J Hum Genet (1998) 43:153–159 © Jpn Soc Hum Genet and Springer-Verlag 1998

ORIGINAL ARTICLE

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Association and linkage of *LDLR* gene variation with variation in plasma low density lipoprotein cholesterol

Received: March 13, 1998 / Accepted: May 11, 1998

Abstract The role of common variation in the low density lipoprotein (LDL) receptor gene (*LDLR*) as a determinant of variation in plasma LDL cholesterol in normolipidemic populations is not well established. To address this question, we used both association and linkage analysis to evaluate the relationship between plasma LDL cholesterol and genetic variation in *LDLR* and in three other candidate genes for lipoprotein metabolism, namely, *APOE*, *PON1*, and *LPL*. We studied a sample of 719 normolipidemic Alberta Hutterites, who comprised 1217 sib pairs. Variation in each of the four candidate genes was significantly associated with variation in plasma LDL cholesterol, but the average effects of the alleles were small. In contrast, sib pair analysis showed that only the *LDLR* gene variation was linked with variation in plasma LDL cholesterol ($P = 0.026$). Thus, the common *LDLR* gene variation was both associated with and linked to variation in plasma LDL cholesterol, suggesting that there is a functional impact of structural variation in *LDLR* on plasma LDL cholesterol in this study sample. However, the absence of linkage of variation in LDL cholesterol with the other three candidate genes, in particular *APOE*, is consistent with a lower sensitivity of linkage analysis compared with association analysis for detecting modest effects on quantitative traits. Attributes such as the genetic structure of the study sample, the amount of variance attributable to the locus, and the information content of the marker appear to affect the ability to detect genotype-phenotype relationships using linkage analysis.

Key words Atherosclerosis · Intermediate trait · Complex disease · Small genetic effects · Lipids

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Introduction

Most individuals who suffer myocardial infarction have plasma lipoprotein concentrations within the reference range for the normal population (Sacks et al. 1996). Thus, it remains a priority to identify the genetic determinants of variation of serum lipoproteins within the normolipidemic range. The low density lipoprotein (LDL) receptor is one of several gene products that play an integral role in plasma lipoprotein metabolism (Dammerman and Breslow 1995). The LDL receptor gene (*LDLR*) is approximately 45 kb in length, consists of 18 exons and 17 introns, and has been mapped to chromosome 19p13.2 (Südhof et al. 1985). Mutations in the *LDLR* gene, of which over 150 have been described (Hobbs et al. 1992), underlie familial hypercholesterolemia (FH). FH heterozygotes have markedly elevated total and LDL cholesterol and frequently develop early coronary heart disease (CHD). However, the heterozygous FH phenotype is relatively rare, with a frequency of about 1 in 500 Caucasians (Hobbs et al. 1992). Less well established is the possible importance of common *LDLR* variation as a determinant of plasma LDL metabolism, particularly within the normolipidemic range.

There have been some reported associations between *LDLR* gene variants and plasma lipoprotein variation. Disparate results have been found when *LDLR* gene markers in exon 8, exon 13, intron 15, and exon 18 were tested for

associations with plasma LDL cholesterol concentrations in several European population samples (Pedersen and Berg 1988; Taylor et al. 1988; Schuster et al. 1990; Humphries et al. 1991; Poledne et al. 1993; Klausen et al. 1993; Wiseman et al. 1993; Ahn et al. 1993; Gudnason et al. 1995). Some of these inconsistencies might have been due to the limitations of association studies in heterogeneous populations (Lander and Schork 1994). These limitations might be overcome, in part, by studying the genetic determinants of plasma lipoproteins in samples from genetically isolated populations, such as the Alberta Hutterites (Hegele et al. 1994, 1995). The Alberta Hutterites have proven to be a useful population for the study of genetic determinants of plasma lipoproteins because their genetic background is more homogeneous, their indices of relatedness are higher, and they share more environmental factors than non-isolated, outbred populations (Hegele et al. 1995).

