabolic syndrome. The AST, ALT, and γ-GTP levels gradually increased as the number of components of metabolic syndrome increased (AST, P = .0003; ALT, P < .0001; γ-GTP, P < .0001, ANOVA). Subjects with 2 or more components of the metabolic syndrome

Table 3. Comparison of Alcohol Intake and Smoking Habit of Subjects Who Did or Did Not Have Liver Dysfunction

	Subjects With Liver Dysfunction	Subjects Without Liver Dysfunction
Alcohol intake	n = 21	n = 113
Days/week	3.1 ± 2.3	3.9 ± 2.5
Amount/day		
0	4 (19.0%)	17 (15.0%)
<30 g ethanol	9 (42.9%)	46 (40.7%)
=30 g ethanol	4 (19.0%)	34 (30.1%)
>30 g ethanol	4 (19.0%)	16 (14.2%)
Smoking	n = 22	n = 112
Never	8 (36.4%)	36 (32.1%)
Former	4 (18.2%)	7 (6.3%)
Current		
≤20 cigarettes/d	8 (36.4%)	56 (50.0%)
>20 cigarettes/d	2 (0.9%)	13 (11.6%)

Table 4. Accumulation of Risk Factors and The Levels of AST, ALT, and γ-GTP

	No. of Risk Factors				
	0	1	2	≥3	*P* Value
No. of subjects	49	49	35	15	
AST (IU/L)	19.8 ± 4.7	20.4 ± 6.3	30.3 ± 23.4*	28.8 ± 11.0	.0003
ALT (IU/L)	18.3 ± 8.3	22.5 ± 14.5	41.5 ± 28.8†	45.9 ± 24.2‡	<.0001
γ-GTP (IU/L)	17.8 ± 9.3	28.4 ± 20.0	47.5 ± 32.8§	40.5 ± 33.7¶	<.0001

NOTE. Each value represents mean ± SD. Risk factors were defined by obesity, hypertension, dyslipidemia, and impaired glucose tolerance as the components of metabolic syndrome.

*P = .0038 *v* subjects without risk factors by Scheffe's test.

†P < .0001 *v* subjects without risk factors by Scheffe's test.

‡P < .0001 *v* subjects without risk factors by Scheffe's test.

§P < .0001 *v* subjects without risk factors by Scheffe's test.

¶P = .0124 *v* subjects without risk factors by Scheffe's test.

had higher values on the liver function test than those without risk factors.

Association of Liver Dysfunction With Frequency of ALDH2 and β3-AR Polymorphisms

Table 5 shows the association of liver dysfunction with the ALDH2 and β3-AR genotypes. Active ALDH2 tended to be seen more frequently among those with liver dysfunction than among those who did not have liver dysfunction, although the difference was not significant (P = .0745, χ^2 test). No relationship between β3-AR gene polymorphism and liver function was evident among the subjects.

Association of Liver Dysfunction With Frequency of ALDH2 and β3-AR Polymorphisms Among Those With Normal BMI

Subjects with normal BMI (19.8 ≤ BMI < 24.2) and elevated ALT level (>40) had active ALDH2 and the Arg/Arg or Trp/Arg genotype of β3-AR (Table 6). In the lean and obese BMI groups, there was no correlation between liver dysfunction and prevalence of β3-AR or ALDH2 polymorphism.

Table 5. Prevalence of Genotype of ALDH2 and β3-AR Genes in Subjects with or without Liver Dysfunction

