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Genetic factors in cholesterol gallstone disease

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ABSTRACT

Most common diseases are strongly influenced by inheritance, but, to date, relatively few genes have been identified that are responsible for the familial clustering of these diseases. In the great majority of cases, no Mendelian inherited trait can be demonstrated. With the exception of cholesterol gallstones associated with low phospholipid level, induced by mutations in the gene of the multidrug resistant protein (MDR3/ABCB4), and of the mutations in the gene of cholesterol 7 a hydroxilase (CYP7A1), recently identified, no single genetic mutation or polymorphism has been found in association with cholesterol gallstone disease in humans.

This happens because common diseases, such as cholesterol gallstone disease, are complex disorders, where multiple genes and environmental factors collaborate to their development. Cholesterol gallstone disease is characterized by a genetic predisposition to lithogenesis, involving multiple genes and gene-gene interactions plus interaction with a "lithogenic" environment. The combination of these factors leads to gallstone formation if a threshold of susceptibility is reached, since each susceptibility allele only confers a modest increase in risk.

There are six major classes of candidate genes which, by encoding hepatobiliary lipid regulators and transporters, could contribute to bile suprasaturation in cholesterol and the formation of gallstones. These have been identified in experimental model. The quantitative trait locus (QTL) mapping is a powerful genetic technique for the identification of genes determining complex traits. Subsequently mapping studies can be undertaken in humans for identification of orthologous LITH genes, because of the exceptional man-mouse chromosome homology.

Keywords: genetics, cholesterol gallstones, lithogenic genes, risk factors, QTL mapping

INTRODUCTION

Prevalence of cholesterol gallstone disease is rising in the industrialized countries in Europe and North America. More than 20 million people in the United States have gallstones (1). Given the higher incidence at advanced ages, the longer life expectancy of the population and the high costs of cholecystectomy, gallstone disease represents a significant burden for these societies. The annual costs of gallstone disease in the United States are higher than 6.5 billion USD (2). In Romania, necroptic (3) and sonographic (4) studies have shown a prevalence of 11-12% of gallstone disease, with an estimated 2.3 million gallstone carriers, and an increasing trend in the last decades (5).

Sustained efforts are presently directed to elucidate the etiology of this common disease, with the goal of preventing gallstone formation. Ethnicity and family history are among the major risk factors for cholesterol lithogenesis. Furthermore, monozygotic twins are much more likely to be concordant for gallstones than dizygotic twins. Gallstone susceptibility might be caused by a genetic predisposition involving multiple genes and gene-gene interactions plus interaction with a "lithogenic" environment including diet, obesity, weight loss, multiple pregnancies etc. The combination of these factors leads to gallstone formation if a threshold of susceptibility is reached, since each susceptibility allele only confers a modest increase in risk (6, 7). ❑

PATHOGENESIS OF CHOLESTEROL GALLSTONE DISEASE

A brief description of the current information regarding cholesterol gallstone pathogenesis is necessary in order to analyze the role of environment and of genetics in the development of the disease.

Three types of abnormalities have been classically considered to be responsible for cholesterol gallstone formation (Table I). The primary and major abnormality consists of bile supersaturation in cholesterol, which is determined by changes in the proportion of the three major lipids in bile: cholesterol, bile acids and phospholipids.

The hepatic cholesterol pool results from *de novo* synthetised cholesterol (key enzyme: HMGCoA reductase), or from plasma lipoprotein particles, especially HDL. Most of the cholesterol derives from the diet, only about 20% results from *de novo* synthesis. Cholesterol is esterified in the liver (key enzyme: ACAT) and is incorporated in VLDL, or it is excreted as free cholesterol in the bile. In the liver, cholesterol is also converted into bile acids, cholesterol 7a hydroxylase (CYP7A1) being the rate limiting enzyme for bile acid biosynthesis. An increased biliary cholesterol saturation might result from an increased *de novo* synthesis, an increased uptake from plasma lipoporteins, a decreased conversion into bile acids or a decreased cholesterol esterification.

