rs2306283 SNPs in *SLCO1B1* were successfully genotyped in 16,664 participants who had classified themselves as having European ancestry. Among those participants, we tested the associations of the two SNPs with the risk of myopathy and with the reduction in LDL cholesterol.

STATISTICAL ANALYSIS

Standard 1-degree-of-freedom (df) (trend) and 2-df (genotypic) tests of association with genotype, and case–control odds ratios for myopathy from logistic regressions, were calculated with the use of SAS (SAS Institute, version 8) and PLINK (version 1.00).[23] For genomewide associations, uncorrected P values smaller than 5×10^{-7} were considered to provide strong evidence of an association, whereas those between 5×10^{-5} and 5×10^{-7} were considered to provide only moderate evidence.[24] Haploview was used to estimate linkage disequilibrium and plot association results.[25] Haplotype frequencies and associated risks were estimated with the use of the haplo.stats package[26] in R.[27] Physical positions and alleles are expressed in terms of the forward strand of the reference human genome (National Center for Biotechnology Information [NCBI] build 36) unless otherwise stated. Ensembl version 46, NCBI dbSNP build 127, and published reports[12,28,29] were used to classify SNP locations and coding status. The attributable risk of myopathy with 80 mg of simvastatin daily was estimated by means of a life-table analysis; participants who were taking amiodarone at baseline were excluded because their simvastatin dose was subsequently reduced, owing to a high risk of myopathy. (Further details are available in the Methods section in the Supplementary Appendix.)

RESULTS

CHARACTERISTICS OF THE PARTICIPANTS

The use of amiodarone at baseline was found to be associated with an increased risk of definite or incipient myopathy among participants who were assigned to receive 80 mg of simvastatin daily in SEARCH,[11] with a relative risk of 8.8 (95% confidence interval [CI], 4.2 to 18.4) during the first year of follow-up (Table 1). After detection of this association early in the trial, participants taking amiodarone who had been assigned to receive 80 mg of simvastatin were switched to treatment with 20 mg of simvastatin, which may explain the less extreme relative risk observed subsequently with concomitant amiodarone use. We also observed small increases in the risk of myopathy among older participants and women, as well as among those with evidence of impaired renal function and those who were taking calcium antagonists at baseline.

GENOMEWIDE ASSOCIATION STUDY

The genomewide association study involved 85 participants with suspected myopathy and 90 controls, all of whom were taking 80 mg of simvastatin daily (Table 1 in the Supplementary Appendix). Single-SNP analysis yielded one strong association of myopathy with the noncoding rs4363657 SNP located within intron 11 of *SLCO1B1* on chromosome 12 ($P=4\times10^{-9}$; $P=0.001$ with the Bonferroni correction). No associations between myopathy and SNPs in any other region yielded an uncorrected P value of less than 10^{-5} (Table 2 and Fig. 1). The prevalence of the rs4363657 C allele was 0.13 among the controls. We calculated that the odds ratio for myopathy was 4.3 (95% CI, 2.5 to 7.2) per copy of the C allele and 17.4 (95% CI, 4.8 to 62.9) among CC homozygotes as compared with TT homozygotes. There was little evidence of deviation from Hardy–Weinberg equilibrium. In addition, the results did not seem to be affected by population substructure or other potential sources of systematic deviation: the chi-square value for rs4363657 was well outside the 95% confidence interval for the quantile–quantile plot, whereas values for all of the other genotyped SNPs were within this confidence interval (see the figure and the Methods section in the Supplementary Appendix). The summary statistics for the genomewide screen are in the repository of the National Institutes of Health Genotype and Phenotype database (dbGaP) (accession number, phs000141.v1.p1).

CANDIDATE GENOTYPING AND HAPLOTYPE ANALYSIS

In light of the strong association between myopathy and rs4363657, we identified additional SNPs within *SLCO1B1* and 20 kb of flanking sequence (10 kb proximal and 10 kb distal to the gene) by resequencing or by imputation from genotyped SNPs. Table 2a in the Supplementary Appendix shows associations with myopathy for a total of 56 genotyped and 141 imputed SNPs in this region. Of these, two genotyped (and nine imputed)

Table 1. Relative Risk of Myopathy Associated with Selected Baseline Characteristics among 6031 Participants Assigned to 80 mg of Simvastatin Daily in SEARCH.

Baseline Characteristic	No. of Participants	Definite or Incipient Myopathy in First Year (N=56)		Definite or Incipient Myopathy in Later Years (N=42)		Definite or Incipient Myopathy at Any Time (N=98)	
		No.	Relative Risk (95% CI)	No.	Relative Risk (95% CI)	No.	Relative Risk (95% CI)
Age							
<65 yr	3019	17	1.0	14	1.0	31	1.0
≥65 yr	3012	39	2.3 (1.3–4.1)	28	2.0 (1.1–3.9)	67	2.2 (1.4–3.4)
Sex							
Male	5005	42	1.0	30	1.0	72	1.0
Female	1026	14	1.6 (0.9–3.0)	12	2.0 (1.0–3.9)	26	1.8 (1.1–2.8)
Estimated glomerular filtration rate							
≥60 ml/min/1.73 m^2	5209	41	1.0	30	1.0	71	1.0
<60 ml/min/1.73 m^2	822	15	2.4 (1.3–4.3)	12	2.6 (1.3–5.1)	27	2.5 (1.6–3.9)
Creatinine							
<85 μmol/liter (1.0 mg/dl)	2731	14	1.0	15	1.0	29	1.0
≥85 μmol/liter (1.0 mg/dl)	3300	42	2.5 (1.4–4.6)	27	1.5 (0.8–2.8)	69	2.0 (1.3–3.1)
Use of amiodarone							
No	5893	47	1.0	39	1.0	86	1.0
Yes	138	9	8.8 (4.2–18.4)	3	3.5 (1.1–11.6)	12	6.4 (3.4–12.1)
Use of calcium antagonists							
No	4459	29	1.0	32	1.0	61	1.0
Yes	1572	27	2.7 (1.6–4.5)	10	0.9 (0.4–1.8)	37	1.7 (1.2–2.6)
Diabetes mellitus							
No	5398	49	1.0	33	1.0	82	1.0
Yes	633	7	1.2 (0.6–2.7)	9	2.3 (1.1–4.9)	16	1.7 (1.0–2.9)

Table 2. Genomic Regions Associated with Myopathy in the Genomewide Association Study.*

Chromosome	SNP	Position	P Value for Trend (1 df)	P Value for Genotypic Test (2 df)	Risk Allele	Other Allele	Risk-Allele Frequency		P Value for Hardy–Weinberg Equilibrium among Controls	Odds Ratio (95% CI) per Risk Allele	Odds Ratio for Heterozygotes	Odds Ratio for Homozygotes	Gene
							Cases	*Controls*					
Strong evidence of association													
12p12	rs4363657	21259989	4.1×10^{-9}	2.5×10^{-8}	C	T	0.46	0.13	1.8×10^{-1}	4.3 (2.5–7.2)	4.4	17.4	*SLCO1B1*
Moderate evidence of association													
1p12	rs2490197	118656353	2.6×10^{-5}	1.4×10^{-4}	A	G	0.39	0.18	7.3×10^{-1}	3.0 (1.7–5.0)	2.7	11.4	
	rs6665507	118661127	2.7×10^{-5}	1.5×10^{-4}	T	C	0.39	0.19	7.3×10^{-1}	3.0 (1.7–5.0)	2.7	11.2	
	rs10494209	118664994	3.4×10^{-5}	1.8×10^{-4}	A	C	0.39	0.19	7.3×10^{-1}	2.9 (1.7–4.9)	2.6	11.2	
	rs6428744	118726966	3.6×10^{-5}	1.1×10^{-4}	T	C	0.48	0.26	4.1×10^{-1}	2.6 (1.6–4.2)	1.9	9.9	
2p11	rs404892	76157421	1.9×10^{-1}	4.0×10^{-5}	T	C	0.54	0.47	6.0×10^{-5}	1.3 (0.9–2.0)	5.4	1.9	
	rs7563058	76196590	1.2×10^{-1}	2.6×10^{-5}	T	C	0.55	0.46	1.1×10^{-4}	1.4 (0.9–2.1)	5.8	2.1	
2q21	rs901225	134444137	1.2×10^{-4}	4.9×10^{-5}	A	G	0.18	0.06	2.3×10^{-1}	4.3 (2.0–9.5)	5.8	0.0	
2q33	rs1975583	200934147	5.2×10^{-5}	3.2×10^{-5}	C	A	0.46	0.25	8.7×10^{-2}	2.5 (1.6–4.1)	4.3	4.4	*DNAPTP6*
	rs295129	200937718	6.7×10^{-5}	1.3×10^{-5}	G	A	0.52	0.31	2.5×10^{-2}	2.4 (1.5–3.8)	5.1	4.6	*DNAPTP6*
6p24	rs1348	10557244	3.9×10^{-5}	1.8×10^{-4}	C	T	0.32	0.13	$1.0\times10^{+0}$	3.0 (1.7–5.1)	2.4	19.4	
7p15	rs17160152	31449058	2.6×10^{-5}	1.5×10^{-4}	T	C	0.25	0.08	$1.0\times10^{+0}$	3.9 (2.0–7.7)	3.6	†	
9p23	rs610789	9578336	1.6×10^{-2}	3.1×10^{-5}	A	G	0.71	0.59	3.1×10^{-2}	1.7 (1.1–2.7)	0.3	1.4	*PTPRD*
	rs598356	9578824	2.6×10^{-2}	2.7×10^{-5}	T	G	0.71	0.59	3.1×10^{-2}	1.6 (1.1–2.6)	0.3	1.3	*PTPRD*
	rs656074	9589879	1.5×10^{-2}	2.3×10^{-5}	T	C	0.70	0.57	3.0×10^{-2}	1.7 (1.1–2.7)	0.3	1.6	*PTPRD*
9p23	rs986974	13805721	4.6×10^{-5}	1.7×10^{-4}	C	T	0.26	0.09	5.7×10^{-1}	3.6 (1.9–6.8)	3.9	6.6	
9q31	rs1516895	107923972	3.7×10^{-5}	1.3×10^{-4}	T	C	0.84	0.65	1.0×10^{-1}	3.2 (1.8–5.5)	1.7	6.1	
10q21	rs10826143	59467137	2.0×10^{-2}	3.6×10^{-5}	C	T	0.55	0.43	5.4×10^{-2}	1.7 (1.1–2.7)	6.3	3.1	
12q24	rs1859836	124791023	1.2×10^{-3}	3.3×10^{-5}	T	C	0.72	0.57	1.2×10^{-3}	2.2 (1.4–3.7)	0.5	2.2	
	rs1558062	124819460	1.1×10^{-3}	2.9×10^{-5}	A	G	0.68	0.51	2.9×10^{-3}	2.2 (1.4–3.5)	0.6	3.1	
15q23	rs6494940	69592172	1.2×10^{-4}	4.5×10^{-5}	A	G	0.88	0.71	1.2×10^{-1}	3.1 (1.7–5.6)	0.5	2.1	*THSD4*
17p12	rs2323178	14242338	2.2×10^{-5}	7.0×10^{-5}	C	T	0.60	0.36	6.5×10^{-1}	2.6 (1.6–4.1)	1.8	7.0	
17q12	rs11870629	28870661	3.9×10^{-5}	2.0×10^{-4}	G	A	0.27	0.10	5.9×10^{-1}	3.5 (1.9–6.6)	3.0	†	*ACCN1*
19q13	rs1618536	50563446	1.7×10^{-5}	5.5×10^{-5}	T	C	0.58	0.35	1.7×10^{-1}	2.7 (1.7–4.4)	2.0	8.2	*ERCC2*
20q11	rs6059106	31188763	4.5×10^{-5}	2.0×10^{-4}	A	G	0.34	0.16	1.1×10^{-1}	3.2 (1.8–5.8)	2.8	†	
20q13	rs1355598	51358916	9.0×10^{-1}	3.3×10^{-5}	A	C	0.30	0.29	4.1×10^{-2}	1.0 (0.6–1.7)	2.5	0.0	*TSHZ2*

