Annals of Hepatology 2006; 5(2): April-June: 97-102

Annals of Hepatology

Original Article

Obesity-related leptin receptor polymorphisms and gallstones disease

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Abstract

Objective: **Investigate the association between polymorphisms in the leptin receptor gene associated with obesity and gallstone disease.** ***Design:*** **We conducted a cross-sectional study, carried out at a tertiary setting.** ***Subjects:*** **We enrolled 97 subjects, comprising 54 subjects with gallstones (cases) and 43 controls (without gallstones).** ***Measurements:*** **Diet was assessed using a validated questionnaire for the Mexican population. Body mass index, waist circumference, serum glucose, insulin, leptin, lipids and lipoproteins levels were measured. Insulin resistance was calculated by HOMA-IR. Genomic DNA was isolated from lymphoblastoid cells, and Q223R and K656N polymorphisms in the leptin receptor gene were typed using polymerase chain reaction. Unconditional univariate logistic regression analysis was conducted to estimate the probability of gallstone disease associated with the polymorphisms as main effect.** ***Results:*** **Cases were different in gender (40.74% males in cases *vs* 74.41% in controls; $p < 0.001$), older (49.74 *vs* 44.83 years; $p < 0.05$), and had more body fat (32.34% *vs* 28.14%; $p = 0.01$). Individuals carrying the polymorphism Q223R exhibited a higher BMI (28.44 ± 6.6 kg/m^2 *vs* 25.94 ± 3.67 kg/m^2, $p < 0.05$) and waist circumference (96.7 ± 16.39 cm *vs* 89.2 ± 11.05 cm, $p < 0.05$). In univariate analysis, we did not observe a relation between the presence of a R223 or N656 genotype and gallstone disease in our population (OR = 0.78, 95% CI 0.35-1.73).** ***Conclusion:*** **Obesity-related leptin receptor polymorphisms are not associated with gallstones disease.**

Key words: Gallstones, obesity, polymorphisms, leptin, receptors.

Introduction

Obesity is associated with many significant comorbidities, including cardiovascular disease, diabetes mellitus, hypertension, stroke, osteoarthritis and gallstone disease (GD).[1] Obesity is thought to be the result of the interplay between environmental and genetical factors. A group of metabolically active proteins produced by adipose tissue, known as adipokynes,[2] had been identified in the last decade, and represent unique and unexplored pathways that unravel the communication between the adipocyte, and other organs.

Leptin is the product of the *ob* gene, which encodes a 167 amino acid protein with a 21 amino acid signal peptide.[3] Its crystal structure indicates that leptin is a member of the cytokine family and has four or possibly five helical segments.[4] The human *ob* gene is localized on chromosome 7 and displays 84% homology with the mouse gene. Leptin have established an exciting pathway for the regulation of food ingestion and energy consumption. Leptin levels adapt to changes in energy balance; during fasting or weight loss,[5] serum concentrations decrease, whereas they increase during overfeeding or weight gain. Leptin mRNA expression in fat cells correlates significantly with body fat mass.[6] Hyperleptinemia is thought to be indicative of leptin resistance, and may play a role in the pathogenesis of obesity.

Leptin receptor (LEPR) is the product of the *1p31* gene and displays a 78% homology with the mouse *db* gene.[7] LEPR spans over 70 kb and includes 20 exons which encode an 1,165 amino acid protein that belongs to the superfamily of cytokine receptors and requires all its extracellular subdomains functional for transmitting the signal. LEPR presents two basic isoforms, a short intracellular domain variant, unable to transmit a signal, which is present in a variety of tissues; and a second one with a

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Manuscript received and accepted: 08 March, 2006.

long intracellular functional domain, capable of activating JAK2 and STAT3, and perhaps others signaling pathways.[8] Leptin receptor gene polymorphisms had been previously associated with obesity[9] and increased leptin levels, and might represent a common form of leptin resistance.

