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BRIEF ARTICLE

ApoB-100, ApoE and CYP7A1 gene polymorphisms in Mexican patients with cholesterol gallstone disease

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Abstract

AIM: To determine the possible association of the ApoB-100 (*Xba* I), ApoE (*Hha* I) and CYP7A1 (*Bsa* I) gene polymorphisms, with the development of cholesterol gallstone disease (GD) in a Mexican population.

METHODS: The polymorphisms were analyzed by polymerase chain reaction followed by restriction fragment length polymorphism, in two groups matched by ethnicity, age and sex: patients with GD (n = 101) and stone-free control subjects (n = 101).

RESULTS: Allelic frequencies in patients and controls were: 34.16% *vs* 41.58% (P = 0.124) for X+ of ApoB-100; 4.46% *vs* 5.94% (P = 0.501) for E2, 85.64% *vs* 78.22% (P = 0.052) for E3, 9.90% *vs* 15.84% (P = 0.075) for E4 of ApoE; and 25.74% *vs* 27.72% (P = 0.653) for C of CYP7A1. Differences in genotypic frequencies between the studied groups were not significant (P < 0.05).

CONCLUSION: These results demonstrated that no association exists between the studied polymorphisms and cholelithiasis in this high prevalent population.

Key words: Apolipoprotein; CYP7A1; Gallstones; Mexicans; Polymorphisms

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INTRODUCTION

Cholesterol gallstone disease (GD) is the major manifestation of gallbladder disease, and is one of the most common digestive disorders worldwide, especially in Western populations[1]. In México, the prevalence of GD is nearly 14.3%, and is a public health problem with high economic impact[2]. The formation of gallstones is accelerated by impaired gallbladder emptying, hypersecretion of cholesterol into bile, or destabilization of bile by kinetic protein factors[3,4]. The pathogenesis of GD is multifactorial, with

environmental and genetic factors involved[5,6]. Several risk factors, such as obesity, diet, female gender, metabolic syndrome and type-2 diabetes, are usually associated with this pathology[7,8]. On the other hand, the high prevalence of GD in American Indians and Hispanic populations, as well as several twin and family studies, suggested that genetic factors play a key role[9-11].

Epidemiological data have shown that disturbances in serum lipids are associated with GD[12-14], suggesting that proteins involved in transport, synthesis and metabolism of lipids are related to lithogenesis. The candidate lithogenic-genes found in humans are: ABC transporters for phosphatidylcholine (ABCB-4) and bile salts (ABCB-11), hepatocanalicular cholesterol transporter (ABCG5/G8), cholesterol-7α-hydroxylase (CYP7A1), cholecystokinine type-A receptor (CCK1R), cholesteryl ester transfer protein (CETP), and apolipoproteins (Apo) A-I, B and E[4,5,15-18].

Lipoprotein particles transport lipids and cholesterol in the bloodstream. ApoB-100 [the sole protein of low density lipoprotein (LDL)] and ApoE [found in VLDL, high density lipoprotein (HDL) and chylomicron remnants] bind to lipids, and are recognized by hepatic and tissue lipoprotein-receptors[19,20]. These proteins are polymorphic in the population, and are considered as genetic determinants of variations in cholesterol and lipids transport and metabolism[19,21].

Bile formation is essential for the removal of excess dietary cholesterol. The first-step and key regulatory enzyme in bile acid synthesis is CYP7A1, catalyzing the formation of 7-α-hydroxycholesterol. Innate deficiency of this enzyme has been related to hypercholesterolemia[12].

Here, we evaluated the association between ApoB-100 (*Xba* I), ApoE (*Hha* I) and CYP7A1 (*Bsa* I) gene polymorphisms with cholelithiasis, in a population from Sinaloa, México, a country with high prevalence of this disease.

MATERIALS AND METHODS

Subjects

We studied two groups matched by ethnicity (natives from Sinaloa, México), age and sex: consecutive symptomatic patients (n = 101) with cholesterol gallstone disease (GD), and healthy stone-free control subjects (n = 101) confirmed by abdominal ultrasonography (EnVisor Ultrasound System, Philips Medical System, Andover, MA, USA). Patients were cholecystectomized at the Division of Gastroenterology of the Regional Hospital of the "Instituto de Seguridad y Servicios Sociales de los Trabajadores del Estado" (ISSSTE, Culiacán, Sinaloa), from May 2008 to April 2009, and only those with cholesterol-stones (≥ 70% of its content) were included in the study. Subjects were also questioned about their past medical history, and their body mass index (BMI) was calculated. In accordance with the World Health Organization's categories, subjects with BMI ≥ 25 kg/m^2 were considered overweight and ≥ 27 as class-I obese. Those with renal or liver malfunction were excluded. The Ethical and Research Committee of ISSSTE approved this study and all subjects signed an informed consent.