	Subjects With Liver Dysfunction (n = 28)	Subjects Without Liver Dysfunction (n = 120)	
ALDH2 genotype			
ALDH2*1/*1	19 (67.9%)	59 (49.2%)	
ALDH2*1/*2	8 (28.6%)	55 (45.8%)	
ALDH2*2/*2	1 (3.6%)	6 (5.0%)	
ALDH2 activity			
Active (ALDH2*1/*1)	19 (67.9%)	59 (49.2%)	χ^2 = 3.18
Inactive (ALDH2*1/*2 + ALDH2*2/*2)	9 (32.1%)	61 (50.8%)	P = .075
β3-AR genotype			
Trp64Trp	17 (60.7%)	85 (70.8%)	
Arg64Trp	11 (39.3%)	28 (23.3%)	
Arg64Arg	0 (0.0%)	7 (5.8%)	
β3-AR allele			
Trp64	45 (80.4%)	198 (82.5%)	χ^2 = 0.14
Arg64	11 (19.6%)	42 (17.5%)	P = .709

Association of ALT Level With the ALDH2 and β3-AR Genotypes in the Normal BMI Group (19.8 ≤ BMI < 24.2)

We analyzed the relationship between ALT level and ALDH2 or β3-AR genotype in the normal BMI group by 2-way ANOVA and post hoc test. The ALT level of the subjects with the Arg/Arg or Trp/Arg genotype of β3-AR was significantly higher than that of the subjects with the Trp/Trp genotype of β3-AR (P = .0390, Scheffe's test) (Fig 1). The ALT level of the subjects with active ALDH2 was significantly higher than that of the subjects with inactive ALDH2 (P = .0286, Scheffe's test) (Fig 1).

The AST level was significantly higher among those with the Arg/Arg or Arg/Trp genotype of β3-AR than among those with the Trp/Trp genotype (β3-AR, P = .0257; ALDH2, p = .1404, Scheffe's test), and the γ-GTP level was significantly higher among those with active ALDH2 than among those with inactive ALDH2 (β3-AR, P = .1556; ALDH2, P = .0351, Scheffe's test).

Logistic Regression Analysis to Predict Factors Associated With Elevated ALT Level

Logistic regression analysis was performed to determine factors that are independently associated with elevated ALT level. Variables included BMI, total cholesterol level, triglyceride level (continuous variables), alcohol and smoking habits, and the ALDH2 and β3-AR genotypes (categorical variables). By forward stepwise variable selection, the BMI, triglyceride level, and active ALDH2 genotype were independently associated with an elevated ALT level (Table 7).

DISCUSSION

The growing epidemic of noncommunicable diseases can be seen not only in developed countries but also in less-developed countries by rapid demographic and lifestyle changes including alcohol drinking habit, high calorie intake, low physical activity, and stress.[21] These changes in lifestyle lead to obesity, hyperlipidemia, hyperglycemia, hypertension, and other metabolic disorders, including NAFLD. It has been suggested that insulin resistance is a basic condition in these systemic metabolic disorders.[22] The subjects with liver dysfunction in the present study had elevated values of BMI, blood pressure, total

Table 6. Relationship Between Elevated ALT and the ALDH2 and β3-AR Genotypes

ALDH2 genotype	Active		Inactive	
β3-AR genotype	Arg64Arg Trp64Arg	Trp64Trp	Arg64Arg Trp64Arg	Trp64Trp
All subjects (n = 148)				
ALT >40 IU/L (n = 23)	6 (26.1%)	9 (39.1%)	3 (13.0%)	5 (21.7%)
ALT ≤40 IU/L (n = 125)	21 (16.8%)	42 (33.6%)	16 (12.8%)	46 (36.8%)
19.8 ≤ BMI < 24.2 (n = 80)				
ALT >40 IU/L (n = 4)	4 (100.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)
ALT ≤40 IU/L (n = 76)	12 (15.8%)	27 (35.5%)	11 (14.5%)	26 (34.2%)

cholesterol, and triglyceride, which are components of the metabolic or X syndrome, compared with the respective values in the subjects without liver dysfunction (Table 2). As shown in Table 4, AST, ALT, and γ-GTP levels gradually increased as the number of components of metabolic syndrome increased. These results suggest that a finding of liver dysfunction among workers in the course of routine physical examination at the workplace is relevant to metabolic syndrome.