* P values lower than 5×10^{-7} were considered to provide strong evidence of an association, whereas those between 5×10^{-5} and 5×10^{-7} were considered to provide moderate evidence of an association.

† Odds ratios for homozygotes were not calculated when there were no rare-allele homozygotes among the controls.

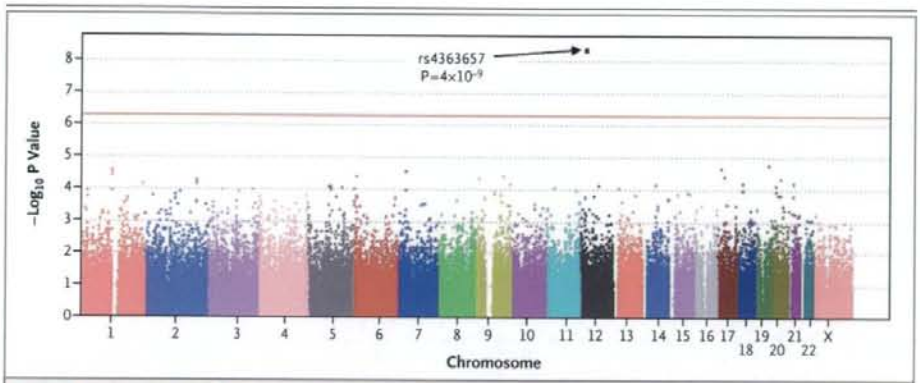


Figure 1. Results of Tests for a Trend in the Association between Myopathy and Each SNP Measured in the Genome-wide Association Study.

P values are shown for each SNP measured among 85 participants with myopathy and 90 matched controls who were taking 80 mg of simvastatin daily. Analyses are based on 316,184 of the 318,237 SNPs (99.4%) on the Sentrix HumanHap300-Duo BeadChip (Illumina). A result above the horizontal red line indicates strong evidence of an association ($P<5\times10^{-7}$).

SNPs were in nearly complete linkage disequilibrium with rs4363657 ($r^2>0.95$ for each). But among them, only rs4149056 (Val174Ala) in exon 6 was "nonsynonymous" (i.e., altering the encoded protein): the prevalence of its C allele was 0.13 among controls, with odds ratios for myopathy of 4.5 (95% CI, 2.6 to 7.7) per copy of the C allele and 16.9 (95% CI, 4.7 to 61.1) among CC homozygotes as compared with TT homozygotes ($P=2\times10^{-9}$, with four missing results imputed).

We identified five other nonsynonymous variants in *SLCO1B1*, including three that were relatively common: rs2306283 (44% frequency of the G allele in controls), rs11045819 (18% frequency of the A allele), and rs34671512 (8% frequency of the C allele) (Table 2a in the Supplementary Appendix). There was only moderate linkage disequilibrium between rs4149056 and these three variants ($r^2<0.20$ for each pairwise comparison). In haplotypes with rs4149056, both the rs2306283 G allele and the rs3471512 C allele were associated with lower risks of myopathy that were of borderline significance ($P=0.03$ and $P=0.06$, respectively), whereas rs11045819 did not appear to influence the risk.

SUBGROUP FINDINGS FOR rs4149056

The odds ratios for myopathy associated with rs4149056 did not differ significantly according to whether the myopathy was definite or incipient or according to baseline age, sex, estimated glomerular function, or use or nonuse of amiodarone, although the study had limited power to detect modest differences (Fig. 2). When we excluded the four participants who were identified as having potentially different ancestry, the P value for rs4149056 changed only slightly, from 2.4×10^{-9} to 2.0×10^{-9} (and there was no alteration in the genomic regions classified as strongly significant).

ATTRIBUTABLE RISK OF MYOPATHY

We selected controls on the basis of the fact that myopathy had not developed in them, and hence, there was a lower probability that they had the rs4149056 C allele, which is associated with an increased risk of myopathy. After allowing for this selection bias, the population prevalence of the C allele was estimated to be 0.15 (which is consistent with the range of 0.14 to 0.22 reported previously among people of European ancestry[27]). On the basis of this prevalence, a life-table analysis was used to estimate the cumulative risk of myopathy among participants taking 80 mg of simvastatin daily, according to their status with respect to the rs4149056 genotype (Fig. 3). CC homozygotes had an 18% cumulative risk, with myopathy occurring primarily during the first year, whereas the CT genotype was associated with a cumulative risk of about 3%. In contrast, the cumulative risk of myopathy was only 0.6% among TT homozygotes who were taking 80 mg of simvastatin. Overall, more than 60% of these myo-

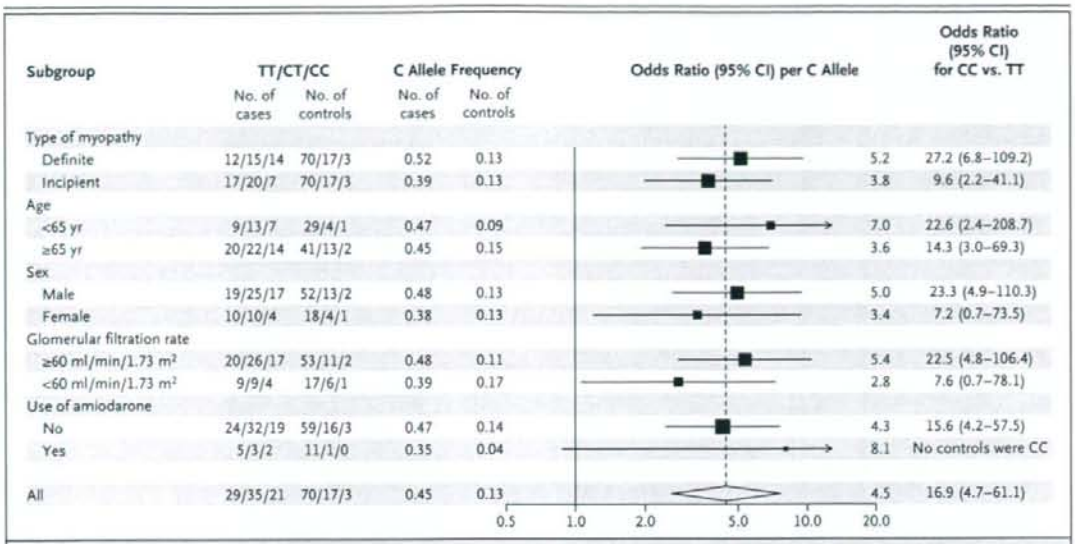


Figure 2. **Odds Ratios for Myopathy Associated with the *SLCO1B1* rs4149056 Genotype among Subgroups of Patients Taking 80 mg of Simvastatin Daily.**

Black squares indicate odds ratios (with area proportional to the amount of statistical information in each subdivision), and horizontal lines indicate 95% CIs (ending with an arrowhead when the CI extends beyond the scale). The overall odds ratio and its 95% CI are indicated by an unshaded diamond.

