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Polymorphisms of MRP2 (*ABCC2*) are associated with susceptibility to nonalcoholic fatty liver disease

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Abstract

Aims: We hypothesized that *ABCC2* gene variants may contribute to susceptibility to nonalcoholic fatty liver disease (NAFLD). Additionally, we tested the hypothesis of a relation between gene variants and disease severity.
Patients and Methods: The study involved 167 individuals: 109 consecutively presenting unrelated patients with features of NAFLD and different stages of disease severity, and a group of 58 healthy individuals. Four tag single-nucleotide polymorphisms (SNPs; rs717620 A/G, rs2756105 C/T, rs2002042 C/T and rs3740066 A/G) representing 46 polymorphic sites (r^2>.8) were genotyped. Furthermore, two additional SNPs (rs17222723 A/T and rs8187710 G/A) were included.
Results: On univariate analysis, after multiple comparison correction by permutation tests, there were significant differences observed in the allele frequencies of rs17222723 and rs8187710 between healthy individuals and NAFLD patients (empirical *P*=.037 and .035, respectively). Allelic odds ratios [95% confidence interval] for rs17222723 and rs8187710 were 2.80 [1.11–7.04] and 2.80 [1.11–7.04], respectively. When we tested the hypothesis of a relation between gene variants and the clinical and histological spectra of NAFLD by multinomial regression analysis, a significant association was observed with the same markers: rs17222723 (*P*=.0029) and rs8187710 (*P*=.015).
Conclusions: Our study suggests a potential role of *ABCC2* in susceptibility to NAFLD and disease severity.

Keywords: Bile; Liver; MRP2; Multidrug-resistance-associated protein; Gene variant; ABCC2; Fatty liver disease; NASH

1. Introduction

Nonalcoholic fatty liver disease (NAFLD) affects 10–24% of the general population in various countries [1] and parallels the frequencies of the obesity and type 2 diabetes epidemics [2]. It is associated with hepatic insulin resistance and is characterized by hepatic accumulation of triglycerides (termed *steatosis*). Although the pathogenesis of human NAFLD is not fully understood, it has been shown that the disease develops from a complex process in which many factors — besides known risk factors such as excessive caloric intake without adequate exercise — are involved. NAFLD represents the hepatic component of metabolic syndrome [3] and is closely linked to nutrition, obesity and consumption of certain types of fats. A study in a rat model of hepatic steatosis showed that saturated fatty acids promote hepatocyte injury [4], and an in vitro study showed that free fatty acids promote hepatic lipotoxicity by stimulating tumor necrosis factor-α expression via a lysosomal pathway [4,5]. Likewise, altered dietary macronutrient composition (for instance, high consumption of fructose syrup in soft drink) may modulate NAFLD even without body weight modification, as elegantly reviewed by Cave et al. [6].

In addition to the abovementioned risk factors, occupational exposure to diverse toxic agents has been implicated as playing a role in the development of fatty liver, leading to

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the suggestion of a potential interaction between nutrition and hepatic response to xenobiotics [4,6]. In fact, the question of how nutrition influences hepatic response to xenobiotics has begun to receive particular attention, especially following the recognition of NAFLD [6]. For example, a recent large-scale gene-profiling analysis of the liver in a nutrition model of NAFLD induced by a high-carbohydrate diet showed that the expression of a large number of genes involved in liver biotransformation of xenobiotics was down-regulated, suggesting that a fatty liver may have an impaired detoxification/neutralization capacity, thus being more vulnerable to toxic effect [6]. Moreover, previous data have recognized that nutritional factors, including proteins, carbohydrates, fats, vitamins and minerals, may affect the liver's efficiency in processing xenobiotics [7].

Efflux transporters are responsible for the excretion of numerous xenobiotics and endobiotics and play an essential role in proper liver function. The multidrug-resistance-associated protein [MRP2 or ABCC2; gene symbol *ABCC2* (*A*TP-*b*inding *c*assette, subfamily *C*, member *2*)], a member of the multidrug resistance protein subfamily, has been associated with the terminal excretion and detoxification of endogenous and xenobiotic organic anions, including lipid peroxidation products [8]. The localization of ABCC2 to the apical membrane of various polarized cells involved in the secretion of conjugated endogenous and xenobiotic substances favors the function of this efflux pump in the terminal phase of detoxification [9]. In addition, due to this substrate specificity, ABCC2 is probably the most important pump for the elimination of conjugates of various toxins from hepatocytes into bile [9]. Furthermore, the hepatic ABCC2 contributes to the driving forces of bile flow [10].

