Published in final edited form as:

Gastroenterology. 2008 October ; 135(4): 1200–1206. doi:10.1053/j.gastro.2008.06.077.

Primary biliary cirrhosis is associated with a genetic variant in the 3' flanking region of the CTLA4 gene

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Abstract

Background and Aims—Genetic variation is invoked as a strong component underlying primary biliary cirrhosis (PBC) and other autoimmune disorders. Data suggests that some of this genetic risk is shared, affecting function of the immune mechanisms controlling self tolerance. Cytotoxic T-lymphocyte antigen 4 (CTLA4) encodes a coinhibitory immunoreceptor that is a key regulator of self tolerance with established genetic associations to multiple autoimmune diseases, but conflicting evidence of involvement with PBC. We aimed to perform a more comprehensive assessment of CTLA4 genetic variation in PBC using a haplotype-tagging based approach.

Methods—Single nucleotide polymorphisms (SNPs) were genotyped in 402 PBC patients and 279 controls and evaluated for association with PBC, and with antimitochondrial antibody (AMA) status and prior orthotopic liver transplant (OLT) among the PBC patients, both individually and as inferred haplotypes, using logistic regression.

Results—All SNPs were in Hardy Weinberg Equilibrium. We identified a novel and relatively strong association between PBC and rs231725, a SNP in the 3' flanking region of CTLA4 located outside of the area previously investigated in PBC. This SNP tags a common CTLA4 haplotype that contains a number of functionally implicated autoimmune CTLA4 SNPs, which was also found to be associated with PBC and to a lesser extent AMA status and prior OLT.

Conclusions—Our findings suggest that CTLA4 has an impact on the risk of PBC and possibly plays a role in influencing AMA development as well as progression to OLT among PBC patients. Replication in a suitable, independent PBC cohort is needed.

INTRODUCTION

It has become widely acknowledged that genetic variation is likely to play a strong role in the development of primary biliary cirrhosis (PBC), and is thought to underlie many pathogenic facets of this complex autoimmune disease, resulting in the diverse set of clinical phenotypes observed among affected patients^{1,2}. However, in contrast to the strong genetic

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NO CONFLICTS EXIST

effects demonstrated in Mendelian disease, the contribution of individual variants to PBC pathogenesis is likely to be quite small, and widely tempered by epigenetic modification as well as environmental and epistatic interaction. Thus, each genetic variant will merely contribute to the risk of disease, or might work to mediate clinical variation such as rate of progression or response to treatment, but will not directly cause disease development^{3,4}. This genetic characteristic is inherent to complex disorders, and when coupled with the rarity of PBC, has significantly hampered previous efforts to identify the genetic contributors to this disease.

Regardless of disease specific influences, autoimmune disease susceptibility is presumed to result from the collective effect of minor genetic variations in the immune mechanisms that establish and maintain self-tolerance⁴. Thus, individuals can be conceptualized as falling somewhere within a genetically-encoded autoimmune-permissive spectrum that, when confronted with disease specific genetic variation and relevant environmental exposures, governs whether or not the particular autoimmunity will develop.

One vital means by which the immune system maintains self-tolerance is through inhibition of T-cell activation upon T-cell receptor stimulation^{4,5}. A key facilitator of this process is the cytotoxic T-lymphocyte antigen-4 (CTLA4) gene, which encodes a coinhibitory immunoreceptor expressed on activated T-cells, that upon binding to ligands (CD80 and CD86) found on the surface of antigen presenting cells delivers an inhibitory signal in competition with its stimulatory counterpart, CD28⁶⁻⁸. The importance of this gene to immune homeostasis is demonstrated by the CTLA4 knockout mouse, which develops severe lymphoproliferative disease and multi-organ autoimmunity, leading to massive tissue destruction and early death^{9, 10}. In humans, genetic variants of CTLA4 have been associated with a wide array of autoimmune diseases¹¹. However, tight linkage disequilibrium (LD) across the gene has made the identification of the specific genetic culprits difficult, and controversy remains as to which variants contribute to autoimmune disease.