1. Supersaturation of bile in cholesterol
2. Enhanced nucleation of cholesterol crystals
3. Impaired gallbladder emptying with stasis
4. Intestinal hypomotility

TABLE 1. Pathogenetic mechanisms of cholesterol gallstone formation

Obesity
Rapid weight loss
Hypertriglyceridaemia and reduced HDL-cholesterol serum level
Drugs lowering serum cholesterol
Slow intestinal transit
Gallbladder stasis
Hypercaloric and fat-rich (atherogenic) diet
Highly absorbable sugars
Type 2 diabetes mellitus
Low fibre diet
Low calcium low vitamin C diet
Alcohol abstinence
Smoking
Sedentary behaviour

TABLE 2. Modifiable (environmental) risk factors for cholesterol gallstones

Additional abnormalities favoring cholesterol gallstone formation are enhanced nucleation of cholesterol crystals, mucus hypersecretion and gallbladder hypomotility. Enhanced nucleation and mucin hypersecretion favor crystal formation, and gallbladder stasis allows crystals to grow and aggregate, forming stones. When bile is highly supersaturated in cholesterol, gallbladder motility or nucleation might not be too much altered, but in case of a slightly supersaturated bile, hypomotility and enhanced nucleation should be the dominant abnormality.

A fourth abnormality (Table 1) leading to lithogenesis has been recently added: intestinal hypomotility. The small intestine represents the site of absorption and reabsorption of dietary cholesterol, and also plays an important regulatory role in the enterohepatic circulation of bile salts. Slow intestinal transit allows bile salts to remain longer in the intestine, exposed to the action of bacteria, and thus favors their transformation into dehydroxylated and more hydrophobic bile salts (acid deoxycholate). Deoxycholate inhibits synthesis of the bile acids in the liver, increasing cholesterol excretion into bile.

Genes controlling synthesis of transporters and enzymes involved in the secretion of biliary lipids are the major natural candidates to influence lithogenesis. Genes involved in the control of gallbladder motility or mucus secretion represent a second large group of candidate genes (8). ❑

ENVIRONMENTAL RISK FACTORS

Beside the unmodifiable risk factors for cholesterol lithogenesis (female gender, increasing age and genetic susceptibility), there are numerous well documented environmental risk factors for gallstones (summarized in (6, 9) (Table 2).

The main risk factors for cholesterol gallstones, namely obesity, hypertriglyceridaemia, type 2 diabetes mellitus, atherogenic diet and physical inactivity are also major risk factors for the metabolic syndrome. Most of the other risk factors are similar. Therefore, cholesterol gall-

stone disease could be considered as a component of the metabolic syndrome (10). ❑

EVIDENCE FOR THE GENETIC BACKGROUND

Most common diseases are strongly influenced by inheritance, but, to date, relatively few genes have been identified that are responsible for the familial clustering of these diseases. This happens because common diseases are almost all complex disorders, where multiple genes and environmental factors collaborate to their development. Cholesterol gallstone disease is such a complex disease, characterized by a genetic (polygenic) predisposition to lithogenesis.

Ethnic and racial differences in prevalence

Necropsy and population studies have shown differences in worldwide gallstone prevalence, which can not be completely explained by environmental factors. There are African and Asian populations with very low prevalence (<5%), European and American populations with intermediate prevalence (10-30%) and populations of Native American ancestry (Pima Indians in Arizona, Mapuche Indians in Chile) with extremely high prevalence of cholesterol gallstones (30-70%). The ethnic subpopulations living in the same country (United States, Chile), but having different heritage of Amerindian admixture (1, 11, 12), significantly differ in gallstone prevalence. That these high differences in prevalence exist between ethnic groups sharing the same environment can be explained only by a genetic predisposition to gallstone formation.

Familial clustering

The family history is a very robust tool for identifying genetic susceptibility. Family histories can be divided into levels of risk. *Low risk* family histories are those with few affected members, often with only the proband or index case affected. This could be evidence of a single gene recessive disorder, but more likely represents a sporadic non-genetic occurrence of disease in an adult. *High risk* families are those with multiple generations affected in a pattern that is consistent with a single gene disorder, such as some of the cancer syndromes. These families usually are recognized by the various components of the inherited syndrome occurring in family members. The biggest group of families is that of *moderate risk,* and gallstone disease belongs to this group. A disease will be found in more than one individual in these families, but the pattern of expression is incomplete and does not follow strict Mendelian rules of inheritance. These are the most difficult to understand but represent the greatest opportunity for genetic studies, and this is where the powerful new technologies driven by the Human Genome Project will have the greatest impact.

