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Common Genetic Variation and Haplotypes of the Anion Exchanger SLC4A2 in Primary Biliary Cirrhosis

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Abstract

Objectives—Deficiencies of the anion exchanger SLC4A2 are thought to play a pathogenic role in primary biliary cirrhosis (PBC), evidenced by decreased expression and activity in PBC patients and development of disease features in SLC4A2 knockout mice. We hypothesized that genetic variation in SLC4A2 might influence this pathogenic contribution. Thus, we aimed to perform a comprehensive assessment of SLC4A2 genetic variation in PBC using a linkage disequilibrium (LD)-based haplotype-tagging approach.

Methods—Twelve single nucleotide polymorphisms (SNPs) across SLC4A2 were genotyped in 409 PBC patients and 300 controls and evaluated for association with disease, as well as with prior orthotopic liver transplant and antimitochondrial antibody (AMA) status among the PBC patients, both individually and as inferred haplotypes, using logistic regression.

Results—All SNPs were in Hardy–Weinberg equilibrium. No associations with disease or liver transplantation were detected, but two variants, rs2303929 and rs3793336, were associated with negativity for antimitochondrial antibodies among the PBC patients.

Conclusions—The common genetic variation of SLC4A2 does not directly affect the risk of PBC or its clinical outcome. Whether the deficiency of SLC4A2 expression and activity observed earlier in PBC patients is an acquired epiphenomenon of underlying disease or is because of heritable factors in unappreciated regulatory regions remains uncertain. Of note, two SLC4A2 variants appear to influence AMA status among PBC patients. The mechanisms behind this finding are unclear.

Introduction

Primary biliary cirrhosis (PBC) is an autoimmune liver disease in which the intrahepatic bile ducts are gradually destroyed, resulting in cholestasis and often eventual progression to cirrhosis (1). The etiology of PBC remains enigmatic and is considered to be complex. That

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Conflict of Interest

Guarantor of the article: Konstantinos N. Lazaridis, MD.

Potential competing interests: None.

Specific author contributions: Brian D. Juran designed the study with input from Konstantinos N. Lazaridis, analyzed the data with the help of Elizabeth J. Atkinson and Joseph J. Larson, and wrote the paper. Konstantinos N. Lazaridis recruited subjects and Erik M. Schlicht mailed questionnaires and blood kits to enrolled participants. Elizabeth J. Atkinson and Joseph J. Larson provided statistical analysis and support. Konstantinos N. Lazaridis is the principal investigator of the laboratory and provided input on the study design and analysis, and editorial comments on the paper.

is, a combination of inherited genetic risks and environmental exposures is likely required for disease development. Evidence for the genetic component is strong, supported by high disease concordance in monozygotic twins (2), and increased familial disease prevalence (3,4). However, few robust genetic associations have been reported to date (5). Environmental risk is supported in concept by variable geographic prevalence (6) and disease clustering (7,8), but no specific environmental candidates have been identified or proposed from these findings. Specific environmental risks such as smoking (3,9), history of urinary tract infection (3), hormone replacement therapy (3), frequent nail-polish use (3), and certain microorganisms (10–12) have been implicated with PBC.

Owing to its complex nature, advancement in the understanding of PBC pathogenesis has been painstakingly slow. However, new mouse models have shed some light on the processes potentially underlying human disease. Most recent among these is the solute carrier 4, anion exchanger 2 (SLC4A2; commonly known as AE2) deficient mouse (13). With age, these mice develop a number of PBC-like features, including increased alkaline phosphatase and alanine aminotransferase levels, portal inflammation with damage to the bile ducts, development of antimitochondrial antibodies (AMAs) that are reactive to the pyruvate dehydrogenase complex E2 subunit (PDC-E2), and CD4 + CD25 + FoxP3 + regulatory T-cell deficiency (13).

SLC4A2 is expressed in a wide array of cell types and is involved in the regulation of intracellular pH through exchange of intracellular bicarbonate for extracellular Cl^- (14). In cholangiocytes, the cells targeted for destruction in PBC, this transporter also plays an important role in maintenance and modification of the bile acid pool through secretin-stimulated bicarbonate secretion (15). About 15 years ago it was postulated that altered function of SLC4A2 might play a role in PBC, and subsequently its expression was shown to be decreased in PBC patients at both the mRNA (16) and protein (14) levels. Follow-up study has also shown decreased basal-state and cAMP-stimulated SLC4A2 activity in cholangiocytes of PBC patients (17).