In a sample of 793 Alberta Hutterites, we previously showed that common variation in several candidate genes, including *LDLR*, was associated with a modest degree of variation in plasma LDL cholesterol (Hegele et al. 1995). We were interested to determine whether quantitative trait linkage analysis would also demonstrate a significant relationship between candidate genes in lipoprotein metabolism, particularly *LDLR*, and plasma LDL cholesterol in these subjects. We report a comparison of the results of two analytic strategies, association analysis and linkage analysis, to define the genetic determinants of plasma LDL cholesterol and apolipoprotein (apo) B concentrations in a sample of 719 Alberta Hutterites.

Methods

Subjects

Twenty-one Alberta Hutterite colonies, representing two different leute, or sects, the Dariusleut and the Lerherleut, participated in the Canadian Heart Health Survey for CHD risk factors (MacLean et al. 1992). Medical histories were taken, physical examinations were performed, and anthropometric variables were measured in all individuals. Fasting blood samples for biochemical and genetic analyses were obtained as previously described (Hegele et al. 1995). The study was approved by the ethical review panels of the Universities of Alberta and Toronto.

Biochemical analysis

All biochemical determinations were made in the J. Alick Little Lipid Research laboratory at St. Michael's Hospital. Plasma concentrations of lipids and lipoproteins were determined as described (Hegele et al. 1994, 1995). Plasma concentrations of apo B were determined by nephelometry (Hegele et al. 1995).

Genetic analysis

Four candidate genes were chosen for the comparison of the results of the association and linkage analyses. The *LDLR* gene encodes the LDL receptor and *Hinc*II detects a bi-allelic restriction fragment length polymorphism (RFLP) in exon 12 due to a silent C1773T substitution at codon 570 (Leitersdorf and Hobbs 1988). The *LPL* gene encodes lipoprotein lipase (LPL) and *Pvu* II detects a bi-allelic RFLP in intron 6 (Johnson et al. 1991). The *APOE* gene encodes apo E, and restriction isotyping with *Hha*I detects arginine-to-cysteine substitutions at residues 112 and 158, which underlie the three common isoforms of apo E, namely, E4, E3, and E2 (Hixson and Vernier 1990). The *PON1* gene encodes serum paraoxonase and *Alw*I detects the R192Q amino acid polymorphism (Humbert et al. 1993), which may be the basis for the interindividual differences in serum paraoxonase activity, but which is also in linkage disequilibrium with another functional variant at codon 55 (Blatter-Garin et al. 1997).

Statistical analysis

We previously reported an association analysis of a slightly larger sample of 793 Alberta Hutterites in which we found significant associations with eight candidate genes and several lipoprotein traits (Hegele et al. 1995). A subsample of 719 of these subjects could be placed into defined sibships representing 187 nuclear families: the features of the sibships are documented in Table 1. For comparison, this subset of 719 individuals was analyzed with both association and linkage analysis, using the quantitative variables LDL cholesterol and apo B. Association analysis consisted of multivariate analysis of variance (ANOVA) with genotypes as independent variables. Sib-pair analysis involved regression of squared differences between siblings of LDL and apo B on estimated identical-by-descent (IBD) alleles (Haseman and Elston 1972).

Allele frequencies of each gene were estimated by gene counting, and deviations from expected Hardy-Weinberg equilibrium were determined by χ^2 analysis. Since LDL cholesterol and apo B were not normally distributed in this sample, both were transformed using the natural logarithm ($\log_e$) to create quantitative variables whose distributions were not significantly different from normal (data not shown).