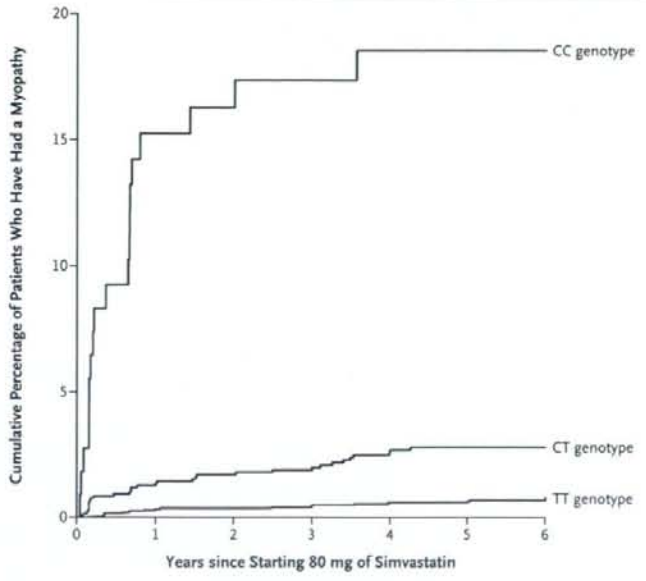
pathy cases could be attributed to the rs4149056 C variant in *SLCO1B1*.

REPLICATION IN THE HEART PROTECTION STUDY

Among 16,664 genotyped participants in the Heart Protection Study,[29] the rs4149056 and rs2306283 variants were not associated with significant differences in pretreatment LDL cholesterol levels (Table 3 in the Supplementary Appendix). Before randomization, all of these participants took 40 mg of simvastatin daily for 4 to 6 weeks, and the mean (±SE) reduction in the LDL cholesterol level was 40.57±0.12%. When both variants were considered together, the reductions were 1.28±0.25% smaller per copy of the rs4149056 C allele ($P<0.001$), and 0.62±0.18% larger per copy of the rs2306283 G allele ($P<0.001$). Overall in the Heart Protection Study, there were 23 definite or incipient cases of myopathy among participants who were taking their assigned 40 mg of simvastatin, as compared with 9 cases among participants who were assigned to take a placebo and were not taking nonstudy statin. Consequently, in contrast with SEARCH, only about half of the myopathy cases among participants taking simvastatin in the Heart Protection Study are likely to have been the result of treatment with statins. Even so, the comparison within the Heart Protection Study between the 21 genotyped participants with myopathy who were taking 40 mg of simvastatin and the 16,643 genotyped controls without myopathy confirmed that the rs4149056 SNP is associated with myopathy ($P=0.004$), albeit with a less extreme relative risk of 2.6 (95% CI, 1.3 to 5.0) per copy of the C allele. This large number of genotyped people without myopathy provides an alternative control population for the SEARCH cases, yielding an odds ratio for myopathy of 4.7 (95% CI, 3.5 to 6.4) per copy of the rs4149056 C allele, with the even smaller P value of 3×10^{-28}.

COMPARISONS WITH PREVIOUS STUDIES

To our knowledge, no previously published study has provided statistically conclusive evidence of associations of genetic variants with statin-induced myopathy (Table 4 in the Supplementary Appendix). In a study of eight candidate genes in 10 subjects with myopathy and 26 controls, an association with *SLCO1B1* SNPs was reported,[17] but those results were not statistically robust after



Genotype	Population Frequency	Cumulative No. and Percentages with Myopathy							
		Year 1				Year 5			
				Attributable to gentoype				Attributable to gentoype	
		no.	%	*no.*	% *of total*	*no.*	%	*no.*	% *of total*
TT	0.730	12	0.34	0	0	21	0.63	0	0
CT	0.249	17	1.38	12.8	75	32	2.83	24.9	78
CC	0.021	16	15.25	15.6	98	19	18.55	18.4	97
All genotypes	1.000	45	0.91	28.4	63	72	1.56	43.3	60

Figure 3. **Estimated Cumulative Risk of Myopathy Associated with Taking 80 mg of Simvastatin Daily, According to *SLCO1B1* rs4149056 Genotype.**

adjustment for multiple comparisons. In the present study, we observed no significant associations between myopathy and SNPs in any of the other genes previously reported to be associated with myopathy (Table 4 in the Supplementary Appendix) or with statin pharmacokinetics (Table 5 in the Supplementary Appendix). In particular, there was no significant association of myopathy with the 20 genotyped and 11 imputed SNPs in the *CYP3A4* gene, which is involved in simvastatin clearance[30] (Table 2b in the Supplementary Appendix). It has been reported that 10% of people with statin-induced myopathy who were referred for genetic testing had, or were carriers for, one of three inherited metabolic myopathies (McArdle's disease, carnitine palmitoyltransferase II deficiency, or myoadenylate deaminase deficiency),[31] but we observed no significant associations between myopathy and SNPs in those genes (Table 5 in the Supplementary Appendix).

DISCUSSION

We provide compelling evidence that at least one common variant in the *SLCO1B1* gene substantially alters the risk of simvastatin-induced myopathy. Among patients taking 20 to 40 mg of simvastatin daily (or standard doses of other statins), the incidence of myopathy is typically only about 1 per 10,000 patients per year,[4] and the effect of these gene variants on the absolute risk of myopathy is likely to be small (as indicated by our results among participants in the Heart Protection Study). In contrast, the risk of myopathy may be substantially

increased in patients who take 80 mg of simvastatin daily (and some other high-dose statin regimens), as well as in those who are also receiving certain other drugs[2-4] (e.g., cyclosporine and, as we found in SEARCH, amiodarone[11]). Hence, the use of those drugs in subjects who are taking such high doses of statins and who have the C allele of the rs4149056 polymorphism may produce particularly high risks of myopathy (Fig. 2).

SLCO1B1 encodes the organic anion–transporting polypeptide OATP1B1, which mediates the hepatic uptake of various drugs, including most statins and statin acids.[12] Several clinical studies have investigated associations between rs4149056 *SLCO1B1* genotypes and statin pharmacokinetics.[12] Although not all those studies yielded significant results, the collective evidence indicates that statin blood concentrations are higher in people with the C allele (Table 6 in the Supplementary Appendix). Five of those studies also examined haplotypes of rs4149056 and rs2306283 and, in aggregate, suggest that the G variant of rs2306283 is associated with lower statin concentrations (data not shown), which is consistent with the lower risk of myopathy observed in SEARCH. Genetic variants that slow the hepatic uptake of a statin might also be expected to reduce its effect on lowering cholesterol. Our data from the Heart Protection Study[22] confirm that these variants do cause small differences in the amount of reduction in LDL cholesterol produced by simvastatin.

The Illumina HumanHap300-Duo panel is estimated from HapMap CEU samples to provide about 75% genomic coverage at $r^2 \geq 0.8$ for common SNPs in people of European descent (see the Methods section in the Supplementary Appendix). Given the numbers of case subjects and controls, the present genomewide association study had only about 50% power to detect odds ratios of about 4 for common variants at an uncorrected P value of less than 5×10^{-7}. Hence, the existence of other genetic variants that carry a relative risk of myopathy of 2 to 4 cannot be ruled out by this analysis. Genes with prior evidence of links with myopathy might instead be regarded as candidates that require less extreme P values to provide good evidence of an association. Tables 4 and 5 in the Supplementary Appendix list approximately 100 such SNPs, which represent about 1/3000 of the genome screen; therefore, a P value of less than 1.5×10^{-3} (i.e., $3000 \times 5 \times 10^{-7}$) might be considered significant for these candidates. No such P values were obtained, however, for any of the SNPs studied in those regions.

In conclusion, this genomewide study has identified common genetic variants in *SLCO1B1* that are associated with substantial alterations in the risk of simvastatin-induced myopathy. These findings are likely to apply to other statins because myopathy is a class effect, and *SLCO1B1* polymorphisms affect the blood levels of several statins. Moreover, these variants may be relevant to the effects of other classes of drugs transported by OATP1B1 (e.g., the oral hypoglycemic agent repaglinide[32]). Consequently, the genotyping of *SLCO1B1* polymorphisms may be useful in the future for tailoring both the statin dose and safety monitoring (especially when statins are used in combination with certain other drugs and during the first year of treatment, when the absolute risk of myopathy is greatest) in order to obtain the benefits of statin therapy more safely and effectively.

Supported by the Medical Research Council, British Heart Foundation, National Health Service Genetic Knowledge Park, and Centre National de Génotypage. The SEARCH trial is funded by a research grant to the University of Oxford (Oxford, United Kingdom) from Merck. The Heart Protection Study was funded by grants from Merck, Roche Vitamins, the Medical Research Council, and the British Heart Foundation.

No potential conflict of interest relevant to this article was reported.

We thank the participants in SEARCH.

APPENDIX

The full SEARCH Collaborative Group is listed in the Supplementary Appendix. *Writing Committee* — E. Link, S. Parish, J. Armitage, L. Bowman, S. Heath, F. Matsuda, I. Gut, M. Lathrop, R. Collins; *Steering Committee* — T. Meade, P. Sleight (co-chairs), R. Collins, J. Armitage (study coordinators), S. Parish (statistician), J. Barton, C. Bray, E. Wincott (administrative coordinators), L. Bowman, R. Clarke, I. Graham, D. Simpson, C. Warlow, D. Wilcken (other members), J. Tobert, T. Musliner (observers); *Data Monitoring Committee* — L. Wilhelmsen (chair), R. Doll (former chair; deceased), K.M. Fox, C. Hill, P. Sandercock (voting members), R. Peto (nonvoting statistician).