One of the limitations of our study is the lack of histological confirmation of the liver disease. However, our patients with elevated liver enzymes were not heavy drinkers, the ratio of AST to ALT was less than 1, and they did not show evidence of viral, metabolic, or autoimmune liver disease, and all them had evidence of fat deposition in the liver by ultrasound. Thus, it is very likely that these patients had NAFLD as the cause of abnormal liver enzymes. Furthermore, although a minimal effect of alcohol cannot be ruled out completely, obesity, but not alcohol drinking, was associated with elevated ALT by logistic regression.

As the present study is a cross-sectional study, we cannot draw any conclusion about a direct relationship between liver dysfunction and obesity. It is now becoming clear that in East Asian populations many subjects with type 2 diabetes with insulin resistance are not obese.[23] And in a preliminary study, workers who gained weight of more than 10% during the past 5 years had a higher ALT level than workers whose body weight remained at nearly the same level during the same period. The above leads to a hypothesis that Asians tend to reveal insulin resistance even with a small increase in body weight.

Other factors, especially genetic factors, may participate in the development of fatty liver, since not all subjects who had gained weight revealed liver dysfunction. We focused on 2 genes that are assumed to be candidates for the development of fatty liver. The β3-AR gene polymorphism has been thought to be related to visceral obesity and insulin resistance.[11-15] Several reports have described a relationship between fatty liver and insulin resistance.[2,24] Therefore, β3-AR gene polymorphism may be involved in the development of fatty liver. We selected the ALDH2 gene as another candidate gene for fatty liver, because the ALDH2 genotype is related to the alcohol drinking habit.[25-27] The present study showed a high prevalence of active ALDH2 among workers with an elevated ALT level. Our results are similar to those of a study that revealed that habitual drinkers with active ALDH2 showed liver dysfunction more often than the subjects with inactive ALDH2.[28] ALDH2 may participate in the metabolism of not only alcohol but also other chemicals with aldehyde radicals, resulting in the alteration of lipid metabolism.

It is interesting to note that the Trp64Arg polymorphism of the β3-AR gene and active ALDH2 were each independently associated with elevated ALT level (Fig 1, β3-AR, $P = .0408$; ALDH2, $P = .0271$, Scheffe's test) among the subjects with normal body weight in the present study. Moreover, all 4 individuals with elevated ALT level and normal BMI had the active ALDH2 genotype and the Arg genotype of β3-AR (Arg genotype means Arg/Arg or Trp/Arg) as shown in Table 6. These results suggest that active ALDH2 and the Arg genotype

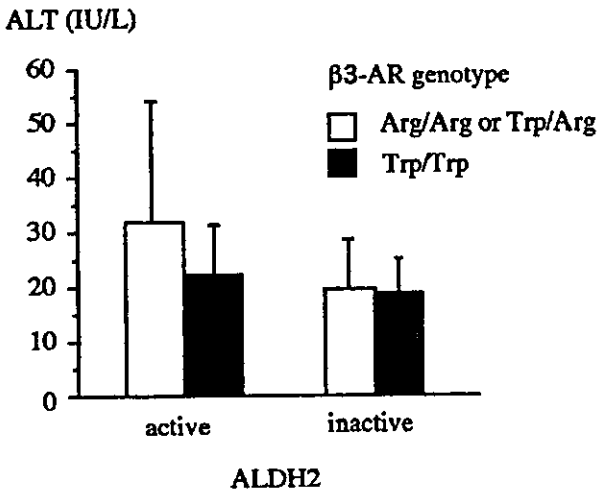

Fig 1. Comparison of the ALT level according to ALDH2 activity and β3-AR polymorphism among workers with normal BMI (19.8 ≤ BMI < 4.2) (n = 80). ALT level was significantly higher among those with active ALDH2 than among those with inactive ALDH2. ALT level was also significantly higher among those with the Arg/Arg or Trp/Arg genotype of β3-AR than among those with the Trp/Trp genotype. (β3-AR, $P = .0390$; ALDH2, $P = .0286$, Scheffe's test).