In order to overcome the LD imposed difficulties with mapping CTLA4, functional studies have been performed on a number of the associated variants. The most widely implicated CTLA4 polymorphism for risk of autoimmunity, commonly referred to as 49AG (rs231775; A/G), appears to affect cell surface expression in response to T-cell activation^{12, 13}. This coding polymorphism is located in the signal peptide that is cleaved from the functional protein, and was shown to affect glycosylation of the autoimmune susceptibility G allele, resulting in diminished processing efficiency and thus decreased trafficking to the cell surface¹³. A polymorphism located at position -318 in the promoter of CTLA4 (rs5742909; C/T) also appears to effect cell surface expression¹⁴, but is suggested to be protective against autoimmunity. The protective T allele was shown to result in increased mRNA levels and cell surface expression of CTLA4 following cellular stimulation¹⁴ suggesting that this promoter polymorphism might increase CTLA4 expression and consequently decrease risk of disease. Interestingly, the autoimmune protective -318 T allele is linked to the non-risk A allele of 49AG, potentially confusing the results. However, the current research suggests that the polymorphisms have separate effects on cell surface expression, but are prone to interaction¹⁴.

Polymorphisms in the 3' untranslated region (UTR) of CTLA4 have also been associated with autoimmunity^{15, 16} and this region of the gene has been shown to regulate mRNA stability and translational efficiency¹⁷. The most extensively studied of the CTLA4 3' UTR polymorphisms is CT60, which appears to affect the expression of a soluble form of the molecule^{15, 18, 19} as well as alter the signaling threshold of CD4+ T cells²⁰, changes which are thought to contribute to autoimmune development.

Due to its apparent importance to autoimmunity in the general sense, CTLA4 has been one of the most widely studied genes in PBC. Early studies of CTLA4 found an association of the minor 49AG allele with PBC in both UK²¹ and Chinese²² populations. But, a follow-up study by the UK group, considering additional SNPs across CTLA4 in a larger patient population with a more appropriate control group, failed to replicate their initial findings, calling into question the relevance of 49AG and other CTLA4 SNPs to PBC²³. Moreover, individual CTLA4 SNPs were not found to be significantly associated with PBC development in studies from Italy²⁴, Germany²⁵ and the US²⁶. However, significant associations of CTLA4 SNPs with anti-mitochondrial antibody (AMA) status^{24,26} and progression to orthotopic liver transplantation (OLT)²⁶ among PBC patients have been reported, suggesting that CTLA4 may indeed play an important role in the pathogenesis of this disease. In light of the recent findings, we aimed to perform a more comprehensive assessment of CTLA4 genetic variation in our PBC patients using a haplotype-tagging based approach.

PATIENTS AND METHODS

Study Participants

The participants in this study, 402 well-documented PBC patients and 279 outpatient-clinic based controls, were previously recruited into our Mayo Clinic PBC Genetic Epidemiology Registry and Biospecimen Repository, which was created with the aim to uncover the genetic and environmental contributors to PBC pathogenesis²⁷. PBC diagnosis was confirmed by chart review supporting persistent biochemical cholestasis (greater than 6 months) in the absence of other known liver disease, compatible liver histopathology, and/or detectable AMA in serum. Controls were recruited from the Mayo Clinic Division of General Internal Medicine during annual visits for preventative medical examination and were matched by age (± 2.5 years), sex, and state of residence to individual PBC patients. Control exclusion criteria included evidence of prior or current liver disease. Demographic characteristics of the patient and control groups are shown in Table 1.

Informed consent was obtained from all study participants. Our registry and present study conform to the ethical guidelines of the 1975 Declaration of Helsinki, and have been approved by the Institutional Review Board of the Mayo Clinic.

Sample Handling and DNA Preparation

The collection of blood specimens from study participants was performed using bar-coded mail-in kits, which were prepared under the direction of the Mayo Central Laboratory for Clinical Trials (MCLCT) as previously described²⁶. The processing of the specimens, assessment, quality control, and distribution was carried out under the supervision of the MCLCT.

The Mayo Clinic General Clinical Research Center (GCRC) performed the isolation of genomic DNA from the blood samples using the PureGene kit (Gentra Systems, MN) as specimens were received. Subsequent handling of the DNA, including quality control and dilution to working concentrations was carried out in our own laboratory.