An increased prevalence of gallstones was found in siblings and among the relatives of gallstone carriers as compared with families of controls (ratio 3:1) (13-15). The greater the number of affected relatives, the greater the risk of gallstones occurring at younger ages, reflecting a stronger polygenic predisposition.

Twin studies

A higher saturation of the bile and a greater correlation of the cholic and deoxycholic acid contents were found in the monozygotic as compared with dizygotic twins, as well as a 40% concordance rate for gallstones in monozygotic as compared with 0% in dizygotic twins (16). A very recent study performed on 43,141 twin pairs in Sweden (17) has indicated a significantly higher gallstone presence in monozygotic versus dizygotic twins (concordance rate 12% versus 6%) confirming the role of the genetic influence (25%), and also stressing the environmental influence: shared (13%) or individual environmental influence (62%). Genetic influence was also found in the phenotype of the disease (symptomatic or asymptomatic) (18-20). The genetic heritability derived from these studies was 0.29± 0.14 (19) and 0.44 ± 0.18 (21) for symptomatic stones, similar to that found in obesity or type 2 diabetes mellitus. ❑

Hepatic lipid regulatory enzymes
Hepatic lipoprotein receptors and related proteins
Hepatic and intestinal intracellular lipid transporters
Hepatic and intestinal membrane lipid transporters
Hepatic lipid regulatory transcription factors
Cholecystokinin, CCK receptors, and gallbladder mucins

TABLE 3. The candidate genes for cholesterol gallstones encode

CANDIDATE GENES FOR CHOLESTEROL LITHOGENESIS

There are six major classes of candidate genes which could contribute to bile supra-saturation in cholesterol and the formation of gallstones (22) (Table 3).

Hepatic lipid regulatory enzymes

The candidate genes encoding the *de novo* synthesis of cholesterol, the bile acid synthesis and cholesterol esterification in the liver are: 3 hydroxy-3-methylglutaryl-coenzyme A (HMGCoA), cholesterol 7a hydroxylase, sterol 27-hydroxylase, sterol O-acyltransferase 2 (formerly Acat 2). The genes encoding these enzymes have been already mapped to the mouse chromosomes (Table 4).

Hepatic lipoprotein receptors and related proteins

The receptors for the HDL lipoproteins (scavenger receptor 1), for the LDL particles (APOB/E receptor) or chylomicron remnants (hepatic lipase) allow plasma lipids to be taken up in the liver. Other genes involved in the extrahepatic metabolism of lipoproteins could be considered as *LITH* candidate genes, such as lecithin-choles-

