Quantitative trait loci mapping for cholesterol gallstones in AKR/J and C57L/J strains of mice

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Cholesterol gallstones are a major clinical as well as public health problem affecting 10–40% of individuals over 60 yr old in the United States (12). Although the high fat and cholesterol diets typical of many Western societies contribute to this disease (2), so does heredity (reviewed in Ref. 27). For example, two large, population-based epidemiological studies in Italy (3, 30) indicated that individuals who have at least one parent with gallstones are three times more likely to have gallstones than individuals with unaffected parents; the risk is significantly higher in those whose parents both have gallstones. Two cross-sectional studies in Germany (14, 25) indicated that first degree relatives of a gallstone patient are more likely to have gallstones than are unrelated individuals. Three other large, carefully controlled studies from Sweden (38), Israel (9), and Northern India (31) established that first degree relatives of gallstone patients are at least twice as likely to have gallstones than nonrelatives.

As with many other genetic diseases, much can be learned about cholesterol gallstones by studying the trait in animal models. The laboratory mouse is a particularly suitable model because of the highly developed genetic resources for the inbred mouse and the discovery that inbred mouse strains fed cholesterol and cholic acid vary considerably in their susceptibility to cholesterol gallstones (1, 8, 13, 26, 37). Therefore, the mouse can be used to dissect the genetic contributions to cholesterol gallstone formation.

Similar to most common diseases, cholesterol gallstone formation is a complex trait and does not yield readily to simple Mendelian analysis for single-gene traits. Rather, locating and estimating the effects of genes that underlie complex traits require quantitative trait locus (QTL) analyses (20). We used recombinant inbred (RI) mouse strains derived from AKR/J (AKR), a gallstone-resistant strain, and C57L/J (C57L), a gallstone-susceptible strain, and the progeny of a backcross between these two strains to determine that susceptibility to cholesterol gallstones is due to several genes (13). We named the major gene, which mapped to chromosome (Chr) 2, Lith1.

Since we identified the Lith1 locus, we systematically characterized the cholesterol gallstone phenotype in the C57L and AKR strains and their F1 progeny. Specifically, Wang et al. (39, 40) showed that C57L mice secrete more cholesterol and bile salts into bile, produce salt-independent bile flow at a higher rate, accumulate more gallbladder mucin, and have larger gallbladders than do AKR mice. Also, Lammert et al. (18) showed that C57L mice possess significantly higher hepatic HMG-CoA reductase activities and lower activities of both bile salt synthetic regulatory enzymes, sterol 27-hydroxylase and cholesterol 7α-hydroxylase, than do AKR mice.

In this study, a QTL analysis of a 231-mouse (AKR × C57L) F1 × AKR backcross was carried out. We con-
firmed the polygenic nature of cholesterol gallstone disease in the mouse and identified Lith2, a second major QTL playing a major role in the disease. By introgressing the C57L alleles for these two loci into congenic strains with an AKR genetic background, we demonstrated that susceptible alleles at both Lith1 and Lith2 can independently cause cholesterol gallstone formation.

MATERIALS AND METHODS

Animals and diet. The inbred mouse strains AKR and C57L were obtained from the Jackson Laboratory; AKXL RI strains were a kind gift from Dr. Benjamin Taylor (Jackson Laboratory), and (AKR × C57L) F1; progeny and F1 × AKR backcross progeny were bred in our colony. Because male C57L mice display significantly higher gallstone prevalence rates than female mice (40), we used only male mice in this study. Mice had free access to food and acidified water and were housed in a temperature-controlled room (22–23°C) with alternating 14:10-h light-dark cycles. We euthanized mice with 500 mg/kg body wt of 2,2,2-tribromoethanol (Sigma, St. Louis, MO). Animals and Use Committees of the Jackson Laboratory and of Harvard University.

Mice were fed laboratory chow (Old Guilford animal diet no. 234; Emory Morse, Guilford, CT), which contains only trace cholesterol (0.02%), or a semisynthetic diet containing 15% butterfat, 1% cholesterol, 0.5% cholic acid, 2% corn oil, 50% sucrose, 20% casein, and essential vitamins and minerals (13). To test for the gallstone phenotype, mice were fed the lithogenic diet for 8 wk beginning when they were 7–8 wk old.