Laboratory tests

Fasting serum glucose and lipids, including total cholesterol, HDL-cholesterol (HDL-C), LDL-cholesterol (LDL-C), and triglycerides, were determined using the HITACHI 917 automatic biochemical analyzer (Hitachi Koki Co. Ltd, Hitachinaka City, Japan). Individuals with the following values were considered above the normal range: glucose > 120 mg/dL; total lipids > 800 mg/dL; triglycerides > 150 mg/dL; cholesterol > 200 mg/dL; and LDL-C > 130 mg/dL. In the case of HDL-C, values > 40 mg/dL were considered normal.

Recovered gallstones were washed with distilled water and dried at 37℃ to determine the cholesterol content using the Liebermann-Burchard reaction, and classified in accordance with their chemical composition[22,23].

DNA amplification and restriction fragment length polymorphism

Genomic DNA was isolated from whole blood containing EDTA, using the salt precipitation method[24]. The polymorphisms were analyzed by polymerase chain reaction followed by restriction fragment length polymorphism (PCR-RFLP). Reaction conditions, primers and restriction fragments are summarized in Table 1.

A region from exon 26 of *apo*B-100 (chromosome 2), containing the C-7673T polymorphism, was amplified and digested with *Xba* I (4 U for 2 h at 37℃), and alleles were identified as X- (normal) and X+[25]. PCR products from exon 4 of *apo*E (chromosome 19) were digested with *Hha*I (2 U for 2 h at 37℃) to determine the codominant alleles E2 (residues Cys112 and Cys158), E3 (Cys112 and Arg158) and E4 (Arg112 and Arg158)[26]. In the same way, a region spanning the polymorphic site A-204C in the promoter region of CYP7A1 (chromosome 8), was amplified and cleaved with *Bsa* I (1 U for 2 h at 37℃) to determine A (normal) and C alleles[27].

Five random samples of each polymorphism were sequenced to confirm the results. Abnormal RFLP's bands were also sequenced.

Statistical analysis

Data are presented as mean ± SD. Mean differences in covariates were analyzed by the Student's t-test. A sample size of 96 individuals per group was calculated to detect differences (delta) of 0.14 in polymorphism frequencies between the groups, with 80% power and 5% significance. Allelic frequencies observed in patients and controls were evaluated for differences using the Mantel-Haenzel χ^2 test, or Fisher's exact test when the number of observations in any cell was ≤ 5. The P values were corrected by Bonferroni test for multiple comparisons, taking into account the number of alleles observed, and considered significant when $P < 0.05$[28]. Odds ratios (OR) with 95% confidence intervals (CI) were used as the measure of association between specific genotypes and alleles with GD[29]. Hardy-Weinberg's equilibrium was calculated by χ^2 test. Multiple logistic regression analysis was performed to investigate the independent factors as-

Table 1 Conditions and products of polymerase chain reaction followed by restriction fragment length polymorphism

Gene	Primers	Tm (℃)	RE	bp	Alleles
ApoB	Forward: GGAGACTATTCAGAAGCTAA Reverse: GAAGAGCCTGAAGACTGACT	55	*Xba* I	710	X-: 710 bp X+: 433 and 277 bp
ApoE	Forward: ACAGAATTCGCCCCGGCCTGGTACAC Reverse: TAAGCTTGGCACGGCTGTCCAAGGA	60	*Hha* I	252	E2: 91 and 84 bp E3: 91, 48 and 36 pb E4: 72, 48 and 36 bp
CYP7A1	Forward: CAGAGCATGGACAGGGAGCAG Reverse: GCAACTCCTCATGGCTGAGGTT	55	*Bsa* I	948	A: 581, 367 bp C: 542, 367 and 39 bp

Forward and reverse primers in 5′ to 3′ directions. Tm: Annealing temperature; RE: Restriction enzyme.