These human studies, along with the recently reported mouse model (13), provide evidence for a pathogenic role of SLC4A2 deficiency in PBC. However, the true nature of these observations as well as the mechanisms giving rise to them remains unclear. For instance, reduced SLC4A2 expression and activity could be the primary etiological components of PBC, or secondary, albeit pathogenic, effects arising because of other physiological features underlying the diseased state. Moreover, the relative strength of SLC4A2-driven pathogenesis might vary widely across the PBC population, depending on other characteristics or risk factors specific to each individual. Genetic variation is one possible mechanism effecting the pathogenic contribution of SLC4A2 to PBC, and has the potential to operate in a wide variety of capacities to facilitate an SLC4A2 pathogenic effect on disease. We hypothesized that genetic variants of SLC4A2 might influence PBC risk, progression, or development of AMA in PBC patients. Thus, we aimed to assess the contribution of the common genetic variation in SLC4A2 to PBC using our large collections of well-characterized PBC patients and suitably matched controls by means of a linkage disequilibrium (LD)-based haplotype-tagging approach.

Methods

Study Participants

The 409 well-documented PBC patients and 300 outpatient-clinic-based controls of this study are participants of our Mayo Clinic PBC Genetic Epidemiology Registry and Biospecimen Repository, which has been described earlier (18,19). Diagnosis of PBC was made on the basis of standard medical criteria, including (a) evidence of persistent biochemical cholestasis (lasting >6 months) without other known liver disease, (b) compatible liver histopathology,

and/or (c) detectable AMA in serum. Controls were recruited from the Division of General Internal Medicine at Mayo Clinic during annual preventative medical examinations and were matched by age (± 2.5 years), sex, and state of residence to individual PBC patients. The exclusion criteria of controls 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 were approved by the Institutional Review Board of the Mayo Clinic.

Sample Handling and DNA Preparation

The collection of blood specimens was performed under the direction of the Mayo Central Laboratory for Clinical Trials as described earlier (18,19). Isolation of genomic DNA was performed by the Mayo Clinic General Clinical Research Center using the PureGene Kit (Gentra Systems, MN) as specimens were received. A total of seven blood samples from PBC patients (1.7%) and no blood samples from controls failed to yield sufficient DNA for genotyping, leaving the 409 patients and 300 controls available for the study.

AMA Testing

The Mayo Clinic Diagnostic Immunology Laboratory performed AMA testing of fresh serum from all study participants at the time of study enrollment using a commercially available ELISA (Diastat AMA, Euro-Diagnostica, Malmo, 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 earlier (18).

SNP selection and typing

Haplotype-tagging single nucleotide polymorphisms (SNPs) for SLC4A2 were identified by means of the LD-select method described by Carlson (20) using the HapMap CEU population data. An r^2 value of 1.0 and minor allele frequency of 5 % were the parameters used to perform the SNP selection. A total of five bins were identified, each of which contained an individual SNP. To achieve better coverage of the gene, additional SNPs were identified using the CEPH population data from the National Institute of Environmental Health Services Environmental Genome Project gene resequencing initiative. For this supplemental selection, LD-select (20) was again used; an r^2 value of 0.85 and minor allele frequency of 5 % were the parameters. Ultimately, a total of 12 SNPs (including the five HapMap SNPs) were selected for genotyping, which tagged all of the additional 19 common SNPs at r^2 >0.85, including all such SNPs 3 kb upstream of the gene, completely covering the known promoter region (21). All analysis was based on gene and SNP coordinates from NCBI build 36 and dbSNP build 127, respectively.

The 12 studied SLC4A2 polymorphisms were typed by commercially available or custom-designed TaqMan allelic discrimination assays by the standard recommended standard protocol provided by the manufacturer (Applied Biosystems, Foster City, CA) using an Applied Biosciences 7300 real-time polymerase chain reaction system. Genotyping calls were made by the laboratory staff blinded to the identity of the patients and controls, and 2% of reactions were repeated to determine allele-calling consistency, with no discrepancies found.

Statistical Analysis

Association with individual SNPs was analyzed by means of logistic regression to determine statistical significance, odds ratios, and 95 % confidence intervals; multiple inheritance models were evaluated. Correction for multiple tests was performed by permutation testing, whereby 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 algorithm and assessed for association using a score test and simulated *P*-values, assuming multiple inheritance models. For both the individual SNP and haplo-type analyses, association with PBC was determined using all members of the case and control groups, whereas association with prior orthotopic liver transplantation (OLT) and AMA status was determined using the appropriate subgroups among the PBC patients. All analyses were two-sided; age and sex were included as adjustment factors in all models. Analysis was performed by members of the Division of Biostatistics at the Mayo Clinic College of Medicine in Rochester, MN using Splus, version 8.0 (Tibco, Palo Alto, CA).