VanPatten *et al* reported that leptin-receptor-defective Zucker (fa/fa) rats have a decreased biliary cholesterol secretion.[10] The same group demonstrated that intracerebroventricular administration of leptin in mice, indirectly promotes reverse cholesterol transport, while diminishing its transformation into bile acids.[11] Our group observed a positive correlation between plasma leptin levels and biliary cholesterol saturation, in a group of obese women following a modest degree of weight loss.[12]

From these data, it is clear that leptin plays a role in the secretion of cholesterol into the bile.[13] High leptin levels in obese have been associated with GD[14,15] but leptin-resistant mice had been showed to be protected against GD.[16] The aim of this study was to investigate the association between obesity-related polymorphisms in the LEPR gene and the presence of GD.

Methods

Populations and sample

We conducted a cross-sectional study in the check-up unit of the Diagnostic Clinic at the Medica Sur Clinic & Foundation, a university hospital with subspecialty care in Mexico City, Mexico, from June 2003 to April 2004. This hospital provides private care for mainly middle- and high-income individuals from Mexico City and surrounding metropolitan areas. Our sample population was formed from a series of consecutive asymptomatic subjects who were referred to the check-up unit by their companies as an annual requirement, not for symptomatic disease. Ninety-seven asymptomatic subjects undergoing routine check-ups were included. The study was approved by the Human Subjects Committee at the Medica Sur Clinic & Foundation as conforming to the ethical guidelines of the 1975 Declaration of Helsinki, and written informed consent was obtained from all participants before entry. After informed consent was obtained, all patients were asked to complete a questionnaire that included demographic and medical variables. The primary demographic and medical variables were gender, age, date of birth and previous surgery. Cases were defined as subjects with an ultrasonographic diagnosis of GD (below); controls were defined as patients with no such evidence (sensitivity and specificity > 96%).[17]

Gallstone disease

Abdominal ultrasound was performed on all subjects using a Sonoline Elegra equipment (Siemens Medical System, Germany) with a 3.5 MHz transducer. Ultrasound diagnosis of GD was assessed by the presence of strong intraluminal echoes that were gravity-dependent or that attenuated ultrasound transmission (acoustic shadowing). All ultrasonographic studies were evaluated, blind, by the same radiologist on two different occasions. Subjects with previous cholecystectomies due to GD were included in the study.

Questionnaire

All subjects completed a questionnaire that included age and gender, previous history of high blood pressure, diabetes, GD (or cholecystectomy due to GD) and a food frequency questionnaire in order to study diet as an environmental confounding factor. The answers was subsequently electronically scanned and the daily intake of various nutrients was determined using SNUT software-a program developed by the National Institute of Public Health, Mexico City, and appropriate to the Mexican population[18] in order to estimate dietary energy, protein, carbohydrate, and total, saturated, monounsaturated and polyunsaturated fat.

Physical examination

Body weight was measured, in light clothing and without shoes, to the nearest 0.10 kg. Height was measured to the nearest 0.5 cm. Body mass index (BMI) was calculated as weight in kilograms divided by height in meters squared. Waist circumference (to the nearest 0.1 cm) was measured at the midpoint between the lower border of the rib cage and the iliac crest, and hip circumference was similarly obtained at the widest point between hip and buttock. Body fat composition was measured by bioelectrical impedance (Omron body fat analyzer model HBF-306INT).

Analytical procedures

Insulin levels were measured using an immunoenzymometric assay (MEIA; Abbott Diagnostics), with inter- and intra-assay coefficients of variation less than 3%.

Plasma glucose in the fasting state was measured in duplicate with an automated analyzer. The coefficient of variation for a single determination was 1.5%. Cholesterol, HDL-cholesterol, and triglycerides concentrations were measured by enzymatic colorimetric methods, using CHOL, HDL-C plus (second generation) and TG assays (Roche Diagnostics Co., Indianapolis, IN) respectively. Low-density lipoprotein cholesterol (LDL) concentrations were calculated using the Friedewald formula.[19]

Assessment of insulin resistance was made using the Homeostatic Model Assessment (HOMA-IR). Insulin resistance was assessed using the HOMA-IR, originally described by Matthews *et al*,[20] based on the following formula: HOMA-IR = fasting insulin (μU/mL) × fasting glu-

cose (mmol/L)/22.5 (values > 2.522 indicate a high index of insulin resistance).

Plasma leptin levels were determined by radio immunoassay using RIA kits (Linco Research, St. Charles, MO, USA). The intra- and interassay coefficients of variation are both less than 5%.