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Comprehensive Psychiatry 49 (2008) 503–507
Comprehensive PSYCHIATRY
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Association study between reward dependence temperament and a polymorphism in the phenylethanolamine *N*-methyltransferase gene in a Japanese female population

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Abstract

Cloninger's theory is that specific dimensions of temperament are associated with single neurotransmitter systems, and it is based on neurophysiologic and genetic approaches to the human traits. It suggests that overexpression of temperament could cause psychiatric illness. Based on this theory, we examined the correlation between reward dependence (RD) trait, measured with the Temperament and Character Inventory, and 5 polymorphisms in genes of norepinephrine pathways, *ADRB1*, *COMT*, *PNMT*, *SLC18A1*, and *SLC6A2*, in 85 Japanese female nursing students. We found that rs3764351 in *PNMT* was significantly associated with RD on Fisher's exact test ($P = .029$, $P_{corr} = .236$). When haplotype analysis was performed for rs3764351 and rs876493 polymorphisms in the 5′ flanking region of *PNMT*, 3 haplotypes were identified. Rs3764351 itself appeared to be correlated with RD in the present study of a specific population, although we could not demonstrate an association between RD and any of the haplotypes. Our findings have implications for the understanding of temperament using neurophysiologic approaches.

1. Introduction

Correlations between traits as behavioral predispositions and mental disorders have recently been increasingly studied. However, it has been difficult to assess the effect of psychologic factors on mental disorders. In this context, Cloninger's theory is unique in suggesting that specific dimensions of temperament are associated with single neurotransmitter systems on the basis of neurophysiology and genetic findings [1,2]. Based on this psychobiologic model of personality, the Temperament and Character Inventory (TCI) was developed [1]. The questionnaire comprises 4 dimensions of temperament: novelty seeking (NS), harm avoidance (HA), reward dependence (RD), and persistence (P). Novelty seeking is hypothesized to be a tendency to be impulsive, exploratory, fickle, excitable, quick tempered, and extravagant. Harm avoidance is related to be cautious, to be tense, to be fearful, and to exhibit apprehensive worrying. Reward dependence refers to the preference for acting in a group, loathing isolation from others, and the concern about evaluation by others. These dimensions are suggested to be correlated with neurotransmitter pathways [1,2]. Although persistence appears to involve a tendency toward perseverance despite frustration and fatigue, its associated neurotransmitter pathways remain unclear. Moreover, overexpression of temperament has been suggested to be associated with mental disorders [3], and studies to investigate such associations have been reported [4-6]. Indeed, regarding RD, a study revealed a significant association between RD and eating disorder [5]. Based on

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0010-440X/$ – see front matter © 2008 Elsevier Inc. All rights reserved.
doi:10.1016/j.comppsych.2008.03.001

physiologic and pharmacologic studies using animal models [7,8] and by measurement of norepinephrine metabolites in human [9,10], the RD behavioral pattern was shown to refer to norepinephrine pathways.

The studies on the effect of interaction with specific genes on such traits are increasing [11,12]. Among them, many studies using TCI examined correlations between genetic variations in specific genetic loci involved in neurotransmission and NS and HA. Indeed, the associations of D4 dopamine receptor gene (*D4DR*) polymorphisms with NS were demonstrated [13]. In addition, Katsuragi et al [14] reported the association between the serotonin transporter gene promoter region (*5-HTTLPR*) and HA. As for RD, the association between RD and norepinephrine pathways is still controversial partly because of limited number of studies, differences in study design, insufficient number of subjects, and ethnic differences [12,15].

On the basis of Cloninger's original theory, we focused on the correlation between RD and genetic polymorphisms of norepinephrine metabolism-related genes. Investigation of the genetic association of single nucleotide polymorphisms (SNPs) in these genes with RD could further the understanding of temperament as a behavioral predisposition at a molecular level. We hope this has implications for the use of TCI as a tool for measuring temperament, which is the basis of behavioral patterns as phenotypes, and detecting susceptibility genes or SNPs for mental disorders with characteristically inappropriate behavioral patterns.

2. Materials and methods

2.1. Subjects

A total of 85 healthy unrelated female volunteers with a mean age of 22 ± 2.7 years (mean ± SD) were recruited from among nursing students. All participants were ethnically Japanese and gave written informed consent to voluntarily participate in this study after receiving full explanation of the procedures. They completed a family and medical questionnaire and were ascertained to be free of major mental disorders among themselves and their families. The participants themselves were also confirmed to have no history of physical illnesses by responding to the questionnaire. The study protocol was approved by the Institutional Review Board and was carried out in accordance with the latest version of the Declaration of Helsinki.

2.2. Candidate genes and SNPs

We checked the genes related to systems for metabolism of norepinephrine using the NCBI db SNP (build 126; http://www.ncbi.nlm.nih.gov/SNP/) and JSNP (http://snp.ims.u-tokyo.ac.jp/) [16,17]. We used SNPs in the target genes as candidates, drawing on previous studies and the reference pages of them at the above websites on Online Mendelian Inheritance in Man (http://www.ncbi.nlm.nih.gov/sites/entrez?db=omim), which exhibit correlations between the SNPs and mental disorders such as affective, anxiety, and panic disorders as well as schizophrenia, which met the *Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition, Text Revision*, criteria [18].

2.3. Genotyping

Genomic DNA was extracted from peripheral blood leukocytes of the volunteers using a DNA extraction kit (QIAamp DNA Blood Kit; QIAGEN, Hilden, Germany). Polymerase chain reaction (PCR) was carried out using 10 to 20 ng of genomic DNA. Genotyping of polymorphisms was performed by the TaqMan method, using the ABI PRISM 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA). PCR amplification was carried out on 10 to 20 ng of DNA using 2.5 μL of 2× TaqMan universal PCR master mix (no AmpErase UNG), 0.25 μL of 20× SNP Genotyping Assay Mix, and 2.25 μL of DNase-free water in a 5-μL reaction volume. The ABI 9700 dual plate thermal cycle (Applied Biosystems) was programmed as follows: 1 cycle of 95°C for 10 minutes, followed by 60 cycles of 92°C for 15 seconds and 58°C for 1 minute.

2.4. Psychometric evaluation

The Japanese version of the TCI has 4 dimensions of temperament and 3 of character. The TCI has been increasingly used to investigate the correlations between genetic polymorphism and personality traits in the Japanese population [14,15,19-21]. It is a self-rating instrument with a 125-item questionnaire, with the original true-false response scale modified to a 4-point scale. Its reliability and validity have been verified [22,23]. In the present study, we used the 125-item, 4-point scale version and applied only with the RD subscale to the subjects. Reward dependence score consisted of RD1, sentimentality; RD3, attachment; and RD4, dependence.

2.5. Statistical analysis

2.5.1. Single SNP analyses

Allele frequencies were calculated and tested for agreement with Hardy-Weinberg equilibrium at each SNP locus. The differences in allele frequencies of each SNP between case and control subjects were compared using Fisher's exact test with one degree of freedom. Odds ratios (ORs) were also calculated with 95% confidence intervals (95% CIs). *P* values of less than .05 (2 tailed) were considered significant.

2.5.2. Haplotype analyses

Single nucleotide polymorphisms exhibiting statistically suggestive findings on Fisher's exact test were subjected to haplotype analyses using Haploview ver 3.2 (http://www.broad.mit.edu/mpg/haploview/). For multiple comparisons, *P* values were corrected by permutation test (no. of iterations = 10 000).

3. Results

3.1. Reward dependence score

The score was not normally distributed as a continuous variable. We observed a tendency toward bimodal distribution with the cutoff point at 31. The mean score was 31, with a SD of 0.5 and a median score of 31. Hence, the participants were divided into 2 groups ($t = 11.25$, $P < .001$). They were the "high-score group" (RD score >31, n = 38) and "low-score group" (RD score ≤31, n = 47).

3.2. Target genes and SNP analyses

The 5 genes we chose were as follows: β_1-adrenergic receptor, *ADRB1*; catechol-*O*-methyltransferase, *COMT*; phenylethanolamine *N*-methyltransferase, *PNMT*; solute carrier family 18, member 1, *SLC18A1*; and solute carrier family 6, member 2, *SLC6A2*.

We tested 5 SNPs in the 5 target genes: rs1801252 (S49G) in *ADRB1* [24], rs4680 (V158M) in *COMT* [25], rs3764351 in *PNMT* [26], rs2270641 (T4P) in *SLC18A1* [27], and rs2242446 in *SLC6A2* [28]. All SNPs were in Hardy-Weinberg equilibrium ($P > .3$). On Fisher's exact test, we found that, among the 5 SNPs, rs3764351 in *PNMT* appeared to be associated with RD (raw $P = .029$, $P_{corr} = .236$). The other 4 SNPs were not statistically significant ($P > .05$), as shown in Table 1. On determination of ORs, we found that rs3764351 genotype (OR, 3.07; 95% CI, 1.16-8.11) and its allele (OR, 2.04; 95% CI, 1.09-3.82) also appeared to be associated with RD.

3.3. Haplotype analyses

On single SNP analyses, we found that rs3764351 in *PNMT* might be correlated with RD. This SNP was consequently subjected to haplotype analyses by constructing a 2-marker haplotype of *PNMT*, with rs3764351 and rs876493 polymorphisms in the 5′ flanking region. As with rs3764351, rs876493 polymorphism in *PNMT* was also reported to be correlated with mental disorders [26,29]. These 2 SNPs as markers and 3 haplotypes identified (C-C, T-T, T-C) were included in the association test (Table 2). Although rs3764351 itself was statistically significant (raw $P = .029$), the other 3 patterns were significantly underrepresented ($P > .035$).