Table 7. Multiple Regression Analysis With Elevated ALT as the Response Variable

Explanatory Variable	Odds Ratio (95% CI)	P Value
BMI	1.564 (1.241-1.991)	<.001
Triglyceride	1.011 (1.004-1.018)	.002
ALDH2 (active)	6.390 (1.031-39.628)	.046

Abbreviation: CI, confidence interval.

of β3-AR are involved in the elevation of ALT level in males by a mechanism other than obesity. Although we did not measure the waist/hip ratio in the present study, visceral obesity in relation to the development of fatty liver in this group should be further investigated.

In conclusion, our study suggests that the genotypes of ALDH2 and β3-AR may be involved in the pathogenesis of abnormal liver enzymes, possibly due to NAFLD. Evaluating ALDH2 and β3-AR genotypes may be useful for predicting and preventing NAFLD as a component of metabolic syndrome.

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Interferon Alfa Down-regulates Collagen Gene Transcription and Suppresses Experimental Hepatic Fibrosis in Mice

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The equilibrium between the production and degradation of collagen is rigorously controlled by a number of growth factors and cytokines. Interferon alfa (IFN-α) is now widely used for the treatment of chronic hepatitis C, which can improve serum levels of fibrotic markers and the degree of hepatic fibrosis, not only in patients who responded to therapy but also in those in whom it is ineffective. These findings may suggest that IFN-α possesses direct antifibrotic effects in addition to its antiviral activity. However, in contrast to IFN-γ, which has been shown to suppress collagen gene transcription, little is known about the mechanisms responsible for the antifibrotic effects of IFN-α. Here, we report that IFN-α, when administered into transgenic mice harboring the α2(I) collagen gene (COL1A2) promoter sequence, significantly repressed promoter activation and prevented the progression of hepatic fibrosis induced by carbon tetrachloride injection. Transient transfection assays indicated that IFN-α decreased the steady-state levels of COL1A2 messenger RNA (mRNA) and inhibited basal and TGF-β/Smad3-stimulated COL1A2 transcription in activated hepatic stellate cells (HSC). These inhibitory effects of IFN-α on COL1A2 transcription were exerted through the interaction between phosphorylated Stat1 and p300. Blocking of the IFN-α signal by overexpressing the intracellular domain-deleted IFN receptor increased basal COL1A2 transcription and abolished the inhibitory effects of IFN-α. In conclusion, our results indicate that IFN-α antagonizes the TGF-β/Smad3-stimulated COL1A2 transcription *in vitro* and suppresses COL1A2 promoter activation *in vivo*, providing a molecular basis for antifibrotic effects of IFN-α. (HEPATOLOGY 2003;38:890-899.)

Abbreviations: IFN, interferon; ALT, alanine aminotransferase; COL1A1 and COL1A2, genes coding for the α1 and α2 chains of type I collagen, respectively; TGF-β, transforming growth factor β; TbRE, TGF-β–responsive element; HSC, hepatic stellate cell(s); kb, kilobase(s); 2,5-AS, 2', 5'-oligoadenylate synthetase; DMEM, Dulbecco's modified Eagle medium; RT-PCR, reverse-transcriptase polymerase chain reaction; IFNAR1 and IFNAR2, the 2 chains of the type I IFN receptor; GAPDH, glyceraldehydes-3-phosphate dehydrogenase.

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Hepatic fibrosis is a pathologic condition characterized by a marked deposition of collagen and other components of the extracellular matrix in the liver.[1] This eventually results in cirrhosis, in which excessive deposition of extracellular matrix proteins causes hepatic failure because of malfunction of hepatocytes and hemodynamic alterations leading to portal hypertension. In addition, the frequent association of hepatocellular carcinoma, especially in cirrhotic patients infected with hepatitis C virus, is also an important clinical issue that has been difficult to deal with.[2,3] Interferon alfa (IFN-α) is now widely used for the treatment of chronic hepatitis C, aiming at the clearance of the virus and a decrease in the serum levels of alanine aminotransferase (ALT). Only a limited number of patients, however, actually respond to IFN-α therapy in terms of viral clearance.[2,3] It is therefore important to suppress the progressive fibrosis and prevent the subsequent occurrence of hepatocellular carcinoma in those patients who did not respond to IFN treatment.

