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Human Pedigree-Based Quantitative-Trait–Locus Mapping: Localization of Two Genes Influencing HDL-Cholesterol Metabolism

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Summary

Common disorders with genetic susceptibilities involve the action of multiple genes interacting with each other and with environmental factors, making it difficult to localize the specific genetic loci responsible. An important route to the disentangling of this complex inheritance is through the study of normal physiological variation in quantitative risk factors that may underlie liability to disease. We present an analysis of HDL-cholesterol (HDL-C), which is inversely correlated with risk of heart disease. A variety of HDL subphenotypes were analyzed, including HDL particle–size classes and the concentrations and proportions of esterified and unesterified HDL-C. Results of a complete genomic screen in large, randomly ascertained pedigrees implicated two loci, one on chromosome 8 and the other on chromosome 15, that influence a component of HDL-C—namely, unesterified HDL_{2a}-C. Multivariate analyses of multiple HDL phenotypes and simultaneous multilocus analysis of the quantitative-trait loci identified permit further characterization of the genetic effects on HDL-C. These analyses suggest that the action of the chromosome 8 locus is specific to unesterified cholesterol levels, whereas the chromosome 15 locus appears to influence both HDL-C concentration and distribution of cholesterol among HDL particle sizes.

Introduction

Genetic analysis of complex traits, which are influenced by multiple genes and their interactions with each other and with environmental factors, has become a major focus of biomedical research during the past decade. Although such studies are yet in their infancy, pessimism has already arisen, and it has been suggested that linkage methods lack power to detect the quantitative-trait loci (QTLs) contributing to risk for complex diseases (Risch and Merikangas 1996). The assessments of the relative lack of power of linkage methods, which sparked this debate, have been based on the assumption of a sibling pair– or nuclear family-based sample ascertained through one or more affected family members. Until recently, linkage-analysis methods that do not require assumptions about the underlying model of inheritance—which, by definition, is difficult to specify for complex phenotypes—were indeed limited to relative pair– and small pedigree–based samples; however, the variance-component linkage method has recently been extended to accommodate pedigrees of arbitrary size and complexity (Almasy and Blangero 1998), and thus linkage studies of complex traits are no longer bound by these methodological limitations.

The pedigree-based quantitative-trait linkage design is applicable to a wide variety of complex phenotypes. Many common complex diseases have known quantitative risk factors or are even diagnosed through quantitative clinical measures. Non–insulin-dependent diabetes mellitus is diagnosed on the basis of glucose and insulin levels. Obesity, a risk factor for many complex diseases and a major health problem in its own right, is often defined by an arbitrary dichotomization of the quantitative phenotypes of body weight or body-mass index. Risk of coronary heart disease is positively correlated with serum levels of LDL-cholesterol and is inversely correlated with HDL-cholesterol (HDL-C) (Miller and Miller 1975; Rhoads et al. 1976; Gordon et al. 1977, 1989; Manninen et al. 1988). Such quantitative risk factors are important not only for the defining of disease in affected individuals but also as indicators of disease susceptibility in the general population.

Identification of genes that contribute to such quantitative risk factors for common complex diseases is the goal of the San Antonio Family Heart Study (Mitchell

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et al. 1996). Forty-one randomly ascertained Mexican American families have been phenotyped for a wide variety of quantitative measures related to heart disease, diabetes, and obesity, all of which are highly prevalent in this population. Members of 10 of the larger families have been genotyped for a complete genomic screen. Here we apply pedigree-based quantitative-trait linkage analysis to a suite of HDL-C–related risk factors for atherosclerosis, all of which have been measured in these family members. These phenotypes include (1) plasma levels of HDL-C, (2) the proportions of HDL-C that are esterified (HDL-EC) and unesterified (HDL-UC), and (3) the distribution of cholesterol among various HDL particle sizes. Multivariate analyses of these HDL phenotypes, as well as simultaneous multilocus analysis of the QTLs identified, yield more information about genetic effects on HDL-C than does analysis of HDL-C alone, and they demonstrate the value that quantitative-trait linkage analysis has for the understanding of the genetic architecture of constellations of traits that contribute to disease susceptibility.