Genetic analyses

DNA was extracted from 300 µL of total blood. Gln223Arg (exon 6) and Lys656Asn (exon 14) polymorphisms in LEPR gene (previously associated with obesity and common in the Mexican population[21] was analyzed using polymerase chain reaction (PCR) restriction fragment length polymorphism. In PCR analysis, a Taq PCR master mix kit (Quiagen) was used in 50 µL of final volume, with the following reaction conditions: 1 cycle of 5 minutes at 95°C, followed by 40 cycles of 30 seconds at 95°C, 30 seconds at 55°C and 30 seconds at 72°C,[22] with a final extension of 10 minutes at 72°C. The PCR products were processed with 5 units of MspI (exon 6) and HaeIII (exon 14) and enzymes during 1 hour at 37°C. The fragments obtained were analyzed in 2% agarose gel stained with ethidium bromide for its visualization in a UV transiluminator. Oligonucleotide sequences are shown in *Table I*.

Statistical analysis

Mean values and their standard deviations (SD) were used to resume the distribution of continuous variables, comparing cases *vs* controls. The non-parametric Mann-Whitney U test was applied to compare these variables due to the non-normal distribution of some variables. Differences were considered significant with p values of < 0.05. Odds ratios (OR) were calculated with 95% confidence interval (95% CI). Unconditional univariate logistic regression analysis was conducted to estimate the probability of gallstone disease associated with the polymorphisms as main effect. All the analysis was carried out with the statistic program SPSS/PC v 12.0 (Chicago, IL).

Results

We enrolled 97 subjects, comprising 54 subjects with gallstones (cases) and 43 controls (without gallstones).

Table I. Oligonucleotides sequences used in PCR-restriction fragment length polymorphism.

Exon 6	
Sense	5- ACCCTTTAAGCTGGGTGTCCCAAATAG-3
Antisense	5-AGCTAGCAAATATTTTTGTAAGCAATT-3
Exon 14	
Sense	5- CACATTGTACAATGGAAGCACAAAGTT-3
Antisense	5- TGTTAAAATCATAGCCATAAGACATCT-3

Cases were different to controls in gender (59% females in cases *vs* 26% in controls; $p = 0.001$), older (49.74 *vs* 44.83 years; $p < 0.05$), higher percentage of body fat (32.34 *vs* 28.14%; $p = 0.01$), and more subjects with high blood pressure (26 *vs* 1; $p < 0.001$). The mean HOMA-IR was higher in cases (1.99 ± 1.76 *vs* 1.41 ± 1.03; $p < 0.05$), however there were no other significant difference among the biochemical variables between cases and controls. Including leptin levels the cases were found to consume higher amounts of polyunsaturated fats per day (19.82 ± 4.32 *vs* 12.47 ± 7.77 g; $p < 0.001$), and lower amounts of saturated (15.08 ± 5.4 *vs* 20.45 ± 8.86; $p = 0.001$) and monounsaturated fats per day (17.42 ± 5.54 *vs* 28.88 ± 12.31; $p < 0.001$) *(Table II)*.

Both polymorphisms were in Hardy-Weinberg equilibrium. We did not observe any statistical differences in the distribution of polymorphisms between cases and control subjects *(Table III)*. When analyze each exon (normal *vs* homozygote or heterozygote polymorphism) we observe significant differences in individuals carrying the heterozygote mutation R223 only in BMI, waist and hip circumference (all $p < 0.05$); homozygote N656 subjects had higher body fat ($p < 0.05$) *(Table IV)*.

In univariate analysis we did not observed a relation between the presence of GD and R223 (OR 0.78, 95% CI 0.35-1.74, $p > 0.05$) or N656 polymorphism (OR 0.81, 95% CI 0.33-1.97, $p > 0.05$) *(Figure 1)*.

Discussion

In this study, we analyze the relation between two polymorphisms LEPR gene, Q223R and K656N GD. Our results show no differences in the prevalence of GD between individuals carrying the LEPR polymorphisms and normal subjects.

Current evidence is consistent with a role for genetics in GD. In family studies, history of gallstones in first-degree relatives increases 3-fold the risk for GD.[15] The heritability of GD has been estimated in several studies. Katsika *et al* reported a heritability component of 25% in Swedish twins[23] and Nakeeb *et al* a 29% in the USA.[24] These studies might underestimate the prevalence of GD because both considered only symptomatic disease. Duggirala *et al* reported a 44% heritability in Mexican-American,[25] but the results could be overestimated since they did not consider shared environmental factors.