Although, to our knowledge, there have been no reports on the role of *ABCC2* in differential individual susceptibility to toxic liver injuries and NAFLD occurrence, this protein was related to the development of fatty liver in the genetically obese Zucker rats, which show a faulty leptin receptor in the brain. Interestingly, the liver expression of ABCC2 was markedly reduced in these animals, suggesting that defective hepatobiliary transport capacity may be a contributory factor in rendering obese Zucker rats more susceptible to liver injury [11]. Other elegant reports that evaluated the expression and function of organic anion transporters in obese Zucker rats also showed that ABCC2 expression is decreased in fatty liver [12].

Genetic variation influences gene expression, and this variation in gene expression can be efficiently mapped to specific genomic regions and variants. In fact, it has been recently shown that single-nucleotide polymorphisms (SNPs) capture 83.6% of the total detected genetic variations in gene expression [13].

These polymorphisms that alter gene expression are becoming important in complex traits such as metabolism and transport of xenobiotics that require a dynamic response to environmental changes [14].

Thus, genetic variants in *ABCC2* may affect individual susceptibility to toxic metabolites in hepatocytes, either by impairing the clearance of xenobiotics and its metabolites or by causing the accumulation of bile acids and other endogenous toxic substances in the liver, as recently shown by Choi et al. [15].

Consequently, as NAFLD is a complex disease that develops from the interplay between genes and the environment, in view of the evidence mentioned above, we hypothesized that *ABCC2* gene variants may contribute to susceptibility to NAFLD. Additionally, we tested the hypothesis of a relation between gene variants and clinical disease severity. Here, then, we performed a candidate gene case–control association study.

2. Patients and methods

We performed a cross-sectional study on NAFLD in a county hospital of the city of Buenos Aires. This study involved a total of 167 individuals of "self-reported" European ancestry, including 109 consecutively presenting unrelated patients (29 males and 80 females) with features of NAFLD and ultrasonographic (US) examinations (performed by the same operator) suggestive of fatty infiltration [16].

Secondary causes of steatosis, including alcohol abuse (≥30 g of alcohol daily for men and ≥20 g of alcohol daily for women), total parenteral nutrition, infection with hepatitis B and hepatitis C viruses, and use of drugs known to precipitate steatosis, were always excluded. By using standard clinical and laboratory evaluation, as well as liver biopsy (LB) features where applicable, autoimmune liver disease, metabolic liver disease, Wilson's disease and α-1-antitrypsin deficiency were likewise ruled out in all patients.

For the evaluation of clinical and biochemical disease severities, NAFLD cases were classified as follows: fatty liver with persistently normal liver function test (FL-NLFT) during 12 months of follow-up; fatty liver with persistently abnormal liver function test (FL-ALFT); and nonalcoholic steatohepatitis (NASH) proven through biopsy, as described below. Patients were defined to have abnormal liver function test in the presence of at least one of the following criteria: (a) elevated serum alanine aminotransferase (ALT) and/or aspartate aminotransferase (AST), defined as >41 U/L; (b) γ-glutamyl-transferase (GGT) >50 U/L; and (c) alkaline phosphatase (AP) >250 U/L.

Additionally, 58 healthy individuals (18 males and 40 females) with the same demographic background and who underwent an annual health examination during the same study period were included in the study as an additional control group. All healthy controls were subjected to ultrasonography. None of them evidenced fatty change, biochemical abnormalities or features indicative of metabolic syndrome.

2.1. Physical, anthropometric and biochemical evaluations

Health examinations included anthropometric measurements, a questionnaire on health-related behaviors and biochemical determinations.

Body mass index (BMI) was calculated as weight/height^2 (kg/m^2) and was used as the index for relative weight. Additionally, waist circumference and hip circumference were also assessed. Blood was drawn from fasting subjects who had lain in supine resting position for at least 30 min. Serum insulin, total cholesterol, high-density lipoprotein (HDL) cholesterol, low-density lipoprotein (LDL) cholesterol, triglycerides, plasma glucose and liver function tests were measured by standard clinical laboratory techniques. Homeostasis model assessment (HOMA) was used to evaluate insulin resistance index and was calculated as: fasting serum insulin (μU/ml)×fasting plasma glucose (mmol/L)/22.5. Elevated blood pressure was defined as systolic arterial blood pressure (SABP) ≥130 mmHg and/or diastolic arterial blood pressure (DABP) ≥85 mmHg, or receipt of antihypertensive medications.

All investigations performed in this study were conducted in accordance with the guidelines of The Declaration of Helsinki. Written consent was obtained from individuals in accordance with the procedures approved by the ethical committee of our institution.