Genetic Effects on HDL-C

Plasma levels of HDL-C, like many quantitative risk factors for complex disease, have been extensively characterized through epidemiological, family, and association studies, which have demonstrated a strong genetic component and have provided clues about which subphenotypes may be most closely related to risk for disease. Heritabilities of total HDL-C have been estimated to be .20–.61 (Rao et al. 1982; Whitfield and Martin 1983; Namboodiri et al. 1985; Hamsten et al. 1986; Austin et al. 1987; Bucher et al. 1988; O'Connell et al. 1988; Hunt et al. 1989; Rice et al. 1991; Heller et al. 1993; Mahaney et al. 1995; Mitchell et al. 1996; Knoblauch et al. 1997).

Segregation analyses in randomly ascertained samples suggest the presence of a major gene for HDL-C levels (Friedlander et al. 1986; Hasstedt et al. 1986; Mahaney et al. 1995); however, recent studies investigating the roles of candidate genes—such as those for angiotensin-converting enzyme, hepatic lipase, lipoprotein lipase, cholesterol ester–transfer protein, and apolipoproteins AI, AII, CIII, and B—in the control of HDL-C levels have produced mixed results, with some studies finding effects of these genes on HDL-C and others either failing to find associations with the same polymorphisms or excluding linkage of HDL-C levels to these regions (Amos et al. 1987; Cohen et al. 1994; Gerdes et al. 1995; Jemaa et al. 1995; Mahaney et al. 1995; Mattu et al. 1995; Minnich et al. 1995; Turner et al. 1995; Dupuy-Gorce et al. 1996; Kamboh et al. 1996; McPherson et al. 1996; Guerra et al. 1997; Knoblauch et al. 1997; Bruce et al. 1998; Devlin et al. 1998; Kastelein et al. 1998). Since HDL-C levels are likely to be controlled by the actions of several genes, a number of these candidate loci may yet be shown to be involved in HDL-C regulation; however, none of them has emerged as a clear contender for the major gene for HDL-C levels that has been predicted by segregation analyses. It may be that the candidate polymorphisms currently being studied are not themselves functional but show an imperfect association with HDL-C levels because they are in linkage disequilibrium with functional polymorphisms. Another alternative is that the major genetic determinants of HDL-C levels are unknown loci yet to be identified. Both of these possibilities are best explored through linkage analyses with highly polymorphic markers, a method that is not dependent on either specification of known candidates or linkage disequilibrium between markers and functional polymorphisms. The present study is the first to report the results of a complete genome screen for novel loci influencing not only plasma levels of HDL-C but also quantitative measures of HDL-C components.

Components of HDL-C

Complicating the search for genes influencing HDL-C levels is the fact that this is a complex and compound phenotype. Moreover, total HDL-C may not be the best indicator of risk for atherosclerosis or other disease. HDL particles differ in size and density, depending on lipid and protein composition. HDL particles are thought to play a key antiatherogenic role in a pathway termed "reverse cholesterol transport": small dense HDL_3 particles acquire cholesterol from peripheral tissues, increasing their size and decreasing their density. Surface HDL_3 cholesterol is then esterified by the enzyme lecithin:cholesterol acyl transferase (LCAT), to produce the larger, less-dense HDL_2 particles. EC on HDL_2 particles is transferred to LDLs and very-low-density lipoproteins, via cholesterol ester–transfer protein, for removal by the liver. Although cholesterol esterification is known to be related to particle size and density, little is known about the potential relationship between it and disease risk.