It has been reported that IFN-α therapy results in an improvement in the serum levels of fibrotic markers such as the N-terminal propeptide of procollagen type III, not only in the patients who responded to the therapy but also in those who did not respond.[4,5] In addition, quantitative histopathologic analyses of paired liver biopsy specimens have shown some improvement in the degree of fibrosis following IFN-α therapy irrespective of the initial virologic response.[6-9] These results may suggest that IFN-α has direct antifibrotic effects in addition to its antiviral activity. However, in contrast to IFN-γ, which has been intensively studied regarding its inhibitory actions on collagen gene expression, little is known about the mechanisms responsible for the antifibrotic effects of IFN-α.

Type I collagen, the major component of the extracellular matrix in the fibrotic liver, is a heterotrimer composed of 2 α1 chains and 1 α2 chain. These chains are encoded by 2 distinct genes, COL1A1 and COL1A2, respectively. Collagen gene expression is strictly controlled by a number of growth factors and cytokines, of which transforming growth factor β1 (TGF-β1, henceforth referred to as TGF-β) is the most important factor in stimulating gene transcription.[10] We previously identified a COL1A2 upstream sequence that mediates the stimulatory effect of TGF-β on gene transcription and designated this region the TGF-β–responsive element (TbRE).[11] TGF-β stimulates COL1A2 transcription via the interaction of Sp1 and Smad3, both of which bind to the TbRE.[11-13] In addition, constitutive activation of the TbRE because of increased binding of Sp1 and Smad3 has been correlated with accelerated COL1A2 transcription in activated hepatic stellate cell (HSC) clones derived from a cirrhotic liver.[14,15]

The present study was designed to determine whether IFN-α directly suppresses hepatic fibrosis and, if so, to study the molecular mechanisms responsible for this inhibitory action of IFN-α. The results indicated that administration of murine IFN-α to transgenic COL1A2 promoter/reporter mice significantly suppressed the promoter activation and prevented the progression of hepatic fibrosis induced by carbon tetrachloride injection. Several lines of *in vitro* experimental evidence using cell transfection assays and immunoprecipitation-Western blot analyses demonstrated that the inhibitory action of IFN-α is exerted, at least in part, by antagonizing the TGF-β/Smad3-stimulated COL1A2 transcription via the common pathway with IFN-γ.

Materials and Methods

Transgenic Mice and Acute and Chronic Carbon Tetrachloride Intoxication. All animals used in the present study received humane care in compliance with the National Institutes of Health guidelines. The transgenic mice contain the −17,000 to +54 region of the mouse COL1A2 gene linked to a firefly luciferase gene.[16] The strong enhancer activity present between −17.0 and −15.5 kilobases (kb) of the far-upstream sequence,[16,17] combined with the extremely sensitive luciferase reporter gene, has made it possible to quantify COL1A2 promoter activity in liver tissue.[18] Acute liver injury was induced by a single intraperitoneal injection of 0.1 mL/kg body weight of carbon tetrachloride mixed with olive oil.[18] Either 50,000 units or 5,000 units/d of natural murine IFN-α[19] or recombinant murine IFN-γ[20] or control saline was subcutaneously injected once a day after carbon tetrachloride administration. Livers were excised 72 hours later and subjected to histologic examination and luciferase assay. Hepatocellular necrosis was confirmed by measuring serum ALT levels and by histologic examination of excised liver specimens. Serum 2′, 5′-oligoadenylate synthetase (2,5-AS) activities were measured using a radioimmunoassay kit (Eiken Immunochemical Laboratory, Tokyo, Japan). Hepatic fibrosis was induced in the mice by injecting intraperitoneally the same amount of carbon tetrachloride once a week for 8 weeks.[18] The mice were treated with either 50,000 units/d of IFN-α or control saline 3 times a week throughout the carbon tetrachloride administration. Sections prepared from the excised liver specimens were stained with Sirius red F3BA, and the degree of hepatic fibrosis was semiquantified by measuring the areas of Sirius red-stained collagen fibers

with the aid of computer software as previously described.[21]