Table 2 Clinical characteristics in control subjects and patients with gallstones (mean ± SD)

Variable	Patients (n = 101)	Controls (n = 101)	P
Sex (F/M)	86.10%/13.90%	86.10%/13.90%	1.000
Age (yr)	51.93 ± 11.23	51.74 ± 10.99	0.904
Body mass index (kg/m^2)	29.03 ± 4.19	27.63 ± 4.42	0.024
Serum glucose (mg/dL)	120.24 ± 44.20	100.30 ± 34.64	0.000
Lipids (mg/dL)	622.00 ± 149.77	688.60 ± 160.68	0.003
Cholesterol (mg/dL)	187.32 ± 45.10	208.21 ± 43.14	0.001
LDL cholesterol (mg/dL)	118.58 ± 41.64	130.94 ± 33.80	0.022
Triglycerides (mg/dL)	119.76 ± 87.05	144.45 ± 72.22	0.029
HDL cholesterol (mg/dL)	43.27 ± 13.26	46.56 ± 14.01	0.089
Cholesterol in stones (% by weight)	99.23 ± 2.50		

LDL: Low density lipoprotein; HDL: High density lipoprotein.

sociated with GD. SPSS v16.0 software (SPSS inc., Chicago, IL, USA) was used for data analysis.

RESULTS

Characterization of the population

Patients with cholelithiasis and control subjects with a mean age of 51.93 (± 11.23) years *vs* 51.74 (± 10.99) years (P = 0.904), and a normal distribution according to the Kolmorov-Smirnov test (P = 0.281) were included in this study. In both groups, 86.10% of individuals were female and 13.90% were male (P = 1.000), giving a 6:1 female/male ratio (Table 2). The cholesterol content of recovered gallstones was 99.23% (± 2.50).

There were statistically significant differences in the measured covariates between patients and controls (Table 2), with the exception of HDL-C (P = 0.089). Body mass index (BMI) and serum glucose were higher in patients than in the control group: 29.03 (± 4.19) kg/m^2 *vs* 27.63 (± 4.42) kg/m^2 (P = 0.024) for BMI; and 120.24 (± 44.20) mg/dL *vs* 100.30 (± 34.64) mg/dL (P = 0.0002) for glucose.

In contrast, serum lipids, cholesterol, LDL-C and triglycerides, were lower in patients than in controls: 622.00 (± 149.77) mg/dL *vs* 688.60 (± 160.68) mg/dL for lipids (P = 0.003); 187.32 (± 45.10) mg/dL *vs* 208.21 (± 43.14) mg/dL for cholesterol (P = 0.001); 118.58 (± 41.64) mg/dL *vs* 130.94 (± 33.80) mg/dL for LDL-C (P = 0.022); and 119.76 (± 87.05) mg/dL *vs* 144.45 (± 72.22) mg/dL for triglycerides (P = 0.029).

Table 3 Allelic (af) and genotypic (gf) frequencies of ApoB-100, ApoE and CYP7A1 polymorphisms in patients with gallstones and controls

Polymorphism	Patients (n = 101)		Controls (n = 101)		P value	OR	95% CI
ApoB-100 *Xba* I							
Alleles	n	af	n	af			
X-	133	65.84%	118	58.42%	0.124	1.37	0.92-2.05
X+	69	34.16%	84	41.58%	0.124	0.73	0.49-1.09
Genotypes	n	gf	n	gf			
X-X-	41	40.59%	34	33.66%	0.308	1.35	0.76-2.39
X+X-	51	50.50%	50	49.50%	0.888	1.04	0.60-1.81
X+X+	9	8.91%	17	16.83%	0.093	0.48	0.20-1.14
ApoE *Hha* I							
Alleles	n	af	n	af			
E2	9	4.46%	12	5.94%	0.501	0.74	0.30-1.79
E3	173	85.64%	158	78.22%	0.052	1.66	0.99-2.78
E4	20	9.90%	32	15.84%	0.075	0.58	0.32-1.06
Genotypes	n	gf	n	gf			
E2E2	0	0.00%	1	0.99%	NC	NC	NC
E3E3	74	73.27%	64	63.37%	0.130	1.58	0.87-2.88
E4E4	1	0.99%	2	1.98%	0.561	0.50	0.04-5.55
E2E3	8	7.92%	6	5.94%	0.580	1.36	0.45-4.08
E2E4	1	0.99%	4	3.96%	0.174	0.24	0.03-2.21
E3E4	17	16.83%	24	23.76%	0.221	0.65	0.32-1.30
CYP7A1 *Bsa* I							
Alleles	n	af	n	af			
A	150	74.26%	146	72.28%	0.653	1.11	0.71-1.72
C	52	25.74%	56	27.72%	0.653	0.90	0.58-1.40
Genotypes	n	gf	n	gf			
AA	59	58.42%	56	55.45%	0.670	1.13	0.65-1.97
CA	32	31.68%	34	33.66%	0.764	0.91	0.51-1.65
CC	10	9.90%	11	10.89%	0.818	0.90	0.36-2.22

OR: Odds ratio; 95% CI: 95% confidence intervals; NC: Not calculated.