Gene symbol	Gene name	Mouse chromosome	Human ortholog
I. *Cyp7a1*	Cholesterol 7 -hydroxylase	4	8q11-q12
Cyp7b1	Oxysterol 7 -hydroxylase	3	8q21.3
Cyp27	Sterol 27- hydroxylase	1	2q33-qter
Hmgcr	HMG-CoA reductase	13	5q13.3-q14
Soat2	Sterol O-acyltransferase 2	15	12q13.3-q15
II. *Apob*	Apolipoprotein B	12	2p24-p23
Apoe	Apolipoprotein E	7	19q13
Lcat	Lecithin-cholesterol acyltransferase	8	16q22.1
Hlp	Hepatic lipase	9	15q21-q23
dlr	LDL receptor	9	19p13.3
Lpl	Lipoprotein lipase	8	8p22
Pltp	Phospholipid transfer protein	2	20q12-q13
Srb1	Scavenger receptor 1	5	12pter-qter
III. *Cav*	Caveolin 1	6	7q31
Cav2	Caveolin 2	6	7q31
Fabp1	Fatty acid binding protein 1, liver	6	2p11
Fabp6	Fatty acid binding protein 6, ileal	11	5q23-q35
Npc1	Niemann-Pick type C1	18	18q11
Pctp	Phosphatidylcholine transfer protein	11	17q21-q24
Scp2	Sterol carrier protein 2	4	1p32
IV. *Abcb4 (Mdr2)*	Phosphatidylcholine „flippase“ (multiple drug resistance protein)	5	7q21
Abcb11(Bsep, Spgp)	Bile salt export pump (Sister of p-glycoprotein)	2	2q24.3
Abcc2 (Cmoat, Mrp)	Canalicular multispecific organic anion transporter (multidrug resistance-related protein 2)	19	10q24
FIC1	Familial intrahepatic cholestasis type 1	18	18q21-q22
Slc10a1 (Ntcp)	Na/taurocholate cotransporting polypeptide	12	14q21.1
Slc10a2 (Isbt,Ibat)	Ileal (apical) Na/bile salt transporter	8	13q33
Slc21a1 (Oatp1)	Organic anion transporting polypeptide	X	Xq25
Slc22a1 (Oct11)	Organic cation transporter 1	17	6q26
V. *Nr2b1(Rxra)*	Retinoid X receptor	2	9q34
Nr1h3 (Lxra)	Liver X receptor	2	11p11
Nr1h4 (Fxr)	Farnesoid X receptor	10	12q21-q24
Srebf 1	Sterol regulatory element binding transcription factor 1	8	17p11.2
Srebf2	Sterol regulatory element binding transcription factor 2	15	22q13
VI. *Cck*	Cholecystokinin	9	3pter-p21.3
Cckar	Cholecystokinin A receptor	5	4pter-qter
Muc1	Mucin 1	3	1q21-q22
Muc3	Mucin 3	5	7q22
Muc5ac, 5b,6	Mucin 5ac, 5b, 6	7	11p15.5

TABEL 4. Chromosomal location of candidate gallstone genes in mice and humans (modified after (6, 22)

terol acyl transferase (Table 4). Polymorphisms of the human *APOE* gene (23-25) and of the human gene encoding the cholesteryl ester transfer protein *(CETP)*, which transfers cholesteryl esters between lipoproteins (26) have been found to be associated with cholesterol lithogenesis.

Hepatic and intestinal intracellular lipid transporters

The intracellular transport of biliary lipids is realized by specific transporters, such as the sterol carrier protein 2 (SCP2), the Niemann-Pick type C1 (its mutation leads to the Niemann-Pick type C1 disease) or the phospholipid and bile salt carriers. Location of their genes has been identified on the mouse chromosomes (Table 4). The *SCP2* gene was overexpressed in patients with cholesterol gallstones, indicating that SCP2 may be an important cause of cholesterol gallstones

Hepatic and intestinal membrane lipid transporters

This is the class of transporters most intensely researched in cholestasis, as well as in biliary lithogenesis, because bile formation and secretion critically depends on their activity. The transporters of the biliary lipids from the hepatocyte to the bile against their concentration gradients in the bile (the export pumps) belong to the ATP-binding casesette (ABC) family of membrane transporters. The bile salt export pump (BSEP, ABCB11) transports monovalent bile salts into bile. Mutations in *ABCB11* cause the progressive familial intrahepatic cholestasis type 2 (PFIC type 2). The canalicular multispecific organic anion transporter (multidrug resistance-related protein 2) (ABCC2, MRP2) favors excretion of organic anions including bilirubin. Mutations in *ABCC2* were found in the patients with Dubin Johnson syndrome. The phosphatidylcholine "flippase" (multiple drug resistance protein) (ABCB4/MDR3) promotes transport of phospholipids into bile. Mutations in *ABCB4* cause the progressive familial intrahepatic cholestasis tupe 3 (PFIC type 3) and have also been found in association with cholesterol gallstones (27-29). Point mutations in the gene controlling the ileal (apical) Na/bile salt transporter *(SLC10A2)* lead to bile salt malabsorption and might favor pigment lithogenesis.