Results

SLC4A2 and PBC Risk: Case–Control Analysis

The 12 SNPs were successfully genotyped in each of the patients and controls, and all were found to be in Hardy–Weinberg equilibrium. The counts and frequencies of each SNP were determined and statistical analysis was performed between PBC patients and controls under additive, dominant (data not shown), and recessive genetic models. No significant associations were identified (Table 2). Frequencies of the SLC4A2 haplotypes were inferred from the individual genotypes through the expectation-maximization algorithm and analyzed for association with PBC using score tests under multiple genetic models. Ten common haplotypes (i.e. frequency >1 %) were identified, representing 91.6 % of the inferred haplotypes in the assessed population. None of these haplotypes showed a significant association with PBC (Table 3).

SLC4A2 and PBC Features: Case–Case Analysis

Using the same methodologies as for the case –control analysis above, we assessed the features of PBC within the affected population. Namely, we investigated the effect of SLC4A2 genetic variation on progression to OLT and on AMA positivity. Of the 409 PBC patients in the study, 44 (10.8%) had received an OLT prior to this investigation as determined by questionnaire responses and medical record review. Our genetic analysis uncovered no significant or even borderline associations between individual SNPs or inferred haplotypes and disease progression to OLT (data not shown).

The AMA status of each PBC patient was determined at the time of enrollment by the Mayo Clinical Immunology Laboratory as described above. Definitive AMA results were obtained for 403 of the 409 PBC patients (98.5%) of whom 47 (11.7%) tested negative for the antibodies. Analysis of the individual SNPs identified two associations with AMA status among the PBC patients (Table 4). The minor G allele and GG genotype of the intronic rs3793336 SNP was increased among AMA-negative patients, resulting in significant findings under both the additive and recessive models. However, following correction for multiple testing this association was only of borderline significance (Table 4). Homozygosity for the minor A allele of the coding SNP rs2303929 was also increased in AMA-negative compared with AMA-positive patients (17% vs. 5%), a result that retained significance following correction (Pcor = 0.03).

Discussion

Using an LD-based haplotype-tagging approach, we assessed the common genetic variation of SLC4A2 in our large group of PBC patients and matched controls. No significant association was detected in the case–control analysis at either the individual SNP or the haplotype level,

suggesting that genetic variation of SLC4A2 has little, if any, direct impact on the risk of developing PBC. In addition, we analyzed the genotyping data within the PBC patients grouped by prior OLT and AMA positivity. No significant findings were obtained in the OLT analysis; however, two SNPs were found to be associated with AMA status, one of which retained its significance following correction.

The negative finding in the case–control analysis is an important observation as it seems to imply that in contrast to inherited defects in the gene itself, some other mechanism is responsible for the reduced presence and activity of SLC4A2 observed in PBC cholangiocytes (14,16,17). Whether this feature of disease is an acquired epiphenomenon of the underlying PBC or is because of heritable factors residing in unappreciated regulatory regions located at a large distance from SLC4A2 remains unclear and will be difficult to determine. As this finding is negative, we should note that this study was well powered to detect a minimum odds ratio of 1.5 for the average SNP (2.1 for the rarest, rs35576067). Thus, weaker inherited genetic effects cannot be discounted, although they would not seem sufficient to explain the earlier cholangiocyte findings (14,16,17).

To evaluate the potential effect of SLC4A2 genetic variation on PBC progression, we used prior OLT to divide the patient population into comparison groups. Although not particularly sensitive because of the inclusion of patients who will likely require OLT in the future with the “non-OLT” group, poor disease outcome in the OLT patients is definitive. Thus, our negative finding for association between the OLT and non-OLT groups suggests that genetic variation in SLC4A2 does not significantly affect disease outcome. It remains conceivable that SLC4A2 genetic variants could affect aspects of disease progression, possibly in the context of treatment with ursodeoxycholic acid. However, meaningful stratification of patients is notoriously difficult because of variable presentation and lack of follow-up staging towing to the invasiveness of biopsy.

Interestingly, the only SLC4A2 associations we detected were with AMA status of the PBC patients. First, homozygosity for the minor A allele of rs2303929, a coding SNP that results in a glutamic acid to glycine substitution, was found to be significantly increased among AMA-negative patients (17% vs. 5%, odds ratio 3.79, 95% confidence interval 1.49–9.09, Pcor = 0.03). Besides, the minor G allele of the intronic rs3793336 SNP was also associated with AMA negativity, under both additive and recessive models, but significance was borderline following correction (Table 4).