AMA Testing

The serum of participants was tested for the presence of AMA at the time of study enrollment. The Mayo Clinic Diagnostic Immunology Laboratory performed the AMA testing utilizing a commercially available ELISA (Diastat AMA, Euro-Diagnostica, Malmö, Sweden) specific for the PDC-E2 (M2) antigen. The absorbance units were categorized as

negative (≤ 0.1 units) and positive (≥ 0.2 units) for detection of AMA as described previously²⁷.

SNP Selection and Typing

Haplotype tagging SNPs for CTLA4 were identified by means of the LD-select method described by Carlson et al²⁸ using the HapMap CEU population data. This method bins SNPs based on an LD threshold as measured by r^2 and thus each SNP exceeds the r^2 threshold with all other SNPs in the bin. SNPs 5kb upstream and downstream of CTLA4, and not located in repeat sequences were included in the analysis, which was based on gene and SNP coordinates from NCBI build 36 and dbSNP build 127, respectively. Moreover, an r^2 value of 0.9 and minor allele frequency (MAF) of 1% were the parameters used to perform the SNP selection. A total of 8 bins were identified of which 5 contained individual SNPs. A single SNP was chosen to represent each of the 3 additional bins, with care taken to include the widely published CTLA4 49AG (rs231775) and CT60 (rs3087243) SNPs.

All 8 CTLA4 polymorphisms were typed by commercially available TaqMan allelic discrimination assays (Applied Biosciences, California) using Applied Biosciences 7300 or 7500-fast real-time polymerase chain reaction systems.

Statistical Analysis

Disease association with individual SNPs was performed using logistic regression to determine statistical significance along with odds ratios (OR) and 95% confidence intervals (CI); multiple inheritance models were considered. Permutation tests were performed to account for multiple testing in the single SNP analyses. Adjusted p-values were computed using 1,000 simulations. For each simulation, case-control status was randomly permuted and a new p-value was calculated. The adjusted p-value was computed from the number of times out of 1,000 simulations that the minimum simulated p-value (over all SNPs) was less than the observed p-value. Haplotypes were inferred using the expectation-maximization (EM) algorithm and studied for association with PBC, AMA status, and prior orthotopic liver transplantation (OLT) using a score test and simulated p-values, assuming multiple inheritance models. Age and sex were included as adjustment factors in all the models. All analyses were two-sided, and the analysis was completed using Splus, version 8.0.

RESULTS

Individual SNP Analysis

All genotyped SNPs were found to be in Hardy-Weinberg equilibrium. The counts and frequencies of each SNP were determined and statistical analysis was performed between (a) the AMA positive PBC patients and controls, (b) the AMA positive and AMA negative PBC patients, and (c) PBC subgroups based on prior OLT (Table 2). A novel and relatively strong PBC association signal was detected at rs231725 (Table 2a), which lies in the 3' flanking region of CTLA4 approximately 2kb downstream of the UTR, outside of the area previously investigated for PBC. Homozygosity for the minor A allele was significantly increased in PBC patients compared to controls (17% vs. 9%, $P_{cor}=0.003$). As well, the overall frequency of the A allele was also increased among PBC patients, although the significance was borderline following correction (37% PBC vs. 30% controls, $P_{cor}=0.061$). Of interest, rs231725 allele and genotype frequencies also differed between the AMA positive and AMA negative PBC patients (Table 2b). Frequency of the A allele was increased among AMA positive patients (37% AMA+ vs. 27% AMA-), as was homozygosity (17% AMA+ vs. 4% AMA-). However, neither difference was significant after correction. Concerning OLT status, the frequency of the A allele of rs231725 was quite similar between the groups (35% OLT vs. 36% no-OLT) (Table 2c). However, homozygosity for the allele was

considerably increased among the OLT patients (25% OLT vs. 14% no-OLT), but not to a level of statistical significance.

In addition to the strong association signal at rs231725, significant results were also obtained for the rs231775 (49AG), rs231777 (+923), and rs11571319 SNPs in the case-control analysis (Table 2). Both rs231777 and rs11571319 appeared protective against PBC in the homozygous state; however the p-values did not retain significance following correction. Interestingly, the increase in homozygosity for the G allele of 49AG observed among PBC patients remained significant following correction (19% PBC vs. 12% controls, $P_{cor}=0.049$). This SNP was also marginally associated with AMA positivity among PBC patients (Table 2b), with decreased homozygosity for the G allele found among AMA negative patients (19% AMA+ vs. 4% AMA-, $P_{cor}=0.077$). Moreover, the 49AG GG genotype was increased in PBC patients who had undergone OLT (Table 2C), but this difference did not reach statistical significance (27% OLT vs. 17% no-OLT). None of the other studied SNPs were found to be associated with PBC or the AMA and OLT subgroups under additive, dominant (data not shown), or recessive models (Table 2).