Cell Culture. Rat HSC were isolated from the livers of male Wistar rats (250- to 300 g body weight) as previously described[22] and were maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with 10 % fetal bovine serum (Gibco BRL, Grand Island, NY). CFSC-2G is an activated rat HSC clone established from a carbon tetrachloride-induced cirrhotic liver[23] and was maintained in the same media supplemented with 10% fetal bovine serum and nonessential amino acids. CF37 cells, which are primary cultures of human fetal skin fibroblasts,[11] were grown in DMEM with 10% fetal bovine serum. The regulation of COL1A2 transcription in CFSC-2G[14,15,24] and CF37[11,15,25] cells has been previously described in detail. Human embryonic kidney 293 EBNA cells were purchased from the American Type Culture Collection (Rockville, MD) and grown in DMEM supplemented with 10% fetal bovine serum.

Real-Time RT-PCR Assay. Quantitative analysis of COL1A2 messenger RNA (mRNA) expression was performed using the Taqman Model 7900 sequence detection instrument (Applied Biosystem, Foster City, CA) as previously described.[26] The mouse COL1A2 primers and probe used are as follows: forward primer, 5′-CACCCCAGCGAAGAACTCATA; reverse primer, 5′-GCCACCATTGATAGTCTCTCCTAAC; and probe, 5′-CGCCCAGGCCAACAAGCATGTC. The rat COL1A2 primers and probe used are as follows: forward primer, 5′-ATGGTGGCAGCCAGTTTGA; reverse primer, 5′-CAGGTACGCAATGCTGTTCTTG; and probe, 5′-CTCGCCTTCATGCGCCTGCTAGC. Glyceraldehydes-3-phosphate dehydrogenase (GAPDH) gene primer and probe mixture provided from the manufacturer (Applied Biosystem) were used to normalize the relative expression levels of mouse and rat COL1A2 mRNA.

Cloning of Type I IFN Receptor cDNA. Total cellular RNA was isolated from normal human peripheral blood mononuclear cells obtained using the Ficoll-Paque separation method (Amersham Pharmacia Biotech UK Ltd., Buckinghamshire, England). Coding sequences for the 2 chains of the human type I IFN receptor, IFNAR1[27] and IFNAR2,[28] were amplified by the reverse-transcriptase polymerase chain reaction (RT-PCR) method using specific oligonucleotide primers. The forward primer 5′-ATGATGGTCGTCCTCCTGGGCGCGA-3′ and the reverse primer 5′-ATTGATGCATCTCAAGAAGACTTTC-3′ were used to generate the intracellular domain-deleted IFNAR1 cDNA (GenBank Accession No. J03171). Similarly, the forward primer 5′-ATGCTTTTGAGCCAGAATGCCTTCA-3′ and the reverse primer 5′-ACCAATCCATTTCAGTGTCACTATG-3′ were used to amplify the truncated IFNAR2 sequence (GenBank Accession No. U29584). The intracellular domain-deleted receptor lacks the binding sites for the Jak1 and Tyk2 tyrosine kinases[29,30] and does not contain any tyrosine residues, which may potentially be phosphorylated. The amplified sequences were cloned into the pcDNA3 expression vector (Invitrogen Corp., Carlsbad, CA) and verified by automatic sequencing.