Although some studies have found that HDL particle–size subfractions have no additional clinical predictive value over that provided by total HDL-C (Sweetnam et al. 1994; Wilson 1995), there is evidence to suggest that HDL_2 and HDL_3 may contribute differently to the risk of atherosclerosis (Jiang et al. 1995; Mowat et al. 1997; Barbagallo et al. 1998). One study found that men with atherosclerosis of the lower limbs had reduced HDL_{2b} and increased HDL_{3c} levels, compared with healthy controls (Mowat et al. 1997). It was also shown, in patients undergoing coronary angiography, that narrowing of the coronary artery was more highly correlated with HDL_2-C than with either total cholesterol or

HDL-C (Jiang et al. 1995). To pursue these potential functional differences within the broader HDL-C phenotype, we have differentiated HDL-EC and HDL-UC and have analyzed a variety of HDL particle sizes. In this report, we analyze the concentration of HDL-UC (expressed in mg/dl) in HDL size classes 2b, 2a, 3a, 3b, and 3c. We also consider the distribution of cholesterol among HDL size classes, divorced from the issue of absolute HDL-UC concentration. For distribution-based analyses, we utilize the proportion of the total HDL-UC present in each particle size (i.e., a series of fractions totaling one). We use such a wide variety of phenotypic measures, as well as total HDL-C and HDL-EC, to aid in the further characterization of the inferred function of QTLs identified through the genomic screen of HDL-UC concentration.

Subjects and Methods

Measurements were made in a subsample of the San Antonio Family Heart Study, consisting of 477 individuals in 10 large pedigrees, who have been genotyped for a complete genomic screen. These families were randomly ascertained, without regard to presence or absence of any disease, through 40–60-year-old Mexican American probands. All available first-, second-, and third-degree relatives of both the proband and the proband's spouse who were ⩾16 years old were examined. All subjects gave informed consent, and all procedures were reviewed by the institutional review board.

The families included in this analysis comprised 35–71 examined individuals (mean 48) and included approximately equal numbers of males and females. Each participant was given a physical exam, and blood was drawn for phenotype and genotype determination. Information on demographic, socioeconomic, and lifestyle variables (e.g., smoking, exercise, diet, and alcohol consumption) was obtained by questionnaire and was summarized by Mitchell et al. (1996), along with mean levels and covariate effects for a variety of cardiovascular risk factors. These individuals have been genotyped for a complete autosomal genomic screen consisting of highly polymorphic short tandem repeats at 304 anonymous markers spaced at ~15-cM intervals. In addition, short tandem repeats or RFLPs at 27 genes of known function were also genotyped.

Distributions of UC among HDL size fractions were determined by densitometry of filipin-stained (Lefevre 1988) HDLs resolved by nondenaturing gradient gel electrophoresis (Rainwater et al. 1992). UC profiles were decomposed, by curve-fitting procedures (Verdery et al. 1989; Rainwater et al. 1995), into five HDL size fractions (Blanche et al. 1981): HDL_{3c}, 7.2–7.8 nm; HDL_{3b}, 7.8–8.2 nm; HDL_{3a}, 8.2–8.8 nm, HDL_{2a}, 8.8–9.7 nm, and HDL_{2b}, 9.7–14.0 nm. On the basis of 77 blind duplicate samples, repeatabilities for fractional-absorbance measurements in this study were 86.5%, 90.4%, 92.8%, 76.0%, and 95.3% for HDL_{3c}, HDL3b, HDL_{3a}, HDL_{2a}, and HDL_{2b}, respectively. Fractional distributions were converted to concentrations by multiplication by HDL-UC concentrations, which were measured enzymatically. To investigate a finer scale of particle sizes, absorbance profiles were also expressed, in 0.1-nm units over the range 7.3–14.0 nm.

Quantitative-trait linkage analyses were conducted with the program SOLAR (Almasy and Blangero 1998), incorporating a simultaneous correction for environmental covariates thought to affect HDL-UC concentration. These covariates were sex, sex-specific age and age-squared, diabetic status, postmenopausal status, cigarette smoking, alcohol consumption, use of medications for diabetes, use of lipid-altering drugs, and use of exogenous sex hormones. Incorporation of environmental covariates such as these improves our ability to detect linkage, by both reducing the unexplained residual variance in HDL-UC levels and increasing the relative genetic signal. Only 10 individuals were using lipid-altering medications at the time of assay, and exclusion of these subjects from the analyses does not materially alter the results.

Results

Heritabilities and Genetic Correlations

Although previous studies have shown strong heritabilities for total HDL-C, the genetic contribution to HDL subphenotypes—including HDL-UC and particle size–specific phenotypes—has received less attention. Heritabilities for concentration of HDL-UC in the five particle-size classes, shown in table 1, indicate that there are strong genetic components for most of these traits.