2.2. Liver biopsies and histopathological evaluation

Percutaneous LB was performed in 68 patients who showed US fatty changes plus persistently abnormal liver function tests (in at least three different determinations in a 12-month follow-up period). LB was performed with ultrasound guidance and modified 1.4-mm-diameter Menghini needles (Hepafix, Braun, Germany) on outpatient basis. LB specimens were routinely fixed in 40 g/L formaldehyde (pH 7.4) embedded in paraffin and stained with hematoxylin–eosin, Masson trichrome and silver impregnation for reticular fibers. The same liver pathologist who was blinded to patient details read all biopsies. The diagnosis of NASH was confirmed by liver histology, and grading of necroinflammatory activity or staging of fibrosis was scored according to the system developed by Brunt et al. [17]. NASH was defined as steatosis plus any stage of fibrosis, or as steatosis plus lobular inflammation plus ballooning degeneration [18].

2.3. Genotype and haplotype analyses

Genetic analyses were performed on genomic DNA extracted from white blood cells by a standard method, as previously described [19].

To assess the contribution of *ABCC2* gene variants to NAFLD, we selected tag SNPs (tSNPs) by using a tagger tool to capture alleles of interest [20], based on Phase II genotyping data from the HapMap project for Caucasians (CEU dataset; Utah residents with ancestry from northern and western Europe) with a minor allele frequency (MAF) of 0.10 and a minimum r^2 of .8. TagSNPs represent surrogates for single untyped SNPs and are representative SNPs in a region of the genome with high linkage disequilibrium.

Furthermore, we included two additional SNPs (rs17222723 A/T and rs8187710 G/A) because, in a recent report that studied the extent of interindividual variability in the expression of canalicular ABC transporters, individual susceptibility to developing acquired forms of cholestatic liver diseases was shown (note that rs17222723 is the actual nomenclature for rs8187694 and that both SNPs are identical) [21].

Genotyping was performed by a high-throughput genotyping method involving polymerase chain reaction amplification of genomic DNA with two-tailed allele-specific primers that introduce priming sites for universal energy-transfer-labeled primers, as previously described [22].

PLINK software was used for assessing the association between SNPs and affection status and quantitative traits and for testing Hardy–Weinberg equilibrium [23]. Controlling for multiple testing was performed by permutation test (100,000 permutations) to obtain an empirical *P* value. Permutation procedures provide a computationally intensive approach to generating significance levels empirically [24].

To ensure genotyping quality, we included DNA samples as internal controls, hidden samples of known genotype and negative controls (water). Genotypes with a signal below a negative control were not scored. Analysis error was estimated by replicating a blinded sample (always belonging to the same individual) eight times across the templates of the project. Among 216 genotypes for the "blinded sample," we had only one unmatched genotype (0.46% error), then the observed error rate was estimated to be <0.5%. The overall genotype completion rates were 98.20%, 92.8%, 94.0%, 94.61%, 91.0% and 100% for rs717620, rs2756105, rs2002042, rs17222723, rs3740066 and rs8187710, respectively.

To explore a possible stratification in the population, we used a collection of 13 SNPs at different loci (located in chromosomes 4, 15, 17, 13, 1 and 3) and then analyzed the data with the Structure program, version 2 [25]. We found no evidence of stratification in our sample because cases and controls showed similar *Q* values and were assigned similar distances to clusters by the program Structure, with no further improvement in the fitting model by adding up to four clusters (the ln of likelihood was maximum for K=1).

Differences in genotype frequencies between cases and controls were analyzed as described using PLINK software.

2.4. Statistical analysis

Phenotypic quantitative data were expressed as mean±S. D. For univariate analysis and to avoid any assumption about variable distribution and homoskedasticity, differences

Table 1
Clinical and biochemical characteristics of the studied individuals

Variables	Healthy individuals (mean±S.D.)	NAFLD patients (mean±S.D.)	Nominal *P* value
Number of subjects	58	109	
Age (years)	46.2±10.0	56.0±11.6	.00001
BMI (kg/m^2)	25.6±5.05	36.3±34.38	.00001
Waist circumference (cm)	83.7±16.7	103.0±15.4	.00001
SABP (mmHg)	121.8±14.2	124.1±15.7	NS
DABP (mmHg)	75.5±9.95	78.4±10.6	NS
Fasting plasma glucose (mmol/L)	4.6±0.5	5.7±2.3	.00001
Fasting plasma insulin (pmol/L)	45.83±24.93	94.86±76.39	.00001
HOMA index	1.4±0.96	3.5±3.2	.00001
Total cholesterol (mmol/L)	6.05±1.06	5.59±1.41	NS
HDL cholesterol (mmol/L)	1.10±0.60	1.21±0.48	NS
LDL cholesterol (mmol/L)	2.98±1.74	3.16±1.52	NS
Uric acid (μmol/L)	0.21±0.12	0.23±0.29	NS
Triglycerides (mmol/L)	1.69±1.05	1.95±1.34	NS
ALT (U/L)	19.0±16.0	50.1±58.5	.007
AST (U/L)	19.83±13.12	37.2±24.7	.008
γGT (U/L)	19.66±18.66	53.4±56.0	.043
AP (U/L)	232.47±118.17	233.4±117.1	NS