Chimeric Constructs and Cell Transfection Assays. The −17.0- to −15.5-kb far-upstream enhancer sequence[16,17] linked to the −350 to +54 COL1A2 promoter was generously provided by Dr. George Bou-Gharios. It was cloned into pGL3 basic luciferase vector (Promega, Madison, WI) and designated −17.0-15.5/350COL1A2/LUC. This internally deleted COL1A2 sequence linked to a β-galactosidase gene showed exactly the same pattern of expression during embryogenesis as the whole −17 kb to +54 sequence.[16,17] The −378COL1A2/LUC construct containing the −378 to +58 COL1A2 sequence linked to a firefly luciferase gene was previously described.[11] In addition to the truncated IFNAR1 and IFNAR2 expression plasmids described above, other expression plasmids used were pCMV-Sp1[31] kindly provided by Dr. Elder, pCMV-Smad3[32] from Dr. Derynck, and pcDNA-p300FLAG and pcDNA3-DNp300[33] from Dr. Kawabata. These plasmids express Sp1, Smad3, flag-tagged p300, and the dominant negative form of p300, respectively, under the control of the cytomegalovirus promoter. An empty expression vector, pcDNA3, was used as a negative control. Preparation of plasmid DNA and transfection into culture cells were performed as previously described.[34] In some experiments, transfected cells were placed in medium containing 0.1% fetal bovine serum and treated with 500 units/mL of rat or human IFN in the absence or presence of 2 ng/mL of TGF-β (Becton Dickinson Labware, Bedford, MA). The IFN used were recombinant rat IFN-α (PBL Biomedical Laboratories, New Brunswick, NJ), recombinant rat IFN-γ (Genzyme-Techne, Minneapolis, MN), and natural human IFN-α and IFN-γ (Otsuka Pharmaceutical Co. Ltd., Tokyo, Japan). Incubation with 500 units/mL of the IFN did not affect the cell numbers when the transfectants were grown in a confluent state (data not shown). Dual luciferase assays were carried out according to the manufacturer's protocol (Promega), and the transcriptional activities of the COL1A2 reporter constructs were normalized against those of cotransfected pRLCMV (Promega). To avoid competition between the cytomegalovirus promoter region of the expression plasmids and that of the pRLCMV vector, the latter was

added to the plasmid mixture at a 1:1,000 molar ratio against the former.

Western Blot Analysis. Whole cell lysates were prepared from IFN-α- or IFN-γ–treated CFSC-2G cells. Immunoblotting was performed as previously described[32] using anti-collagen type I antibodies (Polysciences, Inc., Warrington, PA) and anti-phosphoStat1 antibodies (Upstates Biotechnology, Lake Placid, NY).

Immunoprecipitation and Western Blot Analysis. To analyze interactions between phosphorylated Stat1 and p300, 293 EBNA cells were transfected with a flag-tagged p300 expression plasmid. Forty-eight hours later, transfected cells were left untreated or treated with IFN for 1 hour. Whole cell lysates were subjected to immunoprecipitation with antiflag antibodies (Sigma, St. Louis, MO), followed by immunoblotting with anti-phospho-Stat1 antibodies as previously described.[32]

Statistical Analysis. Values were expressed as mean ± SD. Either the Student's *t* test or the Mann-Whitney *U* test was used to evaluate the statistical differences between groups: a *P* value of less than .05 was considered significant.