Recently two genes, *ABCG5* and *ABCG8*, have been identified, encoding sterolin-1 and sterolin-2, respectively, mutations of which cause the human disease sitosterolemia (30-34). The ABCG5 and ABCG8 transporters have a role in the intestinal absorption and biliary excretion of the neutral sterols (34, 35). Experiments in mice confirmed that ABCG5 and ABCG8 are the major hepatobiliary transporters for cholesterol, that they protect against dietary sterol accumulation in the body and that stimulation of cholesterol excretion by LXR (liver X receptor) agonists requires ABCG5 and ABCG8 (30, 33-37).

Hepatic lipid regulatory transcription factors

The sterol regulatory element binding transcription factors (SREBF) 1 and 2 up-regulate the transcription of *Hmgcr* and *Ldlr*.

The nuclear hormone receptors are a superfamily of cellular receptors that regulate gene transcription. In general, these proteins are activated upon binding ligands, namely small hydrophobic molecules like bile salts and steroid hormones. Dimers of the retinoid receptor that binds retinoic acid (RXR) and a ligand specific counterpart bind to a responsive element in the promoter region of a gene and thereby regulate transcription. *BSEP* expression is controlled by the nuclear receptor pair RXR/FXR, in which FXR is the farnesoid X receptor, which has high affinity for bile salts. Binding of bile salts to FXR in the dimer RXR/FXR induces transcription of the *BSEP* gene, leading to enhanced protein levels and increased bile salt secretion. *FXR* might also represent a candidate gene for biliary cholesterol lithogenesis (38, 39).

Cholecystokinin, CCK receptors, and gallbladder mucins

Cholecystokinin is the major regulator of gallbladder postprandial emptying, after binding to the CCKAR receptor. Gallbladder stasis favors gallstone formation. *Cck* and *Cckar* have been mapped to mouse chromosomes and are presently under intensive research (Table 4). Effects of polymorphisms of *MUC* genes on biliary lithogenesis are also under investigation, as mucin hypersecretion favors crystal formation and aggregation in the gallbladder lumen, but no polymorphisms could be correlated to gallstone formation yet. ❑

IDENTIFICATION OF *LITH* GENES

Association studies

Most association studies have focused on a common genetic variation. Common genetic variants (or polymorphisms) are those for which two or more alleles which exist in 1% or more of the population at large. The association studies are performed by comparing the incidence of a particular polymorphism in affected patients with the incidence of disease in a carefully matched control group. Because common variants are present at high frequency, they can be discovered in any modest sized group of individuals. In the case of complex disease, there is the risk of a false positive (by chance) association. Many associations are therefore not consistently reproduced, so it is very important to have large samples of individuals tested in replicative studies (40).

Association of gallstone formation with some gene polymorphisms in humans has been reported. Certain polymorphisms of the apolipoprotein E genes were correlated with cholesterol gallstone formation. *APOE* locus in humans was found to be a risk factor for cholesterol gallstone formation. *APOE* is inherited as three different allelic variants (*E2, E3* and *E4*). The *E4* genotype is associated with a higher incidence of gallstones, a higher cholesterol content of stones and also a higher recurrence rate after extracorporeal shock-wave lithotripsy (23-25). The *E2* genotype protects against gallstone formation. The effect of *E4* genotype could be explained by an increased hepatic uptake of chylomicron remnants. Polymorphisms of *APOB* and *APOA1* and *CETP* (gene of the cholesteryl ester transfer protein) are presently under investigation, some studies indicating a possible association with cholesterol gallstone disease (26).

In complex diseases, haplotype analysis might offer more information than analysis of the individual polymorphisms. A haplotype is a specific set of alleles present on a single chromosome. The reconstruction of haplotypes from genotype data in a population (by Bayesian method) increases the statistical power of an association study by doubling the sample size and simplifying the data structure (41). This type of analysis has also been used to investigate the genetics of gallstone disease. Through screening of the *ABCG5* and *ABCG8* genes for mutations in the sitosterolemia patients, polymorphisms in both genes have been reported (30, 42, 43) and some *ABCG5/ABCG8* polymorphisms have already been reported to contribute to the genetic variation in plasma lipid levels and in cholesterol saturation of the bile (44-46).