Constructing congenic strains. During this study, two new congenic strains were constructed, AKL-Lith1* and AKL-Lith2*, both are available from the Jackson Laboratory. By convention, the nomenclature of a congenic strain follows this order: the symbol of the background strain, a period, the name of the introgressed gene, and either the superscript “s” for susceptible or “r” for resistant. Classically, we would have introgressed the susceptible Lith1 alleles into an AKR background by backcrossing from the C57L strain. Instead, we crossed AKR mice with mice of the RI strain AKXL-29, because the latter carries C57L alleles in the Lith1 region but AKR alleles at the other C57L susceptible loci, identified by D10Mit2 (cM 16), D19Mit19 (cM 26), and DXMit46 (cM 24). We then backcrossed these F1 progeny to AKR and tested the offspring for their alleles at D2Mit7 (cM 28) and D2Mit14 (cM 49), markers on either side of the Lith1 locus. Progeny heterozygous at Lith1 were backcrossed again to AKR for five generations (N5), at which point we had an “incipient” congenic. Normally, an incipient congenic has the genetic region introgressed and an additional ~3% of the genome from the donor strain. However, our incipient congenic had only about an additional 1.5% of the genome (and the Lith1 locus) from C57L, because we initiated our congenic strain with RI strain AKXL-29, which had only 50% of the C57L genome to begin with. A sister-brother pair of N5 progeny, heterozygous at Lith1, were mated to each other, and their progeny that were homozygous for C57L alleles over the Lith1 region were used as progenitors of the congenic strain. In addition to the selected region, this congenic contained about 0.75% of C57L alleles.

To construct the Lith2 congenic strain, we crossed an AKR mouse with a mouse of RI strain AKXL-17, because this RI strain carries gallstone-susceptible C57L alleles in the Lith2 region but gallstone-resistant AKR alleles over the Lith1 region. We created an incipient congenic (N5), selecting for heterozygosity at D19Mit19 (cM 26) and D19Mit71 (cM 54), markers on either side of the Lith2 locus.

Phenotyping backcross progeny. Gallbladder volume was determined as described previously (13). Gallstones, when present, were visible through the gallbladder wall. To collect them, the gallbladder was cut at the fundus, gently squeezed with forceps, and washed out with 1 ml of cold 95% ethanol. Stones were dried overnight at 40°C and weighed. For the study of congenic strains, fresh gallbladder bile was characterized for true and sandy stones under a polarizing light microscope as described earlier (40).

Genotyping. DNA was isolated from tails or spleens of the mice (36) and genotyped with simple sequence length polymorphisms (SSLP) obtained from Research Genetics, Huntsville, AL. The SSLPs were spaced at about 20-cM intervals throughout the genome (list of markers available upon request to corresponding author). In the initial genome-wide scan, we employed 78 SSLP; when we discovered the QTL, we added 8 SSLPs to Chr 2, 10, and 19 to better define the QTL regions. Polymerase chain reaction was carried out as described previously (35).

Statistical analysis. To locate QTL associated with gallstone formation, we performed four statistical analyses and compared the results: 1) simple association analysis of marker locus alleles with gallstone parameters using ANOVA, 2) maximum likelihood interval mapping as contained in the computer program MAPMAKER (21), 3) a combination of multiple regression with maximum likelihood analysis as contained in the program QTL Cartographer (11, 42), and 4) a combination of regression and interval mapping contained in the program MapManagerQT (23). For some methods, we divided the likelihood ratio statistic by 4.6 to obtain approximate LOD scores. A QTL was judged to be significant if the LOD score was above 3.3 (19). To compare the prevalence of gallstones among strains, we used chi-square analysis; to compare allele effects, we used Student’s t-test. Values are expressed as means ± SE; P < 0.05 is used for significance.

RESULTS

Distribution of gallstone weight and gallbladder size among backcross progeny. The stones of the 231 (AKR × C57L) F1 × AKR backcross progeny ranged from 0.1 to 5.7 mg and averaged 1.0 mg ± 0.1 per mouse; 62 mice (26%) had no stones (Fig. 1). In comparison, as reported by Khanuja et al. (13), the stones of AKR, C57L, and F1 mice average 0, 1.0 ± 0.3, and 1.2 ± 0.1 mg/mouse, respectively. As reported earlier, these stones contain >95% cholesterol (40). The fact that the stones of many backcross progeny were heavier than stones of the susceptible C57L parents suggests that the resistant AKR strain may contain some gallstone susceptibility alleles.