Haplotype Analysis

Frequencies of the CTLA4 haplotypes were inferred from the individual genotypes by the EM algorithm and analyzed for association with PBC, AMA status, and prior OLT with score tests under multiple models (Table 3). Seven common haplotypes (i.e. frequency greater than 1%) were identified, representing 99.5% of the inferred haplotypes in the assessed population. Among the evaluated inheritance models, only the recessive model was globally significant for PBC ($p=0.008$), and was also marginally significant for both AMA status ($p=0.092$) and prior OLT ($p=0.080$) (Table 3). These findings were largely driven by the individually associated A allele of SNP rs231725 (described above), which was present on but a single common haplotype (i.e. CCCGCGGA, Haplotype 2) (Table 3). This resulted in significant association of this individual haplotype with PBC under the additive and recessive models (Table 3a). As well, this haplotype was associated with AMA positivity (Table 3b), and OLT (Table 3c) under the recessive model, suggesting a haplotype effect beyond that of the individual rs231725 SNP, although caution should be taken in interpreting this result considering the borderline significance of the global tests. Of interest, the newly identified PBC-risk haplotype tagged by the A allele of rs231725 contains all of the risk alleles of the SNPs that have been studied functionally (described in the Introduction section above), including the common (non-protective) C allele at -318, the minor G allele at 49AG, and the G allele of CT60.

DISCUSSION

We report the association of PBC with rs231725, a SNP in the 3' flanking region of CTLA4, located outside of the area of this gene previously studied in PBC. Notably, this variant is located on but one of the 7 common CTLA4 haplotypes inferred from the eight SNPs of our study, and thus "tags" a haplotype that demonstrates associations with PBC, and to a lesser extent AMA status, and prior OLT (Table 3).

Whether or not the observed association with PBC is due to functional aspects of CTLA4 directly related to the rs231725 SNP (or other strongly linked SNPs in the 3' flanking region) or to variation in gene function arising from the combination of alleles present on the tagged haplotype is unclear. Interestingly, the newly identified PBC risk haplotype contains a number of the "autoimmune predisposing" CTLA4 alleles for which functional analysis has been performed, raising the possibility that the mixture of alleles present on this haplotype might contribute to disease risk. Among these are the non-protective C allele at -318 in the promoter, found to effect expression of mRNA as well as level of the receptor at

the cell surface^{14, 29}, the coding G allele at 49AG that reportedly results in reduced cell surface expression¹³, and the more common G allele of CT60 which appears to affect the expression of a soluble form of the molecule^{15, 18, 19} as well as alter the signaling threshold of CD4+ T cells²⁰. Of note, the rs231725 SNP was recently reported to be associated with systemic lupus erythematosus (SLE) in a family based investigation from the UK¹⁶. However, this study found the PBC risk allele (i.e. A) of this SNP to be under-transmitted in SLE families in contrast to its over-representation in our PBC patients, suggesting that genetic variation in CTLA4 might play a role in delineating between development of systemic or organ-specific autoimmune disease.

In addition to the rs231725 SNP, our study found a significant risk of PBC associated with homozygosity for the G allele of 49AG, which was also weakly associated with AMA positivity among the PBC patients (Table 2). Of interest, early studies of CTLA4 had also found this genotype to be associated with PBC^{21, 22}, but larger follow-up investigations, including an earlier study from our laboratory²⁶, were not able to replicate the significance, even though the frequency of 49AG GG was universally increased among PBC patients^{23–26}. In light of our novel finding of the rs231725 association and considering the haplotype structure of CTLA4, it is likely that the association we see with 49AG is primarily due to linkage with rs231725. In order to address this, we investigated the carriage of the 49AG G (risk) allele on the chromosomes containing the rs231725 G (non-risk) allele, which was possible due to the limited haplotypic presentation of the 49AG G allele and dependence between the 49AG G and rs231725 A alleles. To this end, we found that carriage of the 49AG G allele on rs231725 G-tagged chromosomes did not significantly differ between PBC patients and controls (7.4% vs. 9.4%, $p=0.3310$) or between AMA positive and AMA negative PBC patients (7.6% vs. 6.1%, $p=0.8044$). However, carriage of the 49AG G allele was significantly increased on the rs231725 G-tagged chromosomes of PBC patients who had undergone OLT, compared to those who had not (15.8% vs. 7.3%, $p=0.0390$), indicating that this allele may have an independent effect on disease progression over and above that conferred by the rs231725 A tagged haplotype.

