Primary Biliary Cirrhosis Is Associated With a Genetic Variant in the 3′ Flanking Region of the CTLA4 Gene

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Background & Aims: **Genetic variation is invoked as a strong component underlying primary biliary cirrhosis (PBC) and other autoimmune disorders. Data suggest 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 transplantation (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.**

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 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-tolerance.[4] 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 on 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, CD28.[6–8] The importance of this gene to immune homeostasis is demonstrated by the CTLA4 knockout mouse, which develops severe lymphoproliferative disease and multiorgan autoimmunity, leading to massive tissue destruction

Abbreviations used in this paper: 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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and early death.[9,10] In humans, genetic variants of CTLA4 have been associated with a wide array of autoimmune diseases.[11] 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.

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 surface.[13] A polymorphism located at position −318 in the promoter of CTLA4 (rs5742909; C/T) also appears to effect cell surface expression[14] but is suggested to be protective against autoimmunity. The protective T allele was shown to result in increased messenger RNA (mRNA) levels and cell surface expression of CTLA4 following cellular stimulation,[14] 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 nonrisk 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 interaction.[14] 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 efficiency.[17] The most extensively studied of the CTLA4 3′ UTR polymorphisms is CT60 (rs3087243; A/G), 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 cells,[20] changes that are thought to contribute to autoimmune development.

Because of 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 United Kingdom[21] and Chinese[22] populations. However, a follow-up study by the United Kingdom group, considering additional single nucleotide polymorphisms (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 PBC.[23] Moreover, individual CTLA4 SNPs were not found to be significantly associated with PBC development in studies from Italy,[24] Germany,[25] and the United States.[26] However, significant associations of CTLA4 SNPs with antimitochondrial 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 pathogenesis.[27] 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,

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		
White, %	100	100
Mean age, *y*[a]	60.4 (34.7−84.9)	62.9 (35.6−87.7)
Mean age at diagnosis[a]	51.1 (29−77)	—
Disease duration[a]	9.3 (0−31)	—
Biopsy at diagnosis (n = 272), *y*		—
Stage I-II	67.6	—
Stage III-IV	32.4	—
Liver Transplanted, %	10.9	—
UDCA Therapy, %	86.7	—

UDCA Ursodeoxycholic acid.
[a]Values expressed as mean (range).