Because we are studying normal variation in multiple related risk factors by using a single sample, we are able to address questions about whether these phenotypes are influenced by overlapping sets of QTLs or by QTLs unique to each phenotype. The concentrations of UC in the various size fractions are correlated with one another. Through bivariate variance-component analysis of pairs

Table 1

Heritabilities for Concentrations of UC in Five HDL Size Fractions

HDL-UC Component	Heritability ± Standard Error	*P*
2b	.55 ± .11	$< 5 \times 10^{-7}$
2a	.62 ± .09	$< 5 \times 10^{-7}$
3a	.18 ± .08	.005
3b	.26 ± .09	1.5×10^{-5}
3c	.64 ± .11	$< 5 \times 10^{-7}$

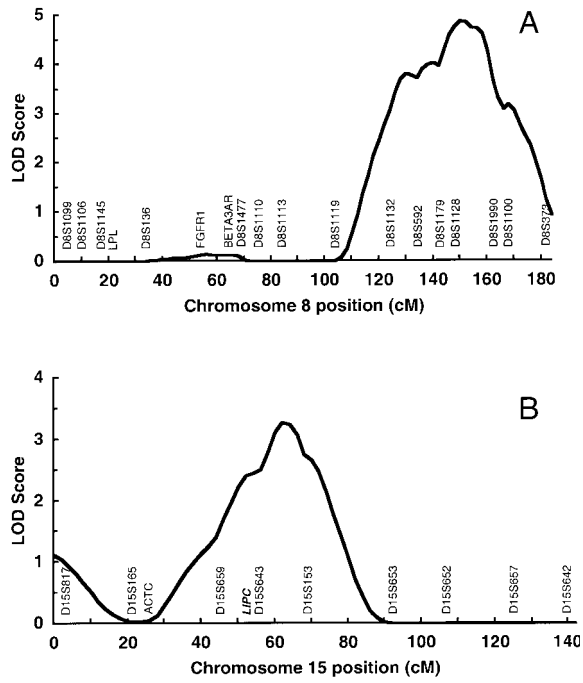

Figure 1 Multipoint linkage analysis of HDL_{2a}-UC on chromosomes 8 (*A*) and 15 (*B*).

of traits, it is possible to decompose these phenotypic correlations into genetic and environmental components, to determine whether the correlations between phenotypes are caused by common genetic influences (i.e., pleiotropy) (Lange and Boehnke 1983; Almasy et al. 1997) or by common environmental influences such as diet. If the traits are genetically correlated, it is also possible to determine whether they share all or only some of their genes in common, by testing whether the genetic correlations differ significantly from 1 (or, for negatively correlated traits, −1).

Concentrations of UC in HDL_{2b} and HDL_{2a} were positively genetically correlated ($\rho_g = .71 \pm .10$; $P < .001$), as were HDL_{3b} and HDL_{3c} ($\rho_g = .86 \pm .09$; $P < .001$). These two groups of traits were negatively genetically correlated with each other ($\rho_g = -.38$ to $-.76$). This pattern of correlations indicates shared genetic influences among the 2b, 2a, 3b, and 3c size fractions; however, all of the correlations were significantly different from 1 (or −1), providing evidence for QTLs unique to each trait. HDL_{3a}-UC did not show significant genetic correlations with any of the other fractions. This may be due, in part, to the low genetic signal (heritability) for HDL_{3a}-UC.

Linkage Analysis

Multipoint analyses were performed across all 22 autosomes, for UC concentration in all five HDL size classes. Only two LOD scores, both for HDL_{2a}-UC, were >3.0. On chromosome 8q, the LOD score for HDL_{2a}-UC reached a multipoint peak of 4.87 (fig. 1*A*; $P = 1 \times 10^{-6}$); on chromosome 15, a peak LOD score of 3.26 (fig. 1*B*; $P = 5.4 \times 10^{-5}$) was detected. HDL_{2b}-UC showed weaker evidence of linkage to this same region of chromosome 15, with a peak LOD score of 2.54 ($P = 3.2 \times 10^{-4}$). Suggestive LOD scores, those in the range of 2–3, were also observed on chromosomes 2, 4, 5, 8p, and 12 (table 2). With the exception of that for the hepatic lipase locus on chromosome 15, no LOD scores >1.0 were observed near the HDL-related candidate genes implicated in previous association studies (i.e., LCAT, ACE, CETP, LPL, APOAI, APOAII, APOAIV, APOCIII, and APOB).