The gallbladder volumes of the 231 (AKR × C57L) F1 × AKR backcross progeny ranged from 5 to 100 μl and averaged 27 ± 1 μl (Fig. 2). Comparatively, as reported by Khanuja et al. (13), the gallbladder volumes of AKR, C57L, and F1 mice average 9 ± 1, 86 ± 12, and 24 ± 5 μl, respectively. Gallbladder volumes were normally distributed, suggesting they are determined by multiple genes. We found no correlation

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between gallstone weights and gallbladder volumes ($r = 0.01, P = \text{not significant}$).

**QTL analysis.** To carry out a genome-wide scan of genetic factors that influence gallstone weight, we used 86 SSLP markers and genotyped only the mice in the upper and lower 16% of the gallstone weight distribution (the percentage was determined by the number of mice that would fill a 96-well microtiter plate, allowing for 2 controls). The 37 mice in the lower 16% of the distribution were selected randomly from the 62 mice that had no gallstones.

The results of all four statistical analyses used to determine QTL from the genome-wide scan are summarized in Table 1. The first analysis, an ANOVA, simply determined a $P$ value for associations between genotype at the SSLP markers used in the genome-wide scan and gallstone formation in the backcross progeny. Four loci with alleles contributed by C57L, on Chr 2, 10, 19, and X, and three loci with susceptibility alleles contributed by AKR, on Chr 6, 7, and 8, were associated with gallstone formation. The most significant association was with the locus on Chr 2.

For our second analysis, we used MAPMAKER, a computer program based on maximum likelihood interval mapping (21). The analysis indicated that three loci on Chr 2, 10, and 19 were associated with gallstone formation. However, the assumptions inherent in this analysis are that the traits 1) are normally distributed, 2) exhibit additive effects, and 3) are not influenced by epistasis (interaction with other genes). However, as displayed in Fig. 1, cholesterol gallstone disease parameters are not normally distributed (e.g., many mice have no stones), the genes appear to have dominance effects, and they may exhibit epistasis. Thus we analyzed our data with three other methods that could accommodate these effects.

For our third statistical analysis, we used QTL Cartographer, a computer program that combines maximum likelihood and regression analyses and that accommodates dominance and epistasis. Again, the same three loci on Chr 2, 10, and 19 and a locus with susceptibility alleles contributed by AKR on Chr 7 were associated with gallstone formation. This analysis did not reveal any epistatic interactions among the loci associated with gallstone formation. Finally, for our fourth analysis, we used the MapManagerQT program, which uses both regression and interval mapping methods. This analysis did not confirm the locus on Chr 10, but it revealed two additional suggestive loci on Chr 6 and 8 with susceptibility alleles contributed by AKR.

All analyses showed that the locus on Chr 2 is the most significant locus (LOD > 3.3) associated with gallstone formation. All analyses also detected an association between a locus on Chr 19 and gallstone formation, although the highest LOD score for that association was only 2.2. Three of the four analyses detected an association between a locus on Chr 10 and gallstone formation, although the significance of that association varied considerably among analyses. Because of differences inherent in the way each analysis computed associations between a locus and gallstone formation, the LOD scores (Table 1) cannot always be compared directly. QTL Cartographer controlled the five most significant loci from a stepwise regression analysis, after which it computed the LOD scores for Chr 7 and 10 from composite interval mapping. MapManagerQT allows one to control for the most significant loci and reanalyze the data; an analysis controlling for the loci on Chr 2 and 19 (Table 1) did increase the significance of the locus on Chr 6.

![Fig. 1. Distribution of gallstone weight in backcross progeny. Values for gallstone weight per mouse (mg) are shown for 231 (AKR × C57L) × F1 male backcross progeny fed for 8 wk with the lithogenic diet containing 15% butterfat, 1% cholesterol, and 0.5% cholic acid. The first group of 116 mice has gallstones in the range of 0–0.49 mg; the second group has gallstones in the range of 0.50–0.99 mg, etc.](http://physiolgenomics.physiology.org/)

![Fig. 2. Distribution of gallbladder volumes in backcross progeny. Values for gallbladder volume in µl for 231 (AKR × C57L) × F1 male backcross progeny fed the lithogenic diet for 8 wk are shown.](http://physiolgenomics.physiology.org/)
Backcross mice heterozygous at any one of the Chr 2, 10, 19, and X loci had significantly ($P < 0.05$) heavier gallstones than mice homozygous for the AKR alleles at the same loci (Fig. 3). The LOD score plots for the QTL on Chr 2 and 19, as determined by interval mapping using the program MapManagerQT, and the location of possible candidate genes are shown in Fig. 4.