Nominal *P* value stands for statistical significance using Mann–Whitney test.
NS: nonsignificant.
All measurements are in SI units.

between groups were assessed by nonparametric Mann–Whitney test. To test the association between markers and disease severity, we used regression analysis for multinomial distribution (logit as the link function), with disease severity as the dependent (response) variable coding controls, FL-NLFT, FL-ALFT and NASH subjects as 0, 1, 2 and 3, respectively; with HOMA and BMI as continuous predictor variables; and with genotype as a grouping variable. We used the CSS/ Statistica program package StatSoft V. 6.0 (Tulsa USA) to perform these analyses.

3. Results

Clinical features, anthropometric variables and laboratory findings on diagnosis that are available for patients and healthy individuals are shown in Table 1. NAFLD patients were older and showed most of the risk factors of metabolic syndrome: elevated BMI, waist–hip ratio, fasting insulin and HOMA index.

In the patients' group, 41 of 109 were classified as having FL-NLFT, 23 were classified as having as FL-ALFT and 45 were classified as having NASH, proven through biopsy. Patients in the FL-NLFT group showed persistently normal ALT, AST, AP and GGT throughout the study period.

3.1. ABCC2 gene variants

To minimize the number of markers selected for genotyping the candidate gene *ABCC2*, we selected four tagSNPs showing a MAF >10% (rs717620 A/G, rs2756105 C/T, rs2002042 C/T and rs3740066 A/G), encompassing 70 kb in chromosome 10 (101.532.567–101.601.283) and representing 46 polymorphic sites (r^2>.8) considering the HapMap project data. Table 2 illustrates the characteristics of tagSNPs, along with the two additional SNPs added to the analysis as described above.

No marker showed departure from the Hardy–Weinberg equilibrium, indicating robust genotyping performance in this study (data not shown).

On univariate analysis, after multiple comparison correction by permutation tests, there were significant differences observed in the allele frequencies of rs17222723 and rs8187710 between the control group and NAFLD patients (empiric *P*=.037 and .035, respectively). Allelic odds ratio (OR) [95% confidence interval (95% CI)] were 2.80 [1.11–7.04] for rs17222723 (T vs. A) and 2.80 [1.11–7.04] for rs8187710 (G vs. A).

Association studies of single tagSNPs of *ABCC2* with NAFLD, using extended Mantel–Haenszel test for trend and genotype counts according to disease status, are shown in Table 3.

When we tested the hypothesis of a relation between gene variants and the clinical and histological spectra of NAFLD (disease severity), a significant association independent of HOMA and BMI was observed with the same markers mentioned above: rs17222723 (*P*=.029) and rs8187710 (*P*=.015).

Finally, when we analyzed the allele frequencies of tagSNPs in patients with NASH, no association was

Table 2
TagSNPs of the *ABCC2* gene genotyped in the study

Location in the *ABCC2* gene	dsSNP rs[a] cluster ID	Function	dsSNP allele	Protein residue	Amino acid position
Exon 1	rs717620	Untranslated	A/G	—	—
Intron 2	rs2756105	Intron	C/T	—	—
Intron 19	rs2002042	Intron	C/T	—	—
Exon 25	rs17222723	Nonsynonymous	A/T	Glu [E] Val [V]	1188
Exon 28	rs3740066	Synonymous	A/G	Ile [I]	1324
Exon 32	rs8187710	Nonsynonymous	A/G	Tyr [Y] Cys [C]	1515

dsSNP rs[a] cluster ID: SNPs on NCBI Reference Assembly.