The pathogenesis of GD is thought to occur as a consequence of three main circumstances; impaired gallbladder emptying, cholesterol supersaturation of bile and accelerated nucleation.[26] Several studies had linked polymorphisms of genes encoding proteins relevant in cholesterol reverse transport. For instance, multidrug resistance protein 3 and 7alpha-hydroxylase deficiencies had been shown to cause GD.[27,28] However, these polymorphisms are rare. It is more likely that common gene polymorphisms in cholesterol metabolism regulating key-proteins could explain the familial

Table II. Anthropometric and biochemical variables of cases and controls.

Variable	Cases (n = 54) Mean ± SD	Controls (n = 43) Mean ± SD	*p* value
Age (years)	49.74 ± 13.86	44.83 ± 8.704	< 0.05
Gender (% male)	40.74%	74.41%	0.001
Weight (kg)	74.19 ± 17.54	76.8 ± 13.96	NS
Height (cm)	162.61 ± 10.72	169.84 ± 8.49	< 0.001
BMI (kg/m^2)	28.17 ± 6.08	26.59 ± 4.18	NS
Waist (cm)	94.2 ± 15.19	93.36 ± 14.51	NS
Hip (cm)	103.3 ± 13.31	104.69 ± 7.9	NS
Waist-to-hip ratio	0.9 ± 0.075	0.88 ± 0.096	NS
Fat body composition	32.34 ± 8.28	28.14 ± 7.35	0.01
Type 2 diabetes mellitus (n)	3	0	NS
High blood pressure (n)	26	1	< 0.001
Insulin (pmol/L)	7.43 ± 4.36	6.05 ± 3.76	NS
HOMA-IR	1.99 ± 1.76	1.41 ± 1.03	< 0.05
Leptin (ng/mL)	14.73 ± 8.23	11.85 ± 7.47	NS
Cholesterol (mg/dL)	208.44 ± 44.54	203.63 ± 34.15	NS
HDL (mg/dL)	40.26 ± 10.79	39.61 ± 11.26	NS
LDL (mg/dL)	135.49 ± 48.49	130.32 ± 25.96	NS
Triglycerides (mg/dL)	186.5 ± 139.58	171.42 ± 97.47	NS
Kilocalories/day	1898.58 ± 398.71	1711.75 ± 506.92	NS
Cholesterol[1] mg	198.1 ± 153.95	193.89 ± 96.18	NS
Saturated fatty acids[1] g	15.08 ± 5.4	20.45 ± 8.86	0.001
Monounsaturated fatty acids[1] g	17.42 ± 5.54	28.88 ± 12.31	< 0.001
Polyunsaturated fatty acids[1] g	19.82 ± 4.32	12.47 ± 7.77	< 0.001

[1]Daily intake
BMI: body mass index, HOMA-IR: Homeostatic Model Assessment insulin resistance, HDL: high-density lipoproteins, LDL: low density lipoproteins.
NS: No significant

Table III. Genotypic characteristics of the leptin receptor among cases and controls.

Variable	Cases (n = 54)	Controls (n = 43)	*p* value
Exon 6			
Normal	22	15	NS
Heterozygote	23	21	NS
Homozygote	9	7	NS
Exon 14			
Normal	40	30	NS
Heterozygote	14	12	NS
Homozygote	0	1	NS

NS: Not significant

association of GD. For instance, polymorphisms in apolipoprotein E and B-100 genes had been associated with GD and cholecystolithiasis in Spain[29] and China.[30]

Obesity is recognized as a major GD risk factor. It has been associated with gallbladder dysmotility[31] and increased biliary secretion of cholesterol from the liver,[32] producing cholesterol-supersaturated bile, a major factor involved in GD. However, the prevalence of these two diseases, apparently parallel, is actually discordant. GD affects non-obese population, and a high percentage of obese subjects do not suffer GD. Bouchard *et al* studied the prevalence of gallstones in five monogenetic models of experimental obesity like this;[33] some models had a decreased prevalence, such as LEPR deficiency model, while others had an increased prevalence compared to wild-type. They stated that rather than being the consequence of obesity, cholesterol gallstones are induced by specific genes that shared susceptibility to obesity.