In addition to determining QTL associated with gallstone weight, we analyzed data using MapManagerQT to detect QTL associated with gallbladder volumes. Again, the locus on Chr 2 was associated with large gallbladder volumes, with the susceptibility allele contributed by C57L, but the LOD score of 2.0 was suggestive rather than significant. Of several other minor loci with LOD scores of 1.0–1.2 associated with large gallbladder volumes, two, identified by D6Mit25 and D7Mit85, were concordant with the QTL for gallstone weight.

### Table 1. Chromosomal markers showing an association with cholesterol gallstone weight in backcross (AKR \times C57L) F1 \times AKR male mice

<table>
<thead>
<tr>
<th>Allele</th>
<th>ANOVA</th>
<th>MAPMAKER</th>
<th>QTL C</th>
<th>MM QT</th>
<th>MMQT Controlled</th>
</tr>
</thead>
<tbody>
<tr>
<td>D2Mit14*</td>
<td>L</td>
<td>&lt;0.001</td>
<td>4.5</td>
<td>5.1</td>
<td>4.6</td>
</tr>
<tr>
<td>D6Mit25*</td>
<td>AK</td>
<td>0.04</td>
<td></td>
<td>1.6</td>
<td></td>
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<tr>
<td>D7Mit85*</td>
<td>AK</td>
<td>0.005</td>
<td></td>
<td>1.7</td>
<td></td>
</tr>
<tr>
<td>D8Mit40</td>
<td>AK</td>
<td>0.006</td>
<td></td>
<td>1.2</td>
<td>1.3</td>
</tr>
<tr>
<td>D10Mit2</td>
<td>L</td>
<td>0.008</td>
<td>1.2</td>
<td>3.2</td>
<td></td>
</tr>
<tr>
<td>D19Mit58</td>
<td>L</td>
<td>0.01</td>
<td>1.8</td>
<td>2.2</td>
<td>1.6</td>
</tr>
<tr>
<td>DXMit46</td>
<td>L</td>
<td>0.06</td>
<td></td>
<td>1.6</td>
<td>1.5</td>
</tr>
</tbody>
</table>

The association of markers with gallstone weight is compared using 4 different statistical methods. L, the C57L allele; AK, the AKR allele; QTL C, QTL Cartographer; MMQT, MapManagerQT. MMQT controlled, the reanalysis controlling for the loci on chromosomes 2 and 19. $P$ values correspond to an ANOVA F test, and the remaining values are LOD scores computed at the marker itself. *Concordance of that quantitative trait locus (QTL) for gallbladder volume.

Confirming the QTL for Lith1 and Lith2 by constructing congenic strains. Although QTL mapping can detect associations between loci and a particular phenotype, the associations must be confirmed, especially
DISCUSSION

As indicated by the distribution of phenotype in the progeny, many genes determine gallstone formation and gallbladder volume. Although our regression analysis detected no significant correlation between these two traits, their overlapping QTL regions, in particular the QTL on Chr 2 and, to a smaller extent, the QTL on Chr 6 and 7, suggest that they may be controlled by some of the same genes.

Each of our statistical analyses revealed a significant locus, Lith1, for gallstone formation on Chr 2, and most revealed a suggestive locus, Lith2, on Chr 19 (LOD scores ranging from 1.6 to 2.2). Each QTL was confirmed to have a strong, independent impact on gallstone risk as shown by the two congenics, one with the C57L allele at the Lith1 locus, the other with the C57L allele at the Lith2 locus introgressed into an AKR background. Mice of the Lith1 congenic strain were susceptible to gallstones and developed the enlarged gallbladders typical of the C57L gallstone phenotype. Mice of the Lith2 congenic strain developed gallstones to the same extent as Lith1 congenic mice, but their gallbladders were not enlarged, suggesting that the development of stones and enlargement of the gallbladder are not inextricably connected. Three of our four statistical analyses detected a suggestive locus on Chr 10 (LOD scores ranging from 1.2 to 3.2), and only one method detected a suggestive QTL on Chr X. We have not yet constructed congenic strains for these loci, and, because they were only suggestive, we have not designated them as Lith genes. However, the Lith2 QTL itself was only suggestive, yet it proved to have a strong independent effect on gallstone susceptibility in the Lith2 congenic, suggesting that a LOD significance threshold of 3.3 may be so high that important QTL are discarded.