Table 2

Results of Linkage Screening of HDL-UC Concentration

Chromosome	Location (cM)	LOD Score[a]	HDL-UC Component	Nearest Marker
2	140	2.28	2a	D2S1790
4	78	2.61	3a	D4S3248
5	186	2.81	2b	D5S1456
8	68	2.07	2b	D8S1477
8	150	4.87	2a	D8S1128
12	96	2.13	2a	PAH
15	62	3.26	2a	D15S643
15	80	2.54	2b	D15S653

[a] All values ⩾2.0 are shown.

When multiple linkage peaks are observed, as in the present case, variance-component linkage analysis is easily expanded to oligogenic models that incorporate two or more candidate loci simultaneously (Blangero and Almasy 1997). Such analyses may provide more-accurate estimates of the relative effect of each locus and may help to narrow the respective candidate regions. The joint two-locus LOD score involves the simultaneous estimation of the effects of both loci and thus has additional degrees of freedom (df), compared with the traditional, single-locus LOD score. The *P* value for a joint two-locus LOD score is still, however, a mixture of χ^2 distributions; specifically, it is distributed as one-quarter of a χ^2 variable with 2 df, one-half of a χ^2 variable with 1 df, and one-quarter of a point mass at 0. In a joint linkage analysis considering the chromosome 8 and chromosome 15 loci for HDL_{2a}-UC, the peak two-locus LOD score was 7.33 ($P < 10^{-8}$).

Figure 2 shows the LOD scores from a joint search of both chromosomal regions. The ring of LOD scores of 6.5 represents an ~95% confidence interval around the joint two-locus peak, which was shifted slightly from the separate single-locus peaks. Whereas the value for the single-locus analysis of chromosome 15 peaked at 62 cM, the joint analysis with chromosome 8 shifted the chromosome 15 peak to 64 cM. The chromosome 8 and

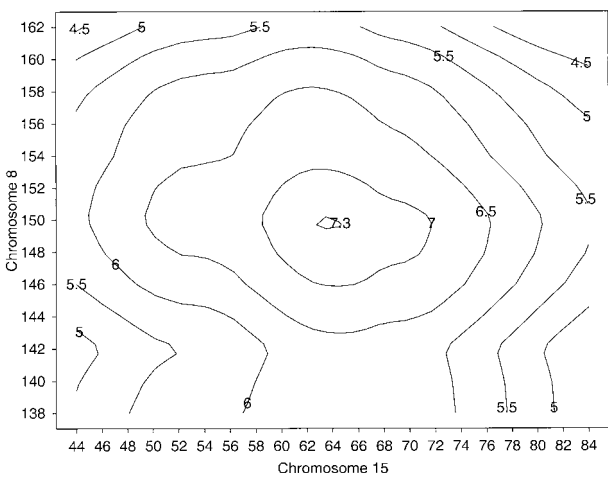

Figure 2 Joint two-locus LOD scores for HDL_{2a}-UC on chromosomes 8 and 15.

chromosome 15 QTLs accounted for 35% and 30%, respectively, of the residual variation in HDL_{2a}-UC concentration, after correction for measured covariates.

To investigate the specificity of the chromosome 8 and chromosome 15 signals for the HDL_{2a} size class, linkage analyses were performed at the location of the chromosome 8 and chromosome 15 multipoint peaks for concentration of HDL-UC, expressed in terms of particle-size classes based on 0.1-nm increments. The linkage signal on chromosome 8 seems to be confined to particles in the HDL_{2a} size region (fig. 3*A*). In contrast, the QTL on chromosome 15 influences variation in a broader spectrum of HDLs (fig. 3*B*). The LOD score at 10.6 nm (in the HDL_{2b} size region) was 3.03 ($P = 9.4 \times 10^{-5}$) whereas the LOD score for the HDL_{2b}-UC size class considered as a whole was only 2.54.