It has been proposed that common functional variants in genes central to immune regulation, such as CTLA4, have arisen and are maintained in the population as the result of balancing evolutionary selection, likely as a mechanism to preserve a diversified immune response to pathogens and infectious disease³⁰. Thus, such variant alleles might prove differentially advantageous by enhancing the response to particular viral or bacterial insults, resulting in increased resistance to infection¹⁵. In this context, the long-term consequence to such a beneficially amplified immune response is an increased risk for development of autoimmunity, predisposition to which is likely to be widespread among the population. The mechanisms controlling whether or not, and which specific autoimmune disease or diseases will develop as a result of this increased risk remain unclear, but are thought to be quite complex, involving interaction between the predisposing genetic variants and environmental exposures to pathogens and toxins, and taking place over a period of many years.

The strength of our study stems from our large collection of meticulously phenotyped PBC patients and well-matched controls, which is sufficiently powered to detect the associations we report and intended to minimize bias due to population stratification. As well, our LD based haplotype-tagging approach afforded good coverage of the genetic variation across CTLA4 without being overly redundant, and our analysis cautiously addressed multiple testing using up-to-date methodology which considers the interdependence of the SNPs and avoids being overly-conservative. Such careful considerations are thought to be important for meaningful association studies at the current time³¹. However, our study is not without faults. Analysis of the AMA and OLT subgroups is somewhat underpowered, and thus caution should be taken in considering the relative impact of CTLA4 associations on these

features of PBC. Moreover, our findings have yet to be replicated in an independent cohort, which is ultimately the true test of any gene association study.

In conclusion, our findings suggest that, contrary to recent opinion, CTLA4 has a noteworthy impact on risk of PBC and may play a role in mediating AMA development as well as disease progression to OLT among PBC patients. The majority of the CTLA4 risk appears to be conferred by the A allele of rs231725, a SNP located in the 3' flanking region that tags a single haplotype which contains the alleles previously identified as associated with a number of organ-specific autoimmune diseases, and for which functional consequences have been demonstrated. Whether it is the rs231725 SNP itself (or other unobserved variants in tight linkage with it) or the combination of alleles on the haplotype tagged by this SNP that are the functional mediators of PBC risk remains unclear.

Acknowledgments

Grant Support: This research was supported by grants to Dr. Lazaridis from the NIH (K23 DK68290, RO3 DK078527 and RO1 DK80670); American Gastroenterological Association (AGA) and Foundation for Digestive Health and Nutrition (FDHN); American Liver Foundation; Palumbo Foundation; Miles and Shirley Fiterman Foundation, and the Mayo Clinic College of Medicine.

Abbreviations

AMA	anti-mitochondrial antibody
CTLA4	cytotoxic T-lymphocyte antigen 4
LD	linkage disequilibrium
OLT	orthotopic liver transplantation
PBC	primary biliary cirrhosis
SNP	single nucleotide polymorphism
UTR	untranslated region

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Table 1

Demographics of PBC patients and controls

	PBC Patients (n=402)	Controls (n=279)
Sex		
Female	91.3%	89.2%
Male	8.7%	10.8%
Race		
Caucasian	100%	100%
Mean Age (years) *	60.4 (34.7–84.9)	62.9 (35.6–87.7)
Mean Age at Diagnosis (years) *	51.1 (29–77)	-
Disease duration (years) *	9.3 (0–31)	-
Biopsy at Diagnosis (n=272)		-
Stage I–II	67.6%	-
Stage III–IV	32.4%	-
Liver Transplanted	10.9%	-
UDCA Therapy	86.7%	-

* Values expressed as mean (range)