The genomic regions harboring the QTL we identified in this study are large (i.e., 10–20 cM), and they contain several candidate genes of interest. One strong candidate for the Lith1 locus is Abcb11 (5, 16), a gene encoding an ABC transporter, previously known as the bile salt export pump (Bsep) or sister to P-glycoprotein (Sppp). This protein is expressed on the canalicular membrane and transports bile salts into the canalicular bile. The glycoprotein receptor gene for megalin (Gp330), a member of the low-density lipoprotein (LDL) receptor gene family (41), also maps to the Lith1 region, but we consider it an unlikely candidate. Although megalin has binding properties for ApoE-containing lipoproteins, it is not expressed in the liver and appears to play no role in hepatic lipoprotein metabolism (15). A candidate gene for Lith2 is Abcc2 (4), another ABC transporter gene that maps to Chr 19 (17), previously known as canalicular multispecific organic anion transporter (Cmoat) or multidrug resistant related protein 2 (Mrp2). The ABC2 protein is expressed on the canalicular membranes of the hepatocyte and is the major transporter of divalent organic anions into bile (10). These anions can uncouple the hepatocyte’s secretion rates of cholesterol and phospholipid from that of bile salts, thereby upsetting cholesterol coupling to other biliary lipids and apparently causing gallstones. Recently, it has been demonstrated, using a subtractive hybridization procedure (24), that Abcc2 is upregulated during diosgenin-induced cholesterol hypersecretion, thus substantiating...
the putative role of Abcc2 in regulation of hepatobiliary cholesterol transport.

The gallstone QTL we identified on Chr 10 is in a region that contains another, recently discovered ABC transporter gene, Abca7 (32), whose function is unknown. The QTL on the X chromosome colocalizes with a mouse QTL for polygenic obesity (28). In humans, obesity dramatically increases susceptibility to cholesterol gallstones, the relative risk increasing in proportion to the degree and perhaps the type of obesity (7, 27). Our X-linked gallstone QTL contains two candidate genes, those for the androgen receptor (Ar) and a 3β-hydroxysteroid dehydrogenase (Nsdhl). Ar is expressed in the liver and plays a role in cholesterol homeostasis; its inhibition with flutamide decreases LDL levels and increases high-density lipoprotein (HDL) levels (6, 29). Nsdhl also plays a role in cholesterol and bile salt biosynthesis and metabolism (22).

The gallstone QTL we found in this study may only be a subset of all the genes that affect the gallstone phenotype; other genes with no polymorphic difference between the AKR and C57L strains may also be important. To identify these other genes and to fully understand the gallstone phenotype, QTL crosses between mice of other gallstone-susceptible and gallstone-resistant strains must be carried out.

The mouse model is becoming a powerful experimental system for finding QTL, the underlying genes, and the physiological pathways for many common, genetically complex diseases. It may also be possible to use the location of a QTL found in an experimental model to predict the location of a QTL in a homologous genomic region in another species, particularly humans. There are several reasons why QTL might be found in the same homologous regions, or be concordant, among species. Of all the proteins that are in a pathway, it is likely that only a subset can affect the final phenotype when mutated. Such proteins would have a nonredundant function or might be key regulatory proteins. If only a limited set of genes can give rise to a major QTL, then QTL in model organisms might predict the location of corresponding QTL in the human genome. For hypertension, QTL found in the rat (33) and the mouse (34) do predict regions containing blood pressure genes in humans. To the extent that it occurs, concordance of QTL across species will provide a major tool in the identification of chromosomal regions and proteins underlying human variation in disease susceptibility. Because cloning the gene underlying a QTL is much more feasible in model organisms than in humans, the concordance of QTL among species would have very significant practical implications for cloning and defining the functions of human disease genes.

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