Until this point, we have been discussing the concentration of UC in various HDL particle–size classes. Another interesting parameterization of the HDL-UC phenotype is the distribution, independent of concentration, of UC among HDL particle sizes; that is, one can analyze the proportion of the total HDL-UC that is carried in each size class, rather than the absolute concentration. If HDL_2 and HDL_3 differ in their predictive value, as has been suggested by a number of studies, it may be that the partitioning of HDL-C between subfractions is as important a risk factor as HDL-C concentration.

The proportion of UC in the 0.10-nm size fractions was analyzed for the regions on chromosome 8 and chromosome 15 that were identified through HDL_{2a}-UC concentration. For the distribution-based phenotypes, the LOD scores on chromosome 8 dropped to <1.0, even for HDL_{2a} (data not shown). This result suggests that the action of the chromosome 8 locus is dependent on HDL-UC *concentration*. An analysis of HDL-UC distribution in the chromosome 15 region, however, showed that this locus affects *distribution* of UC among HDL size classes, independent of UC concentration (fig. 3*C*). The LOD scores in the HDL_{2a} size class were much reduced when distribution of UC was considered; however, the suggestive LOD scores previously seen for UC concentration in the HDL_{3b} and HDL_{2b} size classes (fig. 3*B*) increased to 4.57 at 8.1 nm ($P = 2 \times 10^{-6}$) and 4.32 at 10.7 nm ($P = 4 \times 10^{-6}$), for UC distribution (fig. 3*C*).

Although it would seem logically parsimonious to assume that the proportions of UC in HDL_{2b} and HDL_{3b} and the concentration of UC in HDL_{2a} all show linkage to chromosome 15 because they are influenced by a single QTL, it is possible that there are actually two or more genes within the region, each controlling different traits. The presence of a number of related genes within a single chromosomal region might arise through duplication of an original ancestral gene, followed by mutation and functional divergence of the copies. An example of this would be the apolipoprotein E/CI/CII/CIV gene cluster on chromosome 19. The competing hypotheses—that is, that a single gene influences multiple

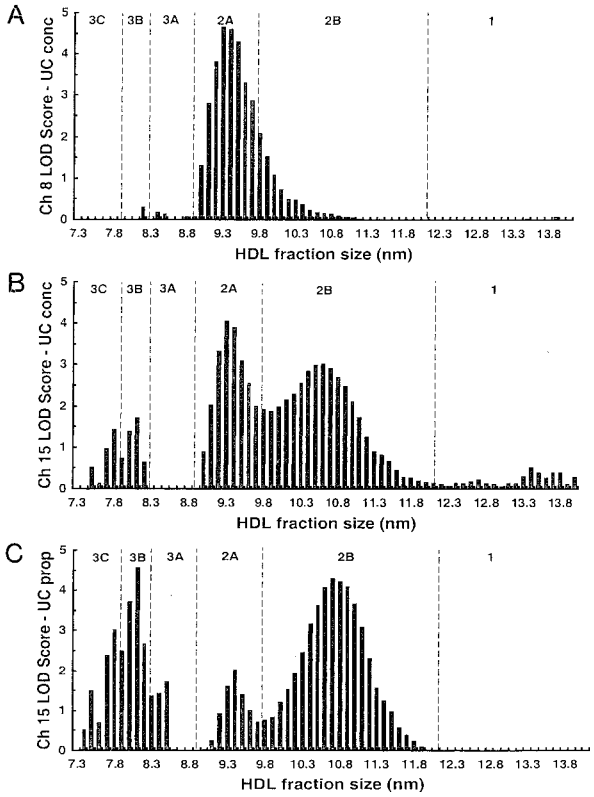

Figure 3 LOD scores for concentration of UC, in 0.1-nm HDL particle–size increments, at multipoint HDL_{2a}-UC linkage peaks on chromosomes 8 (*A*) and 15 (*B*) and for proportion of UC, in 0.1-nm HDL particle–size increments, at the same chromosome 15 location (*C*).