Linkage analysis

Linkage analysis has proved extremely valuable in mapping single gene disorders, but it is much more difficult to use in multifactorial, polygenic disorders, perhaps in part because of a limited power to detect the effect of common alleles with modest effect on disease. Only a reduced number of studies linking genomic regions to gallstone disease have been performed in humans.

An alternative approach would be to identify the cholesterol gallstone susceptibility loci in suitable animal models. This is obtained by mating inbred strains of mice highly susceptible to developing gallstones with inbred strains of mice resistant (with very low susceptibility) to the disease. The F1 offspring are then "backcrossly" mated with the high risk parental strain. By studying the cosegregation of polymorphic markers at multiple loci with the disease, susceptibility regions can be identified. The quantitative trait locus (QTL) mapping is a powerful genetic technique for the identification of genes determining complex traits. Subsequently mapping studies can be undertaken in humans for identification of orthologous *LITH* genes because of the exceptional man-mouse chromosome homology (Table 4) (22, 47-49). ❑

MONOGENIC GALLSTONE DISEASE IN HUMANS

Gallstone disease is a complex disease. In the great majority of cases no Mendelian inherited trait can be demonstrated. With the exception of cholesterol gallstone disease associated with low phospholipid level, induced by mutations in the gene of the multidrug resistant protein (*MDR3/ABCB4*), recently reported by Rosmorduc (27), Jaquemin (28) and Shoda (29, 50), and of the mutations in the gene of cholesterol 7 a hydroxilase (*CYP7A1*) reported by Pullinger (51), no single genetic mutation or polymorphism has been found in association with cholesterol gallstone disease in humans. The mutations in *ABCB4 (MDR3)* are associated with a particular type of cholelithiasis, characterized by intrahepatic sludge, cholesterol gall-

stones and mild cholestasis (increased ãGT)(27, 28, 50).

Some unique genetic defects have also been found in association with pigment stones, such as changes in the CCK receptor (*CCKAR*)(52-54), mutations in the cystic fibrosis gene (*CFTR/ABCC7*) (55) or in the gene controlling the ileal transporter of bile acids (*IBAT/SLC10A2*) (56, 57). ❑

GALLSTONE *(LITH)* GENES IDENTIFIED IN MICE

Until now, at least 20 *Lith* genes have been identified in the experimental model, the best characterized being *Lith1* and *Lith2* (Fig.1. The murine gallstone map). The congenic strains of mice for *Lith1* and *Lith2* develop cholesterol gallstones when fed a lithogenic diet. *Abcb11*, the gene encoding BSEP on the canalicular membrane of the hepatocyte co-localizes with *Lith*1. The congenic strains of mice for *Lith2* have an increased secretion of organic anions because of overexpression of MRP2, encoded by *Abcc2*, which co-localizes with *Lith2*. The gene which encodes the basolateral transport of organic cations *Slc22a1* co-localizes with *Lith3* on mouse chromosome 17 (58). Recently, new QTLs have been identified (by linkage analysis) in mice and named *Lith 4, Lith 5, Lith 10, Lith 6, Lith* (7, 22, 59). The genes encoding *Abcg5-Abcg8* co-localize with *Lith 9* and are located on chromosome 2 (60). The putative list of *Lith* genes remains open. The relevance of the *Lith* genes for human cholelithiasis is under intense research, by means of linkage analysis and association studies in symptomatic and asymptomatic carriers of gallstones. ❑