Table 3
Genotype counts according to disease status and association study of single tagSNP in *ABCC2* with NAFLD

Single tagSNP	Genotype	Disease status		Cumulative OR [95% CI]	*P*
		Control group	NAFLD		
rs7176620	AA	3	6	1.04 [0.53–2.04]	.45
	AG	18	33		
	GG	35	69		
rs2756105	CC	16	29	0.912 [0.48–1.71]	.38
	TC	22	59		
	TT	12	17		
rs2002042	CC	35	50	1.51 [0.76–2.98]	.10
	TC	13	44		
	TT	6	9		
rs17222723	AA	1	0	2.41 [1.01–5.74]	.02
	AT	12	13		
	TT	41	91		
rs3740066	AA	10	15	0.91 [0.49–1.72]	.39
	AG	21	52		
	GG	21	33		
rs8187710	AA	1	0	2.10 [0.88–5.04]	.045
	AG	11	13		
	GG	46	96		

Cumulative OR using proportional odds model is also indicated. *P* value stands for two-sided alternative (cases≠controls) significance from the extended Mantel–Haenszel test for trend.

observed between either necroinflammatory grade or overall fibrosis score and tagSNPs (data not shown).

4. Discussion

We studied the role of *ABCC2* gene variants in both genetic susceptibility to NAFLD and clinical disease severity with a candidate gene association study that involved four tagSNPs, representing 46 polymorphs encompassing 70 kb in chromosome 10. Additionally, we included in the analysis two nonsynonymous *ABCC2* polymorphisms that were previously related to interindividual differences in ABCC2 protein hepatic expression levels. We found that there was a significant association between rs17222723 and rs8187710 variants and NAFLD. The A-allele of both variants confers protection against the presence of NAFLD, meaning that A-allele carriers have an almost threefold lower risk of developing NAFLD when compared with either rs17222723 T or rs8187710 G allele carriers (conversely, T and G alleles confer a threefold higher risk for NAFLD).

Moreover, there was a significant association between the risk allele of the abovementioned SNPs and clinical disease severity, showing that a less severe fatty liver disease was observed in patients who have at least one copy of the A-allele.

Performing liver biopsies on asymptomatic patients who did not show evidence of abnormality in any of the liver function tests (ALT, AST, AP and GGT) during a long follow-up period is, at least, questionable, particularly because no intervention besides lifestyle measures should be recommended. In our study, we preclassify patients for clinical disease severity evaluation based on a panel of liver function tests monitored during a 12-month follow-up period, as it was shown that LB is invasive, costly and prone to severe complications that have been reported to occur in 0.57% [26].

This issue could be a drawback in our study when making conclusions about histological disease severity. However, it is noteworthy that we did not preclassify patients by transaminases only, which may have resulted in underestimation of significant disease [27]. On the contrary, we stratified and selected for biopsy those patients who showed a combination of abnormal liver function tests, which have been previously shown to be strongly associated with disease severity and its complications [28–30].

The potential contribution of *ABCC2* to NAFLD susceptibility has not been described in humans, and our study is the first to provide evidence for association with the disease.

Although the precise molecular events related to the contribution of *ABCC2* gene variants to NAFLD in humans are unclear, several lines of evidence support this association: first is the biological plausibility of this relationship, supported by previous evidence from animal models. In fact, it was postulated that down-regulation of MRP2 is related to insulin resistance and leptin resistance by both transcriptional and posttranscriptional mechanisms [11]. In addition, Pizarro et al. [11] showed that, in the disease model of obese Zucker rats, animals exhibited impaired bile-secretory function with early cholestatic changes even before the occurrence of fatty liver, suggesting a reduced hepatic ability to excrete endobiotics and xenobiotics. Thus, alterations in hepatobiliary transporters may render fatty livers more vulnerable to toxic substances, as shown by Geier et al. [12] in the same disease model of obese Zucker rats. The authors observed that MRP2 expression was decreased in fatty liver, causing impairment of biliary secretion and metabolism of numerous xenobiotics. Therefore, a strong reduction in the expression of genes involved in hepatic bile salt metabolism and transport in the livers of mice was shown after acute inhibition of β-oxidation [31].

Second, previous evidence showing genetic variations in *ABCC2* in humans is an important predisposing factor for herbal-induced or drug-induced toxic liver injuries [15].

Finally, there is evidence of putative functionality for the associated variants in our study. A previous report showed that rs17222723 and rs8187710 are both related to interindividual variability in hepatic canalicular transporter expression [21]. Remarkably, Meier et al. examined ABCC2 expression in human liver tissue and correlated individual expression levels with selected SNPs in several transporter genes. Their results demonstrated that both rs17222723 A and rs8187710 A carriers exhibited high levels of ABCC2 expression and that, on the contrary, rs17222723 T and rs8187710 G carries were low expressors.

Although we cannot rule out the possibility that the association observed in our study is due to another functional

polymorphism in linkage disequilibrium with the reported variants, our results may indicate that *ABCC2* is worthy of consideration as a candidate gene in fatty liver disease.

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