Table 2
Structure and frequencies of 2-loci haplotypes in *PNMT*

Haplotype	rs3764351[a]	rs876493[a]	Haplotype frequencies		χ^2	P^b
			Case	Control		
1	C	C	0.33	0.49	4.44	.035
2	T	T	0.48	0.36	2.43	.119
3	T	C	0.19	0.14	0.85	.356

Haplotypes 1, 2, and 3 consist of 2 SNPs, rs3764351 and rs876493.
[a] refSNP ID: NCBI Build 36.1.
[b] Nominal *P* value before adjustment for multiple testing.

Again, rs3764351 exhibited a weak association with RD (raw $P = .029$; genotype OR = 3.07, 95% CI, 1.16-8.11; allele OR = 2.04, 95% CI, 1.09-3.82). The other 4 SNPs (rs1801252, rs4680, rs2270641, and rs2242446) failed to exhibit associations with RD ($P > .05$), as summarized in Tables 1 and 2.

4. Discussion

Exploring the association between RD and 5 polymorphisms (rs1801252, rs4680, rs3764351, rs2270641, and rs2242446) in genes of the norepinephrine pathways, we found that polymorphism in the 5′ flanking region of *PNMT* (rs3764351) statistically suggests correlation with RD (raw $P = .029$) before correction. When we examined *PNMT* gene haplotypes, rs3764351 itself exhibited some effect on RD, compared with the haplotypes identified. These findings implied that no other SNPs in *PNMT* acted in combination on RD. It, thus, appears that the apparent correlation with rs3764351 in *PNMT* was possibly caused by the single effect of this SNP rather than that of haplotypes. In this regard, Yuan et al [30] reported that a significant difference in

Table 1
Associations of 5 selected polymorphisms in the genes examined

Gene	db SNP ID	Location	Allele frequency[a]				P^b	OR (95% CI)	
			Case (n = 38)		Control (n = 47)			Genotype[c]	Allele[d]
			Ref.	Var.	Ref.	Var.			
ADRB1	rs1801252	Exon 2	0.88	0.12	0.88	0.12	.907	1.11 (0.39-3.11)	0.96 (0.37-2.45)
COMT	rs4680	Exon 4	0.65	0.35	0.59	0.41	.513	1.06 (0.32-3.48)	0.77 (0.40-1.46)
PNMT	rs3764351	5′ flanking	0.33	0.67	0.50	0.50	.029[e]	3.07 (1.16-8.11)	2.04 (1.09-3.82)
SLC18A1	rs2270641	Exon 3	0.71	0.29	0.64	0.36	.402	1.81 (0.45-7.32)	0.74 (0.38-1.46)
SLC6A2	rs2242446	5′ flanking	0.40	0.60	0.35	0.65	.632	1.90 (0.55-6.54)	1.21 (0.65-2.25)

[a] Ref. and Var. were defined by db SNP.
[b] Nominal *P* value before adjustment for multiple testing.
[c] Genotype 11 vs 12 + 22.
[d] Allele 1 vs allele 2.
[e] Raw *P* value, statistically significant.

activity was observed in the haplotype of rs3764351 and rs876493 polymorphisms, in the ability to drive transcription, compared with that of the wild-type haplotype in the 5′ flanking region. They also suggested that this raised the possibility of inherited variation in the ability to form epinephrine from norepinephrine. In a search of the TRANSFAC database (http://www.gene-regulation.com/index.html), the polymorphism rs3764351 of the *PNMT* 5′ flanking region was not located in a putative transcription factor binding element. We proceeded to analyze 5 SNPs based on raw P values. On multiple testing, P_{corr} value of rs3764351 after permutation testing was .236, which did not indicate correlation between the polymorphism and RD at a significance level of .05 (2-tailed). This method of correction is considered conservative, and applying this critical value to the result of an association study reduces the significance of associations that are detected. Our study did not yield a statistically strong association, but it does suggest the possibility of a correlation with RD. Using ORs, weak association with RD was also observed for rs3764351 in *PNMT* (Table 1). PNMT (*PNMT* [MIM* 171190]) catalyzes the synthesis of epinephrine from norepinephrine, the last step of catecholamine biosynthesis, and has been reported to be a candidate gene for a range of mental disorders [26,29]. Therefore, we show the possibility of weak genetic contributions of unidentified polymorphisms in *PNMT* in this specific population of female nursing students. The present study may not exclude true or false negativity with regard to RD because of the small sample size. Mendlowicz et al [31] reported that sex may influence the RD score. As for RD, females tend to score higher than males. Evaluation of the effect of sex on temperament and character could be of importance in further research. It was relatively difficult to measure the definitive temperament to make objective evaluations. An investigation of both nonclinical and clinical populations, as in a case-control study, would enable direct comparisons of differences in clinical phenotype. To recognize phenotype clearly on the basis of clinical information, we need to clearly determine the pathophysiology of mental disorders. Further understanding of the mechanisms of mental disorders will contribute to confirmation of associations between specific neurotransmitters and temperaments based on Cloninger's theory.

Several limitations of the present study must be noted. First, the subjects were all female nursing students, whose mean age was 22 years, with an SD of 2.7 years. The results of the study might have been different for participants with different sex, different backgrounds, and different ethnicities. Second, the previous studies have shown the difficulty in defining an a priori cutoff point, which successfully applies to other studies [5,14,15,19-21]. In the present study, we used the score, 31, as the cutoff point to divide groups into 2 because of the observed score distribution. Third, our findings were based on a small sample size. Further large-scale investigations of other populations will draw clearer conclusions.

In conclusion, we found that rs3764351 in *PNMT* may be a variable in the RD behavioral pattern in the present study of a specific population. Our findings have implications for understanding temperament using neurophysiologic approaches.

Acknowledgment

The authors thank Ryo Yamada for continuous assistance in the statistical analysis.

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Journal of Hepatology 49 (2008) 1038–1045

Journal of Hepatology

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Genetic factors of susceptibility and of severity in primary biliary cirrhosis☆

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See Editorial, pages 881–883

***Background/Aims*: In primary biliary cirrhosis (PBC), pathogenesis is influenced by genetic factors that remain poorly elucidated up to now. We investigated the impact of sequence diversity in candidate genes involved in immunity (*CTLA-4* and *TNFα*), in bile formation (10 hepatobiliary transporter genes) and in the adaptative response to cholestasis (three nuclear receptor genes) on the susceptibility and severity of PBC.**

***Methods*: A total of 42 Ht SNPs were identified and compared in 258 PBC patients and two independent groups of 286 and 269 healthy controls. All participants were white continental individuals with French ancestry.**

***Results*: Ht SNPs of *CTLA-4* and *TNFα* genes were significantly associated with susceptibility to PBC. The progression rate of liver disease under ursodeoxycholic acid (UDCA) therapy was significantly linked to SNPs of *TNFα* and *SLC4A2*/anion exchanger 2 (AE2) genes. A multivariate Cox regression analysis including clinical and biochemical parameters showed that *SLC4A2*/AE2 variant was an independent prognostic factor.**

***Conclusions*: These data point to a primary role of genes encoding regulators of the immune system in the susceptibility to PBC. They also demonstrate that allelic variations in TNFα and *SLC4A2*/AE2 have a significant impact on the evolutive profile of PBC under UDCA therapy.**

***Keywords*: Cytotoxic T-lymphocyte antigen 4 (CTLA-4); Tumor necrosis factor α (TNFα); Anion exchanger 2 (AE2)/SLC4A2; Multidrug resistance 1 (MDR1)/ABCB1; Ursodeoxycholic acid (UDCA); Primary biliary cirrhosis (PBC)**

Received 3 June 2008; received in revised form 4 July 2008; accepted 17 July 2008; available online 1 October 2008
Associate Editor: V. Barnaba

☆ The authors who have taken part in the research of this paper declared that they do not have a relationship with the manufacturers of the materials involved either in the past or present and they did not receive funding from the manufacturers to carry out their research.

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Abbreviations: PBC, primary biliary cirrhosis; CTLA-4, cytotoxic T-lymphocyte antigen 4; TNFα, tumor necrosis factor α; SNP, single nucleotide polymorphism; Ht SNP, haplotype-tagging single nucleotide polymorphism; MDR1, multidrug resistance 1; UDCA, ursodeoxycholic acid; AE2, anion exchanger 2; MDR3, multidrug resistance 3; BSEP, bile salt export pump; CFTR, cystic fibrosis transmembrane conductance regulator; FIC1, familial intrahepatic cholestasis 1; NTCP, Na-coupled taurocholate transport protein; LXRα, liver X receptor α; FXR, farnesoid X receptor; PXR, pregnane X receptor.

0168-8278/$34.00
doi:10.1016/j.jhep.2008.07.027

1. Introduction

Primary biliary cirrhosis (PBC) is an auto-immune disease, characterized by unresolved liver inflammation that affects primarily small bile ducts, cholestasis and anti-mitochondrial antibodies. Liver inflammation, bile duct destruction and cholestasis may lead to cirrhosis and liver failure, necessitating liver transplantation.

There is evidence to indicate that genetic factors are implicated in the pathogenesis of the disease. Increased risk in first-degree relatives, family clustering and concordance between identical twins all show that genetic factors contribute to the life-time risk of PBC [1]. Up to now, candidate gene screening and association studies have been the method used for finding genes that contribute to this complex disease. Because PBC displays features of autoimmunity, most studies have focused on genetic variations in genes related to immunity. A complex and weak association has been found between several major histocompatibility complex loci and susceptibility to PBC [2–5]. Significant associations have also been reported with the polymorphisms of other genes involved in innate or adaptative immunity, e.g. tumor necrosis factor α (TNFα) [2,3]. Allelic variations of cytotoxic T-lymphocyte antigen 4 (CTLA-4), a key regulator of the adaptative immune system have been repeatedly associated with susceptibility to different autoimmune diseases including Graves' disease, type I diabetes, autoimmune hypothyroidism, systemic lupus erythematosus and PBC [6–9]. However, no association between variations in this gene per se and susceptibility to PBC was found in the most recent studies [10,11].