Two single nucleotide substitution, Q223R and K656N, in LEPR gene has been previously associated with obesity.[9] These polymorphisms are localized in exon 6 and 14, respectively, and codify part of the extracellular leptin-binding sequence of the receptor. The amino acid change probably reduces the affinity of leptin for its receptor. Interestingly, in our study, only 38% of the subjects in our sample were Q223 homozygote, so in statistical terms, it was normal to be a carrier of the R223 polymorph in our sample. R223 genotype individuals had higher leptin serum levels, BMI and waist circumference. This finding could represent a genetical susceptibility for obesity in Mexican population, which, combined with a poor balanced diet and life style, could explain the high prevalence of obesity in our adult population (~25%).

Ruhl *et al* showed that high serum leptin levels correlated with the presence of GD in both sexes. However, this effect was not confirmed in multivariate analysis.[14] Ko *et al* recently demonstrated that leptin levels are good indicators of risk of gallstones and biliary sludge in pregnant women.[15] In addition, we demonstrated that the utility of leptin serum levels as predictor of GD are observed only patients with BMI < 30 in univariate and multivariate analysis. Paradoxically, in subjects with BMI ≥ 30 the association of GD with leptin serum levels indicated a protective factor in univariate analysis, which was not confirmed in multivariate analysis.[34]

Table IV. Anthropometric, biochemical variables and gallstones disease according of polymorphism in exon 6 [Q223 homozygotes (normal), R223 heterozygotes and R223 homozygotes] and exon 14 [K656 homozygotes (normal), N656 heterozygotes and N656 homozygotes].

Variable	Exon 6 Normal (n = 37)	Exon 6 Heterozygote (n = 44)	Exon 6 Homozygote (n = 16)	Exon 14 Normal (n = 70)	Exon 14 Heterozygote (n = 25)	Exon 14 Homozygote (n = 2)
Age (years)	46.69 ± 11.88	48.43 ± 12.54	47.31 ± 11.83	48.01 ± 12.08	46.68 ± 12.42	41
Weight (kg)	71.15 ± 12.4	77.54 ± 17.41	79 ± 18.27	76.48 ± 17.05	70.56 ± 11.18	95.5 ± 106
Height (cm)	165.48 ± 9.81	165.79 ± 11.45	166.68 ± 9.16	166.66 ± 10.28	162.82 ± 10.49	174 ± 5.65
BMI (kg/m²)	25.94 ± 3.67	28.44 ± 6.6*	28.35 ± 4.16	27.62 ± 5.72	26.7 ± 4.17	31.7 ± 5.51
Waist (cm)	89.2 ± 11.05	96.7 ± 16.39*	96.62 ± 16.07*	94.99 ± 15.41	89.08 ± 11.67	112.5 ± 9.19
Hip (cm)	100 ± 7.66	106.14 ± 13.33*	106.87 ± 9.57	104.87 ± 12.07	100.88 ± 8.25	108.5 ± 4.95
Fat body composition (%)	29.12 ± 8.1	30.85 ± 8.17	32.6 ± 7.96	30.44 ± 8.21	30.34 ± 8.3	33.6 ± 0.14*
Glucose (mg/dL)	107.29 ± 72.32	94.84 ± 12.13	99.56 ± 30.82	97.38 ± 25.89	108.44 ± 82.52	104 ± 2.82
Insulin (pmol/L)	6.06 ± 3.3	7.14 ± 4.39	7.66 ± 5.1	6.93 ± 4.32	6.14 ± 3.12	11.4 ± 8.62
HOMA-IR	1.7 ± 1.89	1.69 ± 1.14	1.9 ± 1.47	1.68 ± 1.16	1.79 ± 2.2	2.92 ± 2.27
Leptin (ng/mL)	12.02 ± 7.66	13.79 ± 9.43	15.83 ± 7.96	13.5 ± 9.03	13.37 ± 7.67	12.95 ± 2.61
Cholesterol (mg/dL)	198.7 ± 42.81	207.25 ± 36.12	221.31 ± 42.29	206.34 ± 39.49	208.24 ± 43.58	181 ± 9.9
HDL (mg/dL)	38.91 ± 12.15	40.29 ± 9.88	41.53 ± 11.25	40.66 ± 11.58	38.39 ± 9.28	35.6 ± 6.5
LDL (mg/dL)	127.29 ± 32.99	130.47 ± 24.79	154.33 ± 72.65	133.06 ± 43.09	136.2 ± 30.63	105.5 ± 23.3
Triglycerides (mg/dL)	161.86 ± 79.88	185.06 ± 98.07	206.87 ± 226.39	182.65 ± 134.52	168.36 ± 84.41	223.5 ± 98.28
Kilocalories/day	1846.5 ± 508.83	1774.96 ± 407.25	1842.66 ± 494.71	1871.07 ± 466.53	1687.9 ± 397.4	1436.94 ± 07.32
Cholesterol mg/day	202.83 ± 133.44	195.57 ± 138.24	183.21 ± 103.79	196.15 ± 127.31	197.73 ± 144.41	177.56 ± 53.7
Saturated fatty acids g/day	17.24 ± 8.59	17.61 ± 7.24	17.99 ± 6.91	17.88 ± 7.97	16.62 ± 7.03	17.32 ± 5.36
Monounsaturated fatty acids g/day	21.77 ± 1.33	22.83 ± 9.54	24.16 ± 13.44	23.31 ± 11.94	20.88 ± 7.65	23.05 ± 6.87
Polyunsaturated fatty acids g/day	16.68 ± 5.61	16.23 ± 8.2	16.56 ± 7.42	17.07 ± 7.4	15.48 ± 6.21	8.21 ± 1.52**
Gallstones (%)	59.45	52.27	56.25	57.14	56	0