ApoB-100, ApoE and CYP7A1 gene polymorphisms analysis

Allelic and genotypic frequencies of GD patients and healthy controls are shown in Table 3. Statistical analysis showed no differences in genotypic frequencies of ApoB-100 gene *Xba* I polymorphism between patients and controls (P = 0.210): 40.59% *vs* 33.66% (P = 0.308, OR = 1.35) for X-X-; 50.50% *vs* 49.50% (P = 0.888, OR = 1.04) for X+X-; and 8.91% *vs* 16.83% (P = 0.093, OR = 0.48) for X+X+. Frequencies of X+ allele were 34.16% *vs* 41.58% (P = 0.124, OR = 0.73).

In the case of ApoE gene *Hha* I polymorphism, there were no significant differences in the distribution

of alleles between patients and controls ($P = 0.075$). Allelic frequencies were 4.46% *vs* 5.94% ($P = 0.501$, OR = 0.74) for E2; 85.64% *vs* 78.22% ($P = 0.052$, OR = 1.66) for E3; and 9.90% *vs* 15.84% ($P = 0.075$, OR = 0.58) for E4. Genotypic frequencies were 0.00% *vs* 0.99% for E2E2; 73.27% *vs* 63.37% ($P = 0.130$, OR = 1.58) for E3E3; 0.99% *vs* 1.98% ($P = 0.561$, OR = 0.50) for E4E4; 7.92% *vs* 5.94% ($P = 0.580$, OR = 1.36) for E2E3; 0.99% *vs* 3.96% ($P = 0.174$, OR = 0.24) for E2E4; and 16.83% *vs* 23.76% ($P = 0.221$, OR = 0.65) for E3E4.

Frequencies of C allele (CYP7A1 gene *Bsa* I polymorphism) in patients and controls were 25.74% *vs* 27.72% ($P = 0.653$, OR = 0.90). Genotypic frequencies between the groups were similar ($P = 0.911$) with the following distribution in patients and controls: 58.42% *vs* 55.45% ($P = 0.670$, OR = 1.13) for AA; 31.68% *vs* 33.66% ($P = 0.764$, OR = 0.91) for CA; and 9.90% *vs* 10.89% ($P = 0.818$, OR = 0.90) for CC.

The distributions of ApoB-100, ApoE and CYP7A1 gene polymorphisms in both groups were in Hardy-Weinberg equilibrium (all $P \leqslant 0.05$). Multiple logistic regression analysis showed no significant association between the gene polymorphism frequencies and the covariates.

DISCUSSION

In this work, we report on the association of ApoB-100, ApoE and CYP7A1 gene polymorphisms with cholelithiasis in México.

Patients and controls were Mexican Mestizos, natives of the northern state of Sinaloa, who were matched for both age and sex. The female/male ratio (6:1) and mean age (51.93 ± 11.23 year) of the patients were in accordance with the governmental data of the disease in the country, supporting the notion that female gender and age are risk factors.

There were statistically significant differences ($P \leqslant 0.05$) in the covariates between the groups, with the exception of HDL-C (Table 2). BMI and sanguineous glucose were higher in patients than in controls, while serum levels of cholesterol, LDL-C, total lipids, and triglycerides were greater in controls. However, these mean differences were not considered clinically relevant, since both groups were in the class-I obese category, and had normal or borderline levels of serum glucose, cholesterol, LDL-C, total lipids, and triglycerides. In contrast to these results, several studies have demonstrated a clear correlation between BMI and hyperglycemia with GD[30], while other studies found that dyslipidemias, such as decreased HDL-C and increased triglycerides and LDL-C levels, correlated with augmented risk for cholelithiasis[13,14,31].

We found that both genotypic and allelic frequencies of ApoB-100 gene *Xba* I polymorphism did not show significant differences between patients and controls (Table 3). The frequency of the X+ allele in patients was 34.16%, similar to that observed in a Finnish population[18]. A case-control study performed in México, suggested a relationship between the serum concentration of apolipoproteins (B and A-I) and gallbladder disease, however, gene polymorphisms were not analyzed[32]. The *Xba* I polymorphism does not alter the threonine residue at position 2488, however, it may be a marker in strong linkage disequilibrium with several other unknown but functional mutations. It has been reported that the X+ allele is characterized by higher serum levels of cholesterol and LDL-C, and may be a marker for increased risk of GD in the Chinese population[33]. On the contrary, other studies with Polish and India populations did not observe any significant differences between *Xba* I polymorphism and GD[34,35].