One of the challenging features in PBC is that the severity of liver disease is highly variable, ranging from a slow progression to the development of end-stage liver disease within a decade. This variability may reflect differences in the inflammatory destructive process or in the adaptative response to cholestasis. Accordingly, PBC patients also show high variability in the response to therapies that target either the immune system (i.e. anti-inflammatory drugs) or the cholestatic syndrome (i.e. ursodeoxycholic acid) [12,13].

Here, we tested the hypothesis that susceptibility to PBC and/or the severity of the disease could be influenced by the variations in genes involved in immunity (i.e. CTLA-4 and TNFα) or in cholestasis (10 hepatobiliary transporters and three nuclear receptor genes).

2. Study design and methods

2.1. Study participants

Participants in the study, PBC patients and controls, were all French white continental patients with French ancestry recruited specifically for genetic studies. All of them gave informed written consent for genomic DNA analyses. A first group of controls (Group 1), comprised 286 healthy volunteers who were recruited through the Saint-Antoine Clinical Investigation Center, Paris. For validation studies, an independent group of controls (Group 2) consisted of 269 healthy individuals who were recruited at the National Genotyping Center (CNG), Evry. The controls were matched by gender and age (±10.5 years) to individual PBC patients. The PBC patients ($n = 258$) belonged to an observational study of PBC carried out from 1998 to 2002 in France. The diagnosis of PBC was established on the basis of biochemical cholestasis, positivity for anti-mitochondrial antibodies by immuno-fluorescence and compatible histological features at liver biopsy. Patients had the following characteristics at diagnosis: female, 91%; mean age, 51.8 years (range, 20–75 years); serum bilirubin, 1.1 mg/dL (range, 0.1–12 mg/dL); alkaline phosphatase, 2.5× upper normal limit (range, 0.3–32.3); histological stages I–II, 78%. While all 258 PBC patients were compared with controls to identify potential factors of susceptibility and of severity, factors of progression under UDCA treatment were investigated in a sub-group of 147 patients who were followed-up for a mean period of 7.1 years (range, 3–19 years) in one single department (The National Reference Center for Inflammatory Biliary Diseases located at Saint-Antoine Hospital, Paris). The patients in this sub-group had the following main characteristics at diagnosis: female, 91.2%; mean age, 50.2 years (range, 20–68 years); serum bilirubin, 1.2 mg/dL (range, 0.5–10 mg/dL); alkaline phosphatase, 3.5× upper normal limit (range, 1.4–21); histological stages I–II, 52%. The criteria of disease severity at diagnosis were hyperbilirubinemia (>1 mg/dL) or established cirrhosis. Criteria of severe evolution under ursodeoxycholic acid (UDCA) treatment (13–15 mg/kg/day) were the development of hyperbilirubinemia (>2 mg/dL), progression towards cirrhosis, or referral to liver transplantation.

2.2. SNP analyses

Genomic DNA was isolated from human leukocytes using standard methods. The following genes, related to immunity, i.e. *CTLA-4* and *TNFα*, encoding hepatobiliary transporters, i.e. *ABCB1*/MDR1, *ABCB4*/MDR3, *ABCB11*/BSEP, *ABCC2*/MRP2, *ABCC7*/CFTR, *ABCG5*/sterolin 1, *ABCG8*/sterolin 2, *ATP8B1*/FIC1, *SLC4A2*/AE2, *SLC10A1*/NTCP, or nuclear receptors, i.e. *NR1H3*/LXRα, *NR1H4*/FXR, *NR1L2*/PXR, were selected for study. For each one of these genes, the strategy adopted to identify SNPs was to sequence genomic regions of approximately 150,000 bps that included all exons and flanking intron sequences, 5′ and 3′ untranslated regions, as well as promoter regions, in a learning panel of healthy individuals. Using the Genalys software [14], we specified every exon–intron junction and designed primers to amplify fragments of approximately 1000 bps for sequencing. Calculations of probability indicated that a learning panel of 32 DNA samples would allow to detect SNPs of >5% frequency, with a probability >96%. Sixteen pools of 2 equimolecular DNA samples were prepared from 32 healthy French individuals and submitted to sequencing. After the identification of SNPs was completed, we defined Ht SNPs of each gene, i.e. the minimal set of SNPs that allowed to discriminate all different haplotypes with a frequency >5%. Ht SNP genotyping in both control and PBC populations was performed at CNG, using an automated high-throughput Taqman method [15]. All liquid handling was performed robotically in 384-well plates. PCR was accomplished with two primers located on each side of the SNP region and two specific Taqman probes labeled with FAM and VIC fluorophors on the SNP spot (Applera France, Villebon-sur-Yvette, France). End point fluorescence was detected by an ABI SDS 7900 apparatus (Applied Biosystems, Villebon-sur-Yvette, France), which allowed discrimination between homozygous and heterozygous. The method was validated by testing each SNP in the initial learning panel of 32 DNA samples.

2.3. Statistical analyses

Non-parametric tests were used to analyze differences between two groups. The χ^2 and the Fisher exact tests were used to look for associations with different groups. To take into account the multiplicity of comparisons, both permutation tests and Bonferroni method were performed [16]. The effects of alleles on the risks of susceptibility or

severity are expressed by OR (CI 95%). Log-rank analysis and Cox regression model were performed to study allele associations with the progression of liver disease in UDCA-treated PBC patients. Statistical analyses were performed, using the SPSS software (version 11.0.4 for Mac OS X; SPSS Inc. Headquarters, 233 S. Wacker Drive, 11th floor, Chicago, IL 60606, USA).

3. Results

3.1. SNP identification

By screening the 15 candidate genes in the learning panel of 32 individuals, we identified a total of 42 Ht SNPs with a frequency >5% (Tables 1–3). Twelve were newly discovered SNPs at the time of the study. Six were located in coding regions, of which five were synonymous and one resulted in amino acid change, i.e. T17A in *CTLA4* exon 1 (Table 1).

3.2. Case-control analysis

The Ht SNPs that were thus identified, were subsequently examined in a first population of 286 healthy volunteers (Control Group 1) and in the population of 258 PBC patients. The NCBI human ruler sequence version/build 36.1, which is the latest version of major update, was taken as a reference to define variant alleles. Accordingly, the frequency of each variant allele in the two groups is shown in Tables 1–3. All genotype frequencies were in Hardy–Weinberg equilibrium (data not shown).

Statistical analyses showed that the distributions of Ht SNPs in the genes encoding hepatobiliary transporters or nuclear receptors (Tables 2 and 3) were not significantly different between controls and PBC patients (Pc = 0.9–1), whereas significant differences were found in the *CTLA-4* and *TNFα* genes (Table 1).

CTLA-4. SNP identification studies allowed to identify a set of three polymorphisms tagging the most common haplotypes of the CTLA4 gene (i.e. with a frequency >5%). These polymorphisms comprised a non-synonymous SNP located in exon 1, and two SNPs located in the 5′ flanking promoter region. The frequencies of all three variants differed significantly between controls and PBC patients ($p = 0.0003$–0.048). After correction for multiple testing, only Ht SNP A/G in CTLA-4 exon 1, remained significant (Pc < 0.02). The haplotype defined by the three gene variants did not add further discrimination between cases and controls.

TNFα. One of the three polymorphisms of TNFα identified in the initial SNP identification study, C/A located in the 5′ flanking promoter region was also statistically heterogeneous across the two populations (Table 1), the variant allele being more frequent in PBC patients than in controls ($p = 0.006$). Moreover, to validate these results, we used a second independent set of control samples collected from 269 healthy individuals who were recruited as controls for different genetic studies (Control Group 2). While the distributions of Ht SNPs between Control Groups 1 and 2 were not significantly different ($p = 0.24$–0.46), significant differences between cases and controls were confirmed, e.g. A/G in *CTLA-4* exon 1 ($p = 0.0005$), and C/A in *TNFα* 5′ flanking region ($p = 0.0016$).

3.3. Associations with severity of liver disease in PBC

Potential associations between gene polymorphisms and the severity of liver disease were investigated either at diagnosis in the entire cohort of 258 PBC patients or under UDCA treatment in a sub-group of 147 patients who were followed-up for a mean period of 7.1 years at Saint-Antoine Hospital.

At diagnosis, 37% and 4.3% of the 258 patients had hyperbilirubinemia (>1 mg/dL) and cirrhosis, respectively. The distribution of gene alleles was similar in patients with or without criteria of severity with the exception of 2 SNPs, A/G in *ABCB1*/MDR1 exon 3 5′-untranslated region (Table 2, upper box) and C/A in *NR1L2*/PXR exon 9 3′-untranslated region (Table 3, lower box). Genotypes defined by the presence of

Table 1
Ht SNPs of candidate genes and their distribution in PBC patients and controls

Gene symbol (chromosome position)	dbSNP ID	SNP[a]	Location[b] (amino acid residue)	Variant allele (%)		OR	95% CI		P^c
				Controls ($n = 286$)	PBC ($n = 258$)				
CTLA4 (2q33)	rs11571317	C/T	5′ Flanking	10.2	5.7	.53	.34	.85	**.008**
	rs5742909	C/T	5′ Flanking	11.1	7.6	.66	.43	1.00	**.048**
	rs231775	A/G	Exon 1 (T17A)	28.9	39.4	1.60	1.24	2.07	**.0003**
TNFα (6p21.3)	rs1800630	C/A	5′ Flanking	14.6	21.3	1.58	1.14	2.17	**.006**
	rs1799724	C/T	5′ Flanking	11.0	9.7	.87	.58	1.31	.511
	rs1800629	G/A	5′ Flanking	13.5	12.6	.92	.64	1.33	.657

[a] Reference (in the NCBI human ruler sequence build 36.1)/variant nucleotides.
[b] Location of SNPs and corresponding amino acid residue are defined on the following mRNAs: *CTLA4*, NM_005214.2, *TNFα*, NM_000594.2.
[c] Probability values after correction for multiple testing were: CTLA-4 rs11571317, Pc = 0.29; CTLA-4 rs5742909, Pc = 0.95; CTLA-4 rs231775, Pc < 0.02; TNFα rs1800630, Pc = 0.26.