Although PBC-specific AMAs reactive with the inner lipoyl domain of PDC-E2 (22) are clinically detectable in most PBC patients, some 10 % consistently test negative for these antibodies (23). These patients are believed to have a similar clinical course, response to treatment, and prognosis as their seropositive counterparts (23). However, it remains uncertain whether the etio-pathogenic mechanisms leading to this disease variation are also shared, especially considering that detectable levels of AMA often precede the onset of PBC symptoms by years (24,25). Our findings seem to suggest that genetic variants in SLC4A2 might have an effect on the clinical expression of AMA in patients with PBC. Whether this variation functions by protecting against AMA development in patients following a more classical course of PBC development or instead by directly invoking an increased risk of AMA-negative disease, which would signify a more atypical pathogenesis in these patients, is uncertain. However, it is clear that this putative effect is relatively minor, accounting for only a small portion of AMA-negative cases.

In conclusion, the common genetic variation in the SLC4A2 gene does not appear to have a direct effect on the risk of developing PBC or on its clinical outcome. Whether the deficiency of SLC4A2 expression and activity noted in PBC patients (14,16,17) is an acquired

epiphenomenon of the underlying PBC or is because of heritable factors residing in unappreciated regulatory regions remains uncertain and will be difficult to determine. Of interest, two SLC4A2 SNPs appear to have small but significant effects on AMA negativity among patients with PBC. However, the biological mechanism behind this observation is unclear.

Study Highlights

What is Current Knowledge

- SLC4A2 expression is decreased in PBC patients.
- SLC4A2 activity is decreased in cholangiocytes of PBC patients.
- SLC4A2 knockout mice develop PBC-like disease.
- Genetics is thought to play a role in PBC development.

What is New Here

- No genetic association of PBC with SLC4A2 exists.
- The finding narrows the list of potential mechanisms behind observed SLC4A2 decreases.
- One SLC4A2 coding SNP is associated with AMA negativity in PBC patients.
- The finding suggests a possible role for SLC4A2 in the development of AMA-negative disease.

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Table 1
Demographics of PBC patients and controls

	PBC patients (*n*=409)	Controls (*n*=300)
Sex		
Female	91.7%	89.7%
Male	8.3%	10.3%
Race		
Caucasian	100%	100%
Mean age (years)[a]	60.2 (29–85)	62.5 (33–88)
Mean age at diagnosis (years)[a]	50.9 (27–77)	—
Disease duration (years)[a]	9.3 (0–33)	—
Biopsy at diagnosis (n=226)		
Stage I – II	68.6%	—
Stage III – IV	31.4%	—
Liver transplanted	10.8%	—
UDCA therapy	89.0%	—

PBC, primary biliary cirrhosis; UDCA, ursodeoxycholic acid.

[a]Values are expressed as mean (range).

Table 2

SNP frequencies and statistical analysis: PBC patients vs. controls

#	SNP rs ID	Minor allele	Minor allele frequency		Additive model			Recessive model		
			PBC (*n* =409)	Controls (*n* =300)	OR (95% CI)	Pval	Pcor	OR (95% CI)	Pval	Pcor
1	rs33966546	T	0.27	0.26	1.04 (0.82–1.31)	0.774	1.000	1.02 (0.56–1.81)	0.947	1.000
2	rs2303929	A	0.23	0.23	1.03 (0.81–1.32)	0.798	1.000	1.14 (0.61–2.14)	0.684	1.000
3	rs2303930	G	0.35	0.38	0.89 (0.72–1.12)	0.321	0.930	1.06 (0.67–1.66)	0.813	1.000
4	rs3793336	G	0.17	0.17	1.02 (0.77–1.36)	0.892	1.000	1.37 (0.50–3.76)	0.538	0.999
5	rs35576067	T	0.03	0.03	1.27 (0.67–2.42)	0.461	0.980	NA[a]	—	—
6	rs12703112	A	0.21	0.19	1.10 (0.84–1.44)	0.478	0.989	1.28 (0.60–2.74)	0.530	0.999
7	rs13247141	T	0.20	0.19	1.03 (0.80–1.34)	0.812	1.000	1.29 (0.62–2.66)	0.494	0.997
8	rs2303933	A	0.43	0.41	1.08 (0.87–1.34)	0.503	0.993	1.56 (1.03–2.37)	**0.035**	0.231
9	rs2303934	T	0.04	0.04	1.04 (0.59–1.82)	0.894	1.000	NA[a]	—	—
10	rs2303937	A	0.44	0.42	1.08 (0.87–1.33)	0.490	0.991	1.08 (0.74–1.58)	0.682	1.000
11	rs11765015	A	0.26	0.25	1.07 (0.84–1.36)	0.590	0.999	1.58 (0.85–2.93)	0.145	0.689
12	rs11766855	T	0.18	0.17	1.06 (0.79–1.42)	0.687	1.000	1.24 (0.44–3.47)	0.686	1.000

CI, confidence interval; NA, not applicable; OR, odds ratio; PBC, primary biliary cirrhosis; SNP, single nucleotide polymorphism.