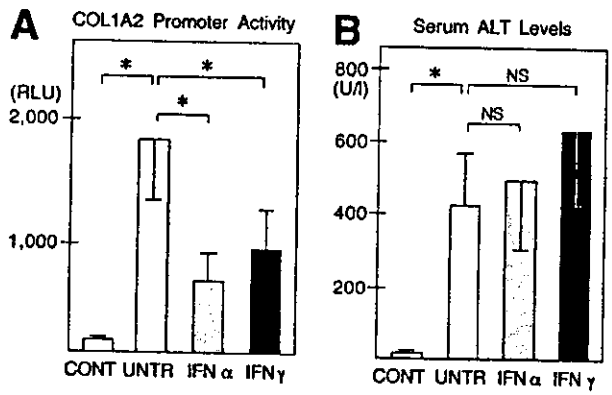

Fig. 1. Effects of IFN treatment on COL1A2 promoter activity in liver tissue following carbon tetrachloride administration. Transgenic mice harboring the COL1A2 upstream sequence linked to a firefly luciferase gene were injected intraperitoneally with 0.1 mL/kg body weight of carbon tetrachloride (CCl_4), then treated with 50,000 units/d of murine IFN-α or IFN-γ or control saline (UNTR). The mice were killed 72 hours after CCl_4 injection and subjected to (A) luciferase assays determining COL1A2 promoter activity in liver tissue and (B) measurement of the serum levels of alanine aminotransferase (ALT). Luciferase activity was normalized against the protein concentration of tissue homogenates. The values are mean ± SD obtained from 7 or 8 mice in each group and expressed relative to those in the control mice without CCl_4 injection (CONT). The **asterisk** indicates that the difference between the groups is significant. NS, not significant; RLU, relative luminescence units.

Results

IFN-α Treatment Suppresses COL1A2 Promoter Activation Following Carbon Tetrachloride Administration. We first examined whether IFN-α directly suppresses activation of COL1A2 promoter *in vivo*. For this purpose, we utilized transgenic mice harboring the −17 kb to +54 COL1A2 upstream sequence linked to a firefly luciferase gene. Activation of this COL1A2 promoter/enhancer region following acute and chronic carbon tetrachloride administration has been previously described.[18] Administration of 50,000 units/d of murine IFN-α into the mice for 3 days without carbon tetrachloride injection did not affect COL1A2 promoter activity in liver tissue or serum ALT levels (data not shown). Seventy-two hours after a single carbon tetrachloride injection, the COL1A2 promoter was activated by approximately 15-fold, and treatment of the mice with 50,000 units/d of murine IFN-α or IFN-γ significantly suppressed this promoter activation (Fig. 1A). On the other hand, there was no significant difference in the mean levels of serum ALT (Fig. 1B) or in the degree of hepatocellular necrosis and mononuclear cell infiltration in liver specimens (data not shown) among the mice treated with IFN-α, IFN-γ, or control saline. In addition, none of the mice in each group died during 72 hours after carbon tetrachloride injection. In the second set of experiments, we compared the inhibitory effects of IFN-α on COL1A2 promoter activity between the mice receiving 50,000 units/d of murine IFN-α and those receiving 5,000 units/d of the cytokine. The results showed that treatment with the lower dose (5,000 units/d) of IFN-α did not suppress activation of the COL1A2 promoter (Fig. 2A). The mean levels of serum 2,5-AS activity in mice treated with 50,000 units/d of IFN-α were significantly higher than those in mice treated with control saline (Fig. 2B). On the other hand, serum 2,5-AS activity in mice treated with 5,000 units/d of IFN-α was not fully elevated (Fig. 2B).

IFN-α Treatment Prevents the Progression of Hepatic Fibrosis. We next examined whether the inhibitory action of IFN-α on COL1A2 promoter activity prevents the progression of hepatic fibrosis induced by repeated carbon tetrachloride injections. Consistent with the results of our previous study,[18] COL1A2 promoter was continuously activated after 8 weeks of carbon tetrachloride intoxication, and treatment of the mice with 50,000 units/d of IFN-α significantly suppressed the promoter activation (Fig. 3A). This inhibition of the promoter activity was paralleled with a significant decrease in the steady-state levels of COL1A2 mRNA (Fig. 3B). In addition, semiquantitative analysis of collagen contents in liver specimens indicated that IFN-α treatment suppressed the progression of hepatic fibrosis induced by repeated carbon tetrachloride injections. The relative area of collagen deposition against the entire observation area was significantly decreased by IFN-α treatment (Fig. 3C).