Table 2
Ht SNPs of candidate genes and their distribution in PBC patients and controls

Gene symbol (chromosome position)	dbSNP ID	SNP[a]	Location[b] (amino acid residue)	Variant allele (%)		OR	95% CI		P
				Controls ($n = 286$)	PBC($n = 258$)				
ABCB1 (7q21.1)	rs2214102	A/G	Exon 3 5′-UTR	92.4	94.2	1.32	.74	2.36	.341
	rs1922242	T/A	Intron 17	43.2	42.7	.98	.73	1.31	.880
	rs2235048	C/T	Intron 27	46.0	50.6	1.25	.93	1.67	.138
ABCB4 (7q21.1)	rs3747806	T/C	Exon 1 5′-UTR	3.2	2.2	.66	.27	1.61	.360
	ss76876405	G/A	Intron 23	8.3	9.3	1.14	.68	1.92	.622
	ss76876555	C/G	Intron 28	5.8	5.6	.98	.52	1.84	.948
ABCB11 (2q24)	rs2287618	A/G	Intron 9	72.8	74.4	1.09	.78	1.52	.625
	rs860510	T/G	Intron 19	54.3	53.2	.96	.71	1.28	.762
	rs473351	A/G	Exon 28 3′-UTR	57.1	55.6	.84	.70	1.26	.684
ABCC2 (10q24)	rs2756109	G/T	Intron 7	62.1	57.4	.82	.61	1.11	.197
	rs2073337	A/G	Intron 12	38.7	36.6	.92	.68	1.24	.568
	rs3047477	GT/–	Intron 19	20.5	23.2	1.17	.82	1.68	.386
ABCC7 (7q31.2)	rs1429566	G/A	Intron 3	20.1	17.8	.86	.63	1.17	.330
	rs1042077	T/G	Exon 15 (T854T)	25.8	28.0	1.12	.86	1.47	.409
ABCG5 (2p21)	rs11887534	C/G	5′ Flanking	5.4	7.0	1.32	.80	2.18	.276
	rs3806471	A/C	5′ Flanking	29.3	29.9	.96	.75	1.27	.851
	ss76876784	C/T	Intron 5	5.7	6.3	1.11	.67	1.85	.691
	ss76876787	G/A	Intron 10	4.4	5.4	1.25	.72	2.17	.434
ABCG8 (2p21)	rs4148213	G/C	Intron 2	37.7	36.7	1.04	.77	1.41	.797
	ss76866107	A/G	Intron 9	12.5	9.1	.70	.44	1.12	.138
	rs4148220	T/C	Intron 10	47.0	48.4	.95	.70	1.27	.709
ATP8B1 (18q21.31)	rs317809	C/T	Intron 11	47.1	47.5	1.01	.76	1.36	.925
	rs160992	C/T	Intron 18	41.0	41.6	1.02	.76	1.38	.878
	rs12958967	C/T	Intron 26	23.0	28.3	1.32	.94	1.85	.107
SLC4A2 (7q35–q36)	rs1050702	C/A	Exon 4 (R95R)	18.4	18.2	.99	.72	1.35	.929
	rs2303932	T/A	Exon 6 (T210T)	59.3	59.3	1.00	.78	1.28	.999
	rs2303939	G/C	Intron 21	45.3	50.4	1.23	.96	1.56	.096
SLC10A1 (14q24.1)	rs4646285	G/A	Exon 1 (T75T)	10.1	11.3	1.14	.71	1.83	.601
	ss76875083	T/C	Intron 3	13.1	13.5	1.03	.67	1.59	.890

UTR, untranslated region.
[a] Reference (in the NCBI human ruler sequence build36.1)/variant nucleotides.
[b] Location of SNPs and corresponding amino acid residue aredefined on the following mRNAs: *ABCB1*, NM_000927.3; *ABCB4*, NM_000443.2; *ABCB11*, NM_003742.2; *ABCC2*, NM_000392.1; *ABCC7*, NM_000492.2; *ABCG5*, NM_022436.2; *ABCG8*, NM_022437.2; *ATP8B1*, NM_005603.2; *SLC4A2*, NM_003040.2; *SLC10A1*, NM_003049.2.

the *ABCB1*/MDR1 variant and the absence of the *NR1L2*/PXR variant were more frequent in patients without hyperbilirubinemia or cirrhosis (81% vs 67%, $p = 0.003$). However, after applying the correction for

Table 3
Ht SNPs of candidate genes and their distribution in PBC patients and controls

Gene symbol (chromosome position)	dbSNP ID	SNP[a]	Location[b] (amino acid residue)	Variant allele (%)		OR	95% CI		P
				Controls ($n = 286$)	PBC ($n = 258$)				
NR1H3 (11p11.2)	rs2279239	T/C	Intron 2	24.1	28.5	1.26	.90	1.76	.183
	rs2279238	C/T	Exon 3 (S99S)	12.2	15.2	1.29	.84	1.99	.249
NR1H4 (12q23.1)	ss76876774	G/T	Exon 3 5′-UTR	2.3	3.5	1.54	.75	3.17	.244
	rs17030285	C/G	Intron 6	2.5	12.9	.97	.67	1.40	.867
NR1L2 (3q12–q13.3)	rs1523127	C/A	Exon 1 5′-UTR	57.6	59.8	1.09	.86	1.40	.469
	rs3732356	G/T	Intron 3	92.4	91.7	.91	.59	1.42	.685
	rs10511395	C/A	Exon 9 3′-UTR	16.8	18.3	1.11	.81	1.52	.519

UTR, untranslated region.
[a] Reference (in the NCBI human ruler sequence build36.1)/variant nucleotides.
[b] Location of SNPs and corresponding amino acid residue are defined on the following mRNAs: *NR1H3*, NM_005693.1; *NR1H4*, NM_005123.1; *NR1L2*, NM_003889.2.

Table 4
Genes with variations related to PBC severity at diagnosis

Gene symbol (chromosome position)	dbSNP ID	SNP[a]	Location[b] (amino acid residue)	Variant allele(%)		OR	95% CI		P^{d}
				Severe[c]	Non-severe				
ABCB1 (7q21.1)	rs2214102	A/G	Exon 3 5′-UTR	87.5	95.7	3.20	1.47	6.95	**.003**
	rs1922242	T/A	Intron 17	37.9	43.6	1.27	.82	1.97	.290
	rs2235048	C/T	Intron 27	49.2	50.7	1.06	.70	1.62	.781
NR1L2 (3q12–q13.3)	rs1523127	C/A	Exon 1 5′-UTR	60.2	59.0	.95	.62	1.47	.830
	rs3732356	G/T	Intron 3	89.0	92.4	1.50	.73	3.07	.266
	rs10511395	C/A	Exon 9 3′-UTR	26.3	15.5	.51	.31	.86	**.011**

UTR, untranslated region.
[a] Reference (in the NCBI human ruler sequence build 36.1)/variant nucleotides.
[b] Location of SNPs and corresponding amino acid residue are defined on the following mRNAs: *ABCB1*, NM_000927.3; *NR1L2*; NM_003889.2.
[c] Severity at diagnosis was defined by hyperbilirubinemia >1 mg/dL (37% of the 258 patients) or cirrhosis (4.3%).
[d] Corrected *p*-values: ABCB1 rs2214102, Pc = 0.14; NR1L2 rs10511395, Pc = 0.38.

multiple testing error, effects of the two polymorphisms were no longer significant (Pc = 0.12) (Table 4).

Then, we determined whether polymorphisms were linked to the progression profile under UDCA treatment (13–15 mg/kg/day), according to our pre-defined criteria (development of hyperbilirubinemia >2 mg/dL, progression towards cirrhosis, or referral to liver transplantation). Two variant alleles, namely A (instead of T) in the *SLC4A2*/AE2 exon 6 (Table 2) and T (instead of C) in the TNFα 5′ flanking region (Table 1), were associated with a reduced risk of PBC progression (log-rank test, $p = 0.009$ and 0.01, respectively). Kaplan–Meier plots of the cumulative rates of progression for these two SNPs are shown in Fig. 1. Severe progression under treatment was virtually absent in patients with the presence of these two variants while it occurred in approximately half of the patients lacking the two variants (data not shown).

A multi-variate Cox regression analysis was performed including all of the following parameters: age, gender, presence or absence of cirrhosis, serum bilirubin, serum albumin, activities of amino-transferases and of alkaline phosphates, platelet counts and presence or absence of the allelic variants of *ABCB1*/MDR1, *NR1L2*/PXR, *SLC4A2*/AE2 and TNFα, that were associated with disease severity at univariate analysis. Four variables were retained as independent factors of severe progression, including the allelic variant of *SLC4A2*/AE2 SNP in exon 6, demonstrating that this genetic marker is a prognostic factor independently of known biochemical markers (Table 5).