In this study, phenotypic and allelic frequencies of ApoE gene *Hba* I polymorphism were similar between the groups (Table 3). E3E3 and E3E4 were the most common phenotypes found in patients (73.27% and 16.83%). The frequencies of E3E3 genotype and the common E3 allele (85.64%) were similar to other reports in Mexican Mestizos[36]. Each of the six possible ApoE phenotypes have particular receptor binding affinities and catabolic rates, as reflected by serum levels and clearance rates of circulating lipoproteins[21]. Previous studies suggested an association between the apoE4 isoform and increased gallstone cholesterol content in cholecystectomized patients from Finland[37], and with a higher risk for gallstones in a case-control study from Spain[38]. In contrast, other studies reported that the E4 polymorphism was not associated with susceptibility to cholesterol GD in Chile and Germany, which have high-risk populations[39]. The E2 allele has been reported as one possible factor in the lithogenesis of cholecystolithiasis[40], but other studies have not yielded consistent findings on this association[41-44].

Statistical analysis showed that CYP7A1 gene *Bsa* I polymorphism frequencies between patients and controls were not different (Table 3). The frequency of C allele in patients was 25.74%, slightly higher than the 37.20% observed in a Chinese population[27]. Other studies have confirmed the relationship between CYP7A1 polymorphism with increased LDL-C levels and gallstone formation in a Chinese population[27]. In addition, CYP7A1 deficiency has been correlated with hypercholesterolemic phenotype[12].

For the three studied genes in this study, the distribution of their genotypes in the groups was not significantly different from the expected distribution for a population in Hardy-Weinberg equilibrium. In addition, there was no correlation of these polymorphisms with BMI, glucose or lipid profile. In this study, we did not take into account type-2 diabetes in the inclusion criteria. However, the stratified analysis of the data (hyperglycemic individuals, with > 140 mg/dL fasting glucose) showed no significant differences (data not shown).

The reason for the inconsistent results obtained from several association studies in different countries, may be due to differences in populations. Mexican populations have a high degree of genetic heterogeneity. HLA analyses have demonstrated that in general, Mexican Mestizos have many Amerindian and few European and African

haplotypes. However, the study population in this work (from Sinaloa state), actually have a particular genetic background, since half of the most common haplotypes found in this population have a proposed European origin[45].

The results of this study showed that no association exists between ApoB-100, ApoE and CYP7A1 gene polymorphisms and cholelithiasis in Mexicans, a population with a high prevalence of this digestive disorder.

COMMENTS

Background

Gallbladder stone disease (GD) is commonly associated with several environmental risk factors such as obesity, diet, gender, metabolic syndrome and type-2 diabetes. However, an increasing number of studies point to genetics factors in the pathogenesis of this important digestive disorder.

Research frontiers

Apolipoproteins (Apo) B and E, as well as cholesterol-7α-hydroxylase (CYP7A1), play a key role in transport, synthesis and metabolism of lipids. Many studies have been performed on the association between these polymorphic genes and lithogenesis, however, the results often differ in populations from different origins.

Innovations and breakthroughs

In México, the prevalence of GD is more than 14% and is a public health problem with a very high economic impact; however, there are very few genetic studies on this disease. Therefore, in this report, the influence of ApoB-100, ApoE and CYP7A1 gene polymorphisms on GD was evaluated in a Mexican population. Results showed that allelic and genotypic distributions were different to those observed in other populations, indicating a particular genetic background in this population. Allelic frequencies in patients and controls were: 34.16% *vs* 41.58% (P = 0.124) for X+ of ApoB-100; 4.46% *vs* 5.94% (P = 0.501) for E2, 85.64% *vs* 78.22% (P = 0.052) for E3, 9.90% *vs* 15.84% (P = 0.075) for E4 of ApoE; and 25.74% *vs* 27.72% (P = 0.653) for C of CYP7A1. Differences in genotypic frequencies between patient and control groups were not significant (P < 0.05).

Applications

These results showed that no association exists between ApoB-100, ApoE and CYP7A1 gene polymorphisms and cholelithiasis in Mexicans. Therefore, the study of more genes involved in disturbances of serum lipids is needed to explain the high prevalence of gallstones in this country.

Peer review

This study reports negative data regarding an association between a number of polymorphisms of proteins involved in transport, synthesis or metabolism of lipids and cholesterol gallstone disease.

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