Table 2

SNP frequencies and statistical analysis

a. PBC											
SNP rs ID	Common Name	Minor Allele	Minor Allele Frequency		Additive Model			Recessive Model			Pcor
			PBC (n=351*)	Con (n=279)	OR (95% CI)	Pval	Pcor	OR (95% CI)	Pval	Pcor	
rs16840252	-1146	T	0.17	0.20	0.83 (0.62-1.12)	0.221	0.687	0.39 (0.14-1.06)	0.065	0.230	
rs11571317	-657	T	0.08	0.06	1.33 (0.86-2.08)	0.202	0.638	1.32 (0.12-14.84)	0.822	1.000	
rs5742909	-318	T	0.08	0.10	0.83 (0.56-1.22)	0.335	0.849	0.23 (0.03-2.06)	0.188	0.601	
rs231775	49AG	G	0.41	0.37	1.22 (0.97-1.53)	0.086	0.328	1.75 (1.12-2.74)	0.015	0.049	
rs231777	+923	T	0.15	0.18	0.79 (0.58-1.07)	0.128	0.468	0.34 (0.12-0.97)	0.043	0.160	
rs3087243	CT60	A	0.42	0.43	0.94 (0.75-1.17)	0.562	0.979	0.96 (0.64-1.45)	0.853	1.000	
rs11571319	-	A	0.17	0.20	0.83 (0.62-1.12)	0.223	0.691	0.36 (0.13-0.96)	0.042	0.158	
rs231725	-	A	0.37	0.30	1.34 (1.06-1.68)	0.014	<i>0.061</i>	2.22 (1.34-3.68)	0.002	0.003	
b. AMA											
SNP rs ID	Common Name	Minor Allele	Minor Allele Frequency		Additive Model			Recessive Model			Pcor
			AMA+ (n=351)	AMA- (n=45)	OR (95% CI)	Pval	Pcor	OR (95% CI)	Pval	Pcor	
rs16840252	-1146	T	0.17	0.20	0.80 (0.45-1.44)	0.464	0.934	0.37 (0.07-1.95)	0.241	0.679	
rs11571317	-657	T	0.08	0.08	1.06 (0.47-2.42)	0.885	1.000	-	0.989	1.000	
rs5742909	-318	T	0.08	0.13	0.58 (0.28-1.18)	0.131	0.434	-	0.992	1.000	
rs231775	49AG	G	0.41	0.31	<i>1.55 (0.97-2.48)</i>	0.064	0.242	5.15 (1.21-21.8)	0.026	<i>0.077</i>	
rs231777	+923	T	0.15	0.19	0.70 (0.38-1.27)	0.243	0.696	0.58 (0.06-5.25)	0.631	0.931	
rs3087243	CT60	A	0.42	0.48	0.78 (0.50-1.21)	0.261	0.737	0.88 (0.40-1.92)	0.741	0.985	
rs11571319	-	A	0.17	0.20	0.80 (0.45-1.44)	0.464	0.934	0.37 (0.07-1.95)	0.241	0.679	
rs231725	-	A	0.37	0.27	<i>1.56 (0.97-2.50)</i>	0.068	0.247	4.37 (1.03-18.6)	0.046	0.142	
c. OLT											
SNP rs ID	Common Name	Minor Allele	Minor Allele Frequency		Additive Model			Recessive Model			Pcor
			OLT (n=44)	No OLT (n=358)	OR (95% CI)	Pval	Pcor	OR (95% CI)	Pval	Pcor	
rs16840252	-1146	T	0.22	0.17	1.34 (0.75-2.39)	0.317	0.816	2.80 (0.54-14.6)	0.221	0.608	
rs11571317	-657	T	0.05	0.09	0.51 (0.18-1.46)	0.210	0.655	-	0.989	1.000	
rs5742909	-318	T	0.10	0.09	1.19 (0.55-2.59)	0.655	0.992	-	0.992	1.000	

c. OLT

SNP rs ID	Common Name	Minor Allele	Minor Allele Frequency		Additive Model			Recessive Model		
			OLT (n=44)	No OLT (n=358)	OR (95% CI)	Pval	Pcor	OR (95% CI)	Pval	Pcor
rs231775	49AG	G	0.43	0.40	1.15 (0.74–1.78)	0.531	0.977	1.93 (0.93–3.99)	0.075	0.191
rs231777	+923	T	0.20	0.15	1.52 (0.84–2.74)	0.168	0.560	4.04 (0.70–23.2)	0.118	0.356
rs3087243	CT60	A	0.35	0.43	0.73 (0.46–1.16)	0.187	0.597	0.83 (0.35–1.95)	0.672	0.960
rs11571319	-	A	0.22	0.17	1.34 (0.75–2.39)	0.317	0.816	2.80 (0.54–14.6)	0.221	0.608
rs231725	-	A	0.35	0.36	1.00 (0.64–1.54)	0.991	1.000	1.98 (0.94–4.18)	0.074	0.190