ANOVA with a general linear model from SAS (version 6.11; SAS Institute 1995) was used to determine the sources of variation for the $\log_e$ transformed biochemical traits. F-

Table 1 Features of sibships in the Alberta Hutterite sample

Total number of subjects	719	
Number of nuclear families	187	
Number (percent) with	2 Sibs:	75 (40%)
	3 Sibs:	41 (22%)
	4 Sibs:	31 (17%)
	5 Sibs:	15 (8%)
	6 Sibs:	25 (13%)
Number of full sib pairs	1217	
Number of half-sib pairs	41	
Number (percent) of sibships with parental genotype and phenotype information:	Both parents	64 (34%)
	One parent	35 (19%)
	Neither parent	88 (47%)

tests were calculated based on the type III sums of squares. Type III sums of squares account for missing values and unbalanced designs, and were used previously to determine sources of variation for quantitative traits in the Alberta Hutterites (Hegele et al. 1994, 1995). Dependent variables were $\log_e$ LDL and $\log_e$ apo B. Independent variables were genotypes of *LDLR*, *APOE*, *LPL*, and *PON1*. Covariates were age, sex, $\log_e$ of the body mass index (BMI, which was defined as weight [in kg]/height [in m^2]), and the colony of origin, which was included in the model to correct for variation that was related to other shared genetic and environmental factors. Since members of the same colony were more highly related than individuals from different colonies, including the colony term would, in part, account for non-independence between members of the same colony, although the relationships between individuals were complex (Fujiwara et al. 1989; Hegele et al. 1994, 1995).

When a significant association was identified with ANOVA, the average effects of the alleles on plasma lipoprotein traits were estimated using the following formulas (Boerwinkle et al. 1987):

$$\alpha_2 = \frac{f_{22}\bar{y}_{22} + 1/2f_{23}\bar{y}_{23} + 1/2f_{24}\bar{y}_{24}}{f_2} - \bar{y}$$

$$\alpha_3 = \frac{f_{33}\bar{y}_{33} + 1/2f_{23}\bar{y}_{23} + 1/2f_{34}\bar{y}_{34}}{f_3} - \bar{y}$$

$$\alpha_4 = \frac{f_{44}\bar{y}_{44} + 1/2f_{24}\bar{y}_{24} + 1/2f_{34}\bar{y}_{34}}{f_4} - \bar{y}$$

where α_2, α_3, α_4 are the average effects of three alleles at one locus; f_{22}, f_{23}, etc. are the expected relative genotype frequencies; $\bar{y}_{22}$, $\bar{y}_{23}$ etc. are the phenotypic means for the genotypes; and $\bar{y}$ is the grand mean of the sample.

We then tested for linkage of these four candidate genes with variation in plasma LDL cholesterol and apo B concentrations. We used the SIBPAL (version 2.8) subroutine of SAGE (version 2.2, 1994), a non-parametric method, which is a model-independent test for linkage between a set of marker loci and a putative trait locus (Haseman and Elston 1972). A negative regression slope of the squared quantitative trait differences between sib pairs on the estimated proportion of alleles that the sib pairs share identical-by-descent (IBD) at a marker locus implies genetic linkage; in the absence of linkage, the slope of the regression line does not differ significantly from zero (Haseman and Elston 1972). Pedigrees were constructed for the subset of 719 Hutterites using the family structure program, FSP subroutine of SAGE 2.2. The marker allele frequencies used in the analysis were taken from the entire Hutterite data set of 793 individuals (Hegele et al. 1995).

Results

Allele and genotype frequencies

Allele frequencies of *LDLR*, *APOE*, *LPL*, and *PON1* in the subsample of 719 subjects are shown in Table 2. The genotype frequencies did not deviate from the expectation under Hardy-Weinberg equilibrium (data not shown). As reported previously, these allele frequencies were similar to those reported in other Caucasian samples, except for the *APOE* alleles, with the allele encoding the E3 allele being more common and the alleles encoding E4 and E2 being less common in the Hutterites than in other European samples (Hegele et al. 1995).