* $p < 0.05$, *versus* normal exon
** $p < 0.005$, *versus* normal exon
BMI: body mass index, HOMA-IR: Homeostatic Model Assessment insulin resistance, HDL: high density lipoproteins, LDL: low density lipoproteins.

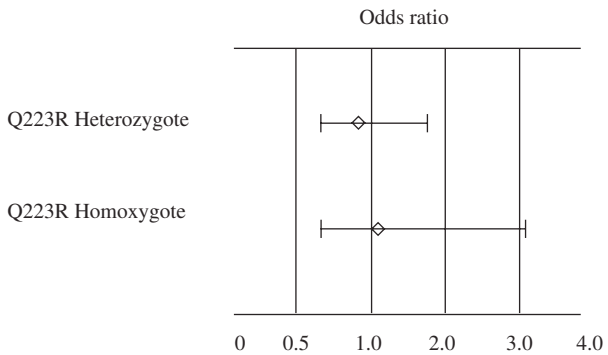

Figure 1. Univariate analysis shows the odds ratio to be a case of GD according to genotype. No polymorphisms showed statistical significance when the probability to be a case was analyzed. The relative risk of being a case of GD was 1.03 (95% CI 0.35-3.03) for R223 homozygotes, 0.78 for R223 heterozygotes (95% CI 0.35-1.74) and 0.78 for the presence of the polymorphism in exon 6 (95% CI 0.34-1.79). The relative risk of being a case was ∞ ($p = 0.2$) for N656 homozygotes, 1.02 for N656 heterozygotes (95% CI 0.41-2.55) and 0.81 for the presence of the polymorphism in exon 14 (95% CI 0.33-1.97).

Tran *et al* showed that LEPR deficient obese mice have decreased biliary cholesterol saturation and a decreased cholesterol crystal formation despite elevated serum cholesterol, and suggest that gallstone formation does not require hypersecretion of biliary cholesterol[16] and instead is the result of the effect of diabetes and hyperlipidemia on gallbladder motility.[35]

Leptin induces reverse cholesterol transport via activation of its hypothalamic long-form receptors in response to weight gain. Therefore, we hypothesized that leptin resistance in obese subjects would produce the loss of leptin-induced anorexigenic effect and protect them against bile cholesterol supersaturation through the loss of prolithogenic leptin effects, and that frequent obesity-associated LEPR polymorphisms could be responsible for this phenomenon. However, our results show that obesity-related LEPR polymorphisms are not associated with GD.

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