traits or that there are several related genes within a chromosomal region—can be formally tested by means of the same types of bivariate genetic analyses that have been used to address the more general question of genetic correlation between traits (Almasy et al. 1997). If two traits have a locus-specific genetic correlation of 0, then they are influenced by separate genes, and we can reject the pleiotropy hypothesis. On the other hand, if their locus-specific genetic correlation is significantly different from 0, we can reject the multiple-independent-genes hypothesis. *P* values for the genetic correlations are calculated by comparison of the likelihood of a model in which the correlation is estimated and the likelihood of a model in which the correlation is constrained to be 0. The difference in $\log_e$ likelihood between these two models is distributed as a χ^2 variable with 1 df. The $\log_e$-likelihood difference between the estimated correlation and a correlation of 1 or -1 (representing complete pleiotropy) is, however, distributed as a mixture of one-half of a χ^2 variable with 1 df and one-half of a point mass at 0, because of the fixation of the nested parameter on a boundary.

The locus-specific genetic correlations at the chromosome 15 region were $-.82$, for concentration of UC in HDL_{2a} and proportion of UC in HDL_{3b}, and $-.90$, for proportions of UC in HDL_{2b} and HDL_{3b}. Both correlations were significantly different from 0 ($P = .0003$ and $P = .0007$, respectively) and were not significantly different from 1 or -1, supporting the hypothesis of a single locus with pleiotropic effects on both HDL-UC concentration and its distribution among HDL size classes.

Discussion

There are no known lipoprotein-related genes in the region of the HDL_{2a}-UC linkage signal on chromosome 8. In contrast, the linkage signals on chromosome 15 occur near the hepatic lipase locus, which codes for a relatively well-characterized enzyme with a known role in lipoprotein metabolism. Since hepatic lipase is thought to catalyze the conversion of HDL_2 to HDL_3 via lipolysis of triglycerides (Silverman et al. 1993), it is not unexpected that it should be an important determinant of distribution of HDL-C among HDL particle–size classes. Other linkage and association studies have also found evidence that the hepatic lipase locus influences plasma HDL-C levels (Cohen et al. 1994; Guerra et al. 1997). Our results, however, raise an interesting question. Why do we see this signal with UC specifically? We have analyzed linkage to the chromosome 15 region, both by means of total HDL-C concentration and by means of concentration and distribution of EC (by use of Sudan-black B stain), in the various HDL particle–size classes. Although our linkage signals are strongest with UC, we do see suggestive signals for other HDL-C traits at the markers that flank our HDL-UC linkage peaks, D15S643 and D15S153. Total HDL-C gave a LOD score of 1.69 at D15S643, and somewhat stronger—although still only suggestive—LOD scores were seen for the proportion of EC in HDL_{3b} and in HDL_{2b}, at D15S153 (LOD scores were 2.35 and 2.27, respectively). Although the decomposition of HDL-C into esterified and unesterified components was crucial to our detection of this linkage, we believe that the chromosome 15 locus has a more general effect on HDL metabolism and is not specific to HDL-UC.

The analysis that we have presented here has several unique aspects. Although quantitative risk factors—such as HDL-C—are commonly studied, they are usually addressed in small sampling units consisting of sib pairs or nuclear families and are often treated in a dichotomous manner. Simulation studies have shown that direct analyses of a quantitative trait are more powerful than analyses of dichotomizations of that trait (Duggirala et al. 1997). Additionally, both simulations and theoretical power calculations demonstrate that large pedigrees provide more power for quantitative-trait analyses than do either nuclear families or sib pairs (Wijsman and Amos 1997). We have also explored a variety of related subphenotypes—some of which are quite uncommon, such as EC and UC. Studying a wide range of phenotypes has allowed us to begin to characterize the genetic relationships among the traits and to formulate specific hypotheses of pleiotropy for the QTLs detected in the linkage screen, providing some potential clues to gene function. Finally, we performed a joint linkage analysis of the chromosome 8 and chromosome 15 QTLs simultaneously, confirming that each locus has independent explanatory power and that the multilocus model fits the data better than does either single-locus model.

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