* Only the AMA+ PBC patients were considered in the case-control analysis

Missing odds ratios (OR) for SNPs rs11571317 and rs5742909 under the recessive model in 2b and 2c are due to lack of minor allele homozygotes in the AMA negative and OLT subgroups.

Table 3

CTLA4 haplotype data and analysis

a. PBC													
Hap#	SNP								Frequency		P-Additive (p=0.099)*	P-Dominant (p=0.157)*	P-Recessive (p=0.008)*
	1	2	3	4	5	6	7	8	All	PBC			
1	C	C	C	A	C	A	G	G	0.348	0.332	0.173	0.115	0.645
2	C	C	C	G	C	G	G	A	0.334	0.365	0.016	0.165	0.002
3	T	C	T	A	T	G	A	G	0.091	0.084	0.324	0.466	0.167
4	C	T	C	A	C	A	G	G	0.074	0.083	0.197	0.186	-
5	T	C	C	A	T	G	A	G	0.066	0.061	0.462	0.310	-
6	C	C	C	G	C	G	G	G	0.057	0.047	0.068	0.081	-
7	T	C	C	A	C	G	A	G	0.025	0.026	0.830	0.846	-

b. AMA													
Hap#	SNP								Frequency		P-Additive (p=0.284)*	P-Dominant (p=0.608)*	P-Recessive (p=0.092)*
	1	2	3	4	5	6	7	8	All	AMA+			
1	C	C	C	A	C	A	G	G	0.340	0.332	0.207	0.189	0.497
2	C	C	C	G	C	G	G	A	0.354	0.365	0.065	0.260	0.030
3	T	C	T	A	T	G	A	G	0.090	0.084	0.138	0.112	-
4	C	T	C	A	C	A	G	G	0.082	0.083	0.919	0.973	-
5	T	C	C	A	T	G	A	G	0.061	0.061	0.975	0.684	-
6	C	C	C	G	C	G	G	G	0.047	0.047	0.992	0.994	-
7	T	C	C	A	C	G	A	G	0.024	0.027	0.340	0.356	-

c. OLT													
Hap#	SNP								Frequency		P-Additive (p=0.569)*	P-Dominant (p=0.108)*	P-Recessive (p=0.080)*
	1	2	3	4	5	6	7	8	All	no OLT			
1	C	C	C	A	C	A	G	G	0.340	0.342	0.724	0.470	0.739
2	C	C	C	G	C	G	G	A	0.354	0.355	0.958	0.118	0.038
3	T	C	T	A	T	G	A	G	0.090	0.088	0.596	0.540	-
4	C	T	C	A	C	A	G	G	0.082	0.086	0.242	0.239	-
5	T	C	C	A	T	G	A	G	0.061	0.058	0.473	0.603	-

c. OLT													
Hap#	SNP								Frequency		P-Additive (p=0.569)*	P-Dominant (p=0.108)*	P-Recessive (p=0.080)*
	1	2	3	4	5	6	7	8	All	no OLT			
6	C	C	C	G	C	G	G	G	0.047	0.042	0.144	0.121	-
7	T	C	C	A	C	G	A	G	0.024	0.025	0.489	0.510	-
* Haplotype Global P-value													
* Haplotype Global P-value													
* Haplotype Global P-value													
Missing p-values under the recessive model is due to lack of haplotype specific homozygotes (i.e. individuals homozygous for all indicated alleles of the specific haplotype) in one or more of the compared groups.													
SNP 1 – rs16840252, S NP 2 – rs11571317, SNP 3 – rs5742909, SNP 4 – rs231775, SNP 5 – rs231777, SNP 6 – rs3087243, SNP 7 – rs11571319, SNP 8 – rs231725													