[a]At least one of the groups had no rare allele homozygotes.

Table 3

SLC4A2 haplotype structure and analysis: PBC patients vs. controls

	SNP												Frequency		P-additive (P =0.913)[a]	P-dominant (P =0.775)[a]	P-recessive (P =0.707)[a]
#	1	2	3	4	5	6	7	8	9	10	11	12	PBC	Control			
1	C	G	G	A	C	G	C	G	C	G	G	C	0.283	0.316	0.182	0.076	0.897
2	C	G	A	G	C	G	C	A	C	G	G	C	0.160	0.155	0.687	0.843	0.450
3	T	A	A	A	C	G	T	G	C	A	A	C	0.131	0.127	0.893	0.987	0.613
4	C	G	A	A	C	A	C	A	C	A	G	T	0.127	0.115	0.523	0.657	0.275
5	T	A	A	A	C	G	C	G	C	A	A	C	0.051	0.047	0.655	0.673	NA
6	C	G	G	A	C	G	C	A	C	G	G	C	0.039	0.045	0.807	0.819	NA
7	C	G	A	A	C	A	C	A	C	G	G	C	0.038	0.034	0.727	0.830	NA
8	T	G	A	A	C	A	C	A	C	A	G	T	0.032	0.028	0.953	0.957	NA
9	T	A	A	A	T	G	T	G	C	A	A	C	0.030	0.027	0.619	0.639	NA
10	C	G	A	A	C	G	T	G	T	A	A	C	0.023	0.028	0.572	0.597	NA

NA, not applicable; PBC, primary biliary cirrhosis; SNP, single nucleotide polymorphism.

[a]Global P-value under the specified model.

Table 4

SNP frequencies and statistical analysis: AMA-positive vs. AMA-negative PBC patients

#	SNP rs ID	Minor allele	Minor allele frequency		Additive model			Recessive model		
			AMA+ (*n* =356)	AMA− (*n* =47)	OR (95% CI)	Pval	Pcor	OR (95% CI)	Pval	Pcor
1	rs33966546	T	0.27	0.29	1.10 (0.68–1.75)	0.711	1.000	2.38 (0.95–5.88)	0.063	0.303
2	rs2303929	A	0.23	0.28	1.27 (0.79–2.04)	0.320	0.933	**3.70 (1.49–9.09)**	**0.005**	**0.030**
3	rs2303930	G	0.36	0.28	0.70 (0.44–1.14)	0.152	0.655	0.64 (0.22–1.85)	0.412	0.983
4	rs3793336	G	0.16	0.27	1.96 (1.16–3.33)	0.011	0.074	4.76 (1.29–16.7)	0.018	0.091
5	rs35576067	T	0.03	0.03	0.89 (0.26–3.13)	0.859	1.000	NA[a]	—	—
6	rs12703112	A	0.22	0.18	0.77 (0.44–1.35)	0.359	0.957	0.39 (0.05–3.03)	0.362	0.958
7	rs13247141	T	0.21	0.17	0.80 (0.46–1.37)	0.421	0.977	0.68 (0.15–3.03)	0.605	1.000
8	rs2303933	A	0.43	0.49	1.25 (0.79–1.92)	0.303	0.924	1.89 (0.95–3.70)	0.067	0.313
9	rs2303934	T	0.04	0.01	0.29 (0.03–1.82)	0.173	0.718	NA[a]	—	—
10	rs2303937	A	0.45	0.41	0.86 (0.56–1.33)	0.509	0.997	1.18 (0.56–2.43)	0.669	1.000
11	rs11765015	A	0.26	0.26	0.99 (0.61–1.59)	0.962	1.000	2.13 (0.87–5.26)	0.098	0.446
12	rs11766855	T	0.18	0.15	0.73 (0.39–1.37)	0.327	0.937	NA[a]	—	—

AMA, antimitochondrial antibodies; CI, confidence interval; NA, not applicable; OR, odds ration; PBC, primary biliary cirrhosis; SNP, single nucleotide polymorphism.

[a]At least one of the groups had no rare allele homozygotes.