Table 2 Candidate gene allele frequencies in Alberta Hutterites

Gene	Allele	Frequency	Average allelic effect	
			LDL-C (mmol/l)	apo B (g/l)
LDLR	1773T	0.61	+0.10	+0.03
	1773C	0.39	−0.16	−0.05
APOE	E4	0.056	+0.39	+0.12
	E3	0.940	−0.02	−0.01
	E2	0.004	−1.22	−0.32
LPL	Present (+)	0.48	−0.09	−0.03
	Absent (−)	0.52	+0.09	+0.03
PON1	Q192	0.71	−0.005	−0.01
	R192	0.29	+0.02	+0.01

LDLR, Low density lipoprotein (LDL) receptor gene; T or C, thymidine or cytidine, respectively, in exon 12, codon 570, nucleotide position 1773 of *LDLR*; *APOE*, apolipoprotein E gene; E4, E3, and E2, are the isoforms determined by restriction isotyping; *LPL*, lipoprotein lipase gene; + or −, presence or absence, respectively, of *Pvu*II restriction site in *LPL* intron 6; *PON1*, serum paraoxonase gene; Q or R, glutamine or arginine, respectively, at position 192 of serum paraoxonase; BMI, body mass index; apo B, apolipoprotein B

Association analysis

Genetic variation of each of *LDLR*, *APOE*, *LPL*, and *PON1* was significantly associated with the variation in plasma LDL cholesterol and apo B concentrations in this subsample of 719 Alberta Hutterites (Table 3). This is consistent with the results reported previously in the larger sample of 793 Hutterites (Hegele et al. 1995). Age, sex, $\log_e$ BMI, and colony of origin were each significantly associated with variation in both plasma lipoprotein traits, as reported previously (Hegele et al. 1995).

The average effects of marker alleles on plasma LDL cholesterol and apo B

The mean ± SD according to genotypes of the genes that were significantly associated with variation in the plasma lipoprotein traits are shown in Table 4. For each gene that was significantly associated with variation in a plasma lipoprotein trait, the average effect of each allele was also calculated, and these are shown in Table 2.

Linkage analysis

The same phenotypes and genotypes in this subsample of 719 Hutterites were studied using non-parametric linkage

Table 3 Comparison of levels of significance (*P*) for association and linkage analysis of candidate genes and quantitative traits in Alberta Hutterites

	$\log_e$ LDL cholesterol		$\log_e$ apo B	
Trait	Association	Linkage	Association	Linkage
Source of variation				
LDLR genotype	<0.0001	0.026	<0.0001	0.16
APOE genotype	<0.0001	0.24	0.0006	0.57
LPL genotype	0.0002	0.67	<0.0001	0.42
PON1 genotype	0.030	0.43	0.030	0.25
Sex	<0.0001	0.024	<0.0001	<0.001
Age	<0.0001	0.062	<0.0001	0.003
Log_e BMI	0.0006	0.39	<0.0001	0.63
Colony of origin	<0.0001		<0.0001	

Colony of origin was not included as a covariate in the linkage analysis
Abbreviations, As in Table 2

Table 4 Clinical and biochemical features (means ± SD) according to candidate genotypes in Alberta Hutterites

Gene	*LDLR*			*APOE*		
Genotype	1773T/T	1773T/C	1773C/C	E3/E2	E3/E3	E4/E3
Number (female/males)	128/137	181/152	63/50	5/1	330/300	37/38
Age (years)	38 ± 15	39 ± 15	36 ± 14	37 ± 16	38 ± 15	39 ± 15
BMI (kg/m^2)	28 ± 4	29 ± 5	28 ± 5	27 ± 7	28 ± 5	29 ± 6
LDL cholesterol (mmol/l)	3.41 ± 0.92	3.20 ± 0.98	2.86 ± 0.82	1.96 ± 0.61	3.18 ± 0.89	3.68 ± 0.86
apo B (g/l)	1.25 ± 0.31	1.20 ± 0.30	1.08 ± 0.27	0.86 ± 0.18	1.19 ± 0.30	1.34 ± 0.31
Gene	*LPL*			*PON1*		
Genotype	−/−	+/−