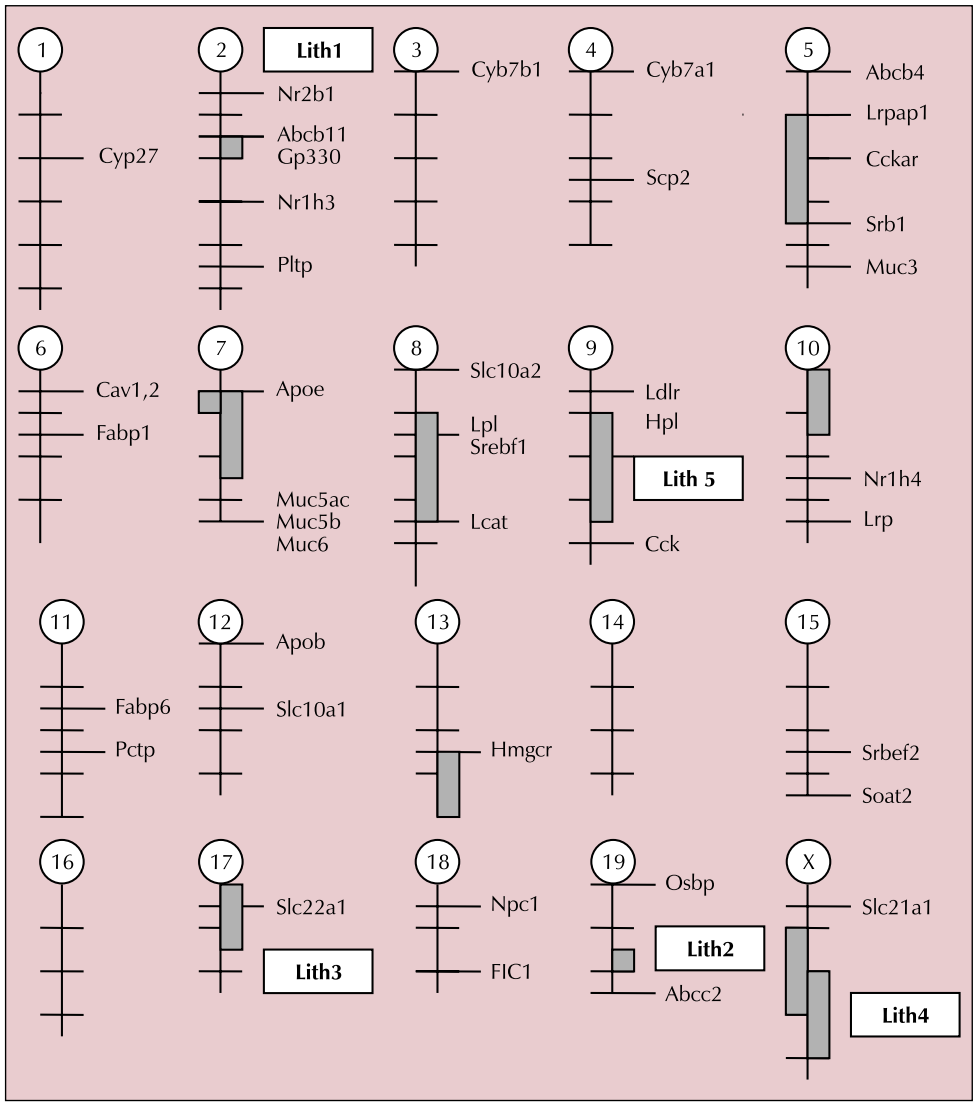

FIGURE 1. The murine gallstone map, containing the candidate genes and QTLs for cholesterol gallstone formation on chromosomes representing the entire mouse genome. Modified from Gastroenterology, vol. 120, Lammert F, Carey MC, Paigen B. Chromosomal organization of candidate genes involved in cholesterol gallstone formation: A murine gallstone map, p.221-238, 2001 (with permission from the American Gastroenterological Association).

Legend: QTLs for gallstones are represented as gray bars.

CONCLUSION

The individual predisposition to cholesterol gallstone disease in the presence of environmental risk factors could be determined by a complex genetic basis. It is probable that a significant proportion of individuals have a genetic predisposition to develop cholesterol gallstones. It is also possible that other individuals have susceptibility alleles for developing obesity and type 2 diabetes mellitus, diseases with a high risk of cholesterol gallstone formation.

By evaluating gene polymorphisms or haplotypes, individual prognostic factors might be identified in subjects with "healthy" phenotypes. Screening of the LITH genes in obese patients or in patients with other metabolic diseases could become important especially when the associated diseases have an increased surgical risk. Elucidation of the molecular genetics of cholesterol gallstone disease might also offer new possibilities of gallstone prevention or therapy. ❑

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