4. Discussion

This study dedicated to common variants of genes involved in immunity and bile secretion shows that susceptibility to PBC is associated with genetic variation in *CTLA-4* and probably also, although to a lesser extent, in *TNFα*. Before any therapeutic intervention, the severity of PBC is linked potentially to *ABCB1*/MDR1 and to *NR1L2*/PXR polymorphisms. The study also reveals that the progression of the disease under UDCA therapy

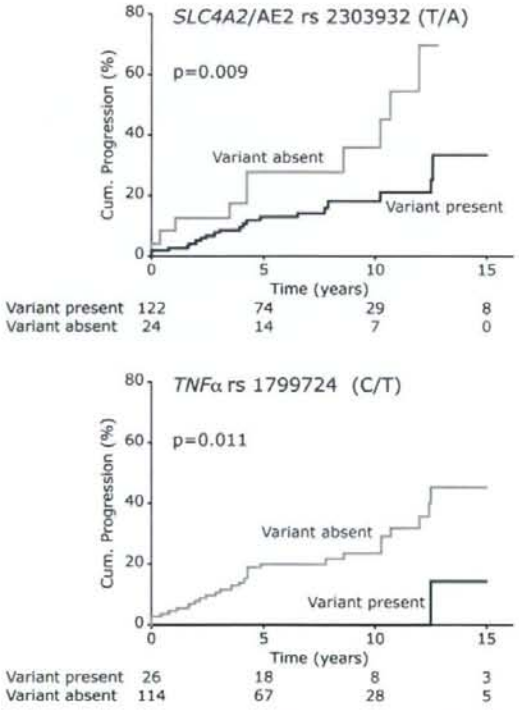

Fig. 1. Kaplan-Meier plots of the cumulative progression under UDCA according to SLC4A2/AE2 and TNFα genotypes. Progression under UDCA treatment, defined as the development of hyperbilirubinemia (>2 mg/dL), evolution towards cirrhosis, or referral to liver transplantation, was investigated in a sub-group of 147 patients who were followed-up for a mean period of 7.1 years at Saint-Antoine Hospital, Paris. NB: Missing data for SLC4A2 and TNFα genotyping in 1 and 7 patients, respectively.

Table 5
Cox regression analysis: independant prognostic variables retained in the model

	Estimate	SE	RR	95% CI		*P*
Serum bilirubin > 1 mg/dL	1.717	.560	5.57	1.86	16.67	**.002**
Alkaline phosphatase > 3N	1.320	.614	3.74	1.12	12.46	**.031**
Serum albumin < 38 g/L	1.425	.586	4.16	1.32	13.10	**.015**
SLC4A2/AE2 (rs2303932) – variant present (A)	−2.151	.622	.12	.04	.38	**<.001**

Progression under UDCA treatment, defined as the development of hyperbilirubinemia (>2 mg/dL), evolution towards cirrhosis, or referral to liver transplantation, was investigated in a sub-group of 147 patients who were followed-up for a mean period of 7.1 years at Saint-Antoine Hospital, Paris.
NB Variables presented in the first column used a scoring of 1.

is associated with allelic variation of *TNFα* and *SLC4A2*/AE2 genes.

The approach used for the selection of variants was based on tagging which exploits the extensive linkage disequilibrium in many parts of the genome. Tagging common alleles susceptible to affect a complex trait is expected to detect common and thus weakly causal variants in contrast to uncommon alleles that may carry substantial relative risks. Investigated patients were from both general and referral centers in France. However, because many variants in association studies depend on ethnic and population background, both the learning panel used to define Ht SNPs and the study populations comprised only French white continental patients with French ancestry. In addition, using a second group of controls with similar ethnic background validated the results. Because data from Haplotypes Map and other databases were not yet available at the time of the initiation of our study, only a low number of candidate genes were selected, thus representing an obvious limit of the present study.

4.1. Variants associated with susceptibility to PBC

CTLA-4 is a negative regulator of T cells and over the past 10 years, the CTLA-4/B7 pathway has emerged as a major determinant of immune tolerance [17]. The *CTLA-4* A to G transition of exon 1 (referred to as A49G in the literature) that results in the amino acid change T17A, was previously found to be associated with the occurrence of several autoimmune disorders [6–8,18]. While two isoforms of CTLA-4, a full-length isoform and a soluble form are generated by alternative splicing, the disease susceptible G haplotype of exon 1 SNP is associated with lower amounts of the transcripts encoding the soluble form [7]. Because the soluble splice form of CTLA-4 is secreted and inhibits T-cell proliferation in vitro [19], it has been proposed that a reduction in this form produced by the disease-susceptible haplotype, could contribute to the emergence of auto-immunity. Likewise, in the present study, the most significant association with PBC, was that of *CTLA-4* A49G. Considering that this SNP is associated with autoimmune thyroid diseases and other autoimmune disorders, we performed new analyses after excluding patients with coexisting autoimmune disorders. Still, the association with PBC remained significant (data not shown). This polymorphism was previously found to be associated with PBC in studies of 200 British patients [6], and of 77 Chinese patients [20]. However, the study of a smaller population from Brazil failed to confirm this association [21], and the most recent studies provided controversial results; in studies of 180 and 80 PBC patients from Germany and Italy/UK, respectively, no evidence for a link between CTLA-4 gene polymorphisms and PBC was found [10,22]. By contrast, in a large study from North America, the GG genotype at position 49 was significantly associated with subsets of PBC patients such as those with positive anti-mitochondrial auto-antibodies, or those requiring liver transplantation [11].

TNFα is a key cytokine to inflammation and apoptosis. SNPs in the promoter region of the gene encoding TNFα, were previously found to be associated with several auto-immune disorders such as systemic lupus erythematosus [23], primary Sjögren syndrome, rheumatoid arthritis [24], or primary sclerosing cholangitis [25]. In PBC, significant associations have also been reported between polymorphisms of the *TNFα* promoter region and disease susceptibility or severity [26–29]. In the present study, we identified a new variant in the 5′ flanking region of TNFα, which was significantly more frequent in PBC patients than in controls, providing further support to the notion that allelic variation in TNFα contributes to autoimmunity in PBC.

4.2. Variants associated with the severity of PBC

Until now, only variations in the genes encoding CTLA-4 [11], TNFα [26–29] or interleukin 1 [30], all involved in immunity, have been reported to affect the progression of PBC liver disease to late stages (III–IV). The present study provides the first evidence that variations in genes involved in detoxification, *ABCB1*/MDR1 and *NR1L2*/PXR, may influence the severity of liver disease in PBC patients, before treatment. MDR1, a major transporter of xenobiotics, confers resistance to drugs, and to environmental/dietary toxins

[31,32]. MDR1 may also provide an alternative route for the transport of bile salts, as suggested by studies in *ABCB11* knock-out mice [33]. In a previous study performed in Italy, no association was found between the silent C3435T polymorphism and PBC [34]. Yet, the present results indicate that *ABCB1* gene should be further considered as a severity-associated locus. In keeping with this view, associations have been reported between the exposure to tobacco or to other environmental chemicals and the risk or the severity of PBC [35,36]. Interestingly *NR1L2*/PXR SNP C/A in exon 9 3′-UTR (Table 3, lower box) was also associated albeit weakly with the severity of PBC at diagnosis. PXR, predominantly expressed in the liver and in the intestine, is a key regulator of many target genes evolved to protect the organism from exposure to environmental and dietary toxins. Polymorphisms altering PXR expression or function have been recently shown to affect the prognosis in primary sclerosing cholangitis [37] but not that of PBC [34]. Taking into account the major role of FXR in the adaptative response to cholestasis and of MDR3 in the pathophysiology of cholestasis, we expected some influence of their Ht SNPs on the behavior of PBC. This would have been in favor of targeting these genes in future therapeutic studies. However, we found no association between polymorphisms of nuclear receptors or of major hepatobiliary transporters and the severity of the disease.

To explore the possible impact of the common gene variants identified here on PBC behavior during long-term UDCA therapy, a subset of 147 patients followed in our department were studied. Two Ht SNPs, namely C/T in *TNFα* 5′ flanking region (Table 1) and T/A in *SLC4A2*/AE2 exon 6 (Table 2), were linked to prognosis. Worsening of PBC was mainly observed in patients lacking these two variants. These findings confirm the importance of allelic variation in the TNFα promoter for both susceptibility and severity of PBC. *SLC4A2*/AE2 gene product ensures alkaline choleresis. AE2 is critical for ductal bicarbonate secretion [38] and also influences acid/base equilibrium and activation status in lymphocytes [39]. Although it remains to be determined if T/A synonymous SNP located in exon 6 of the gene, is directly responsible for the phenotype observed, there is strong evidence for an important role of this gene in PBC pathogenesis. AE2 expression is reduced in the liver and in blood mononuclear cells from PBC patients [40,41] and partially restored in these patients by UDCA, a bile acid that induces bicarbonate-rich choleresis [42]. Furthermore, it has been recently shown that *Ae2a,b−/−* mice develop immunological and hepatobiliary features similar to those found in PBC [43]. The present results showing a strong relation between allelic variation of the gene and the evolution under UDCA, lends support to the assumption that *SLC4A2*/AE2 gene is a target of UDCA.

Acknowledgements

The following centers participated in the study: D. Capron (Amiens), J.P. Miguet (Besançon), T. Dao (Caen), J.P. Zarski (Grenoble), C. Buffet (Kremlin-Bicêtre), V. Canva (Lille), S. Pol (Paris), O. Goria (Rouen) and the members of the "Observatoire de la Cirrhose Biliaire Primitive" in France. We thank V. Godard (Paris) for her technical assistance.

The study was supported by a grant from PHRC 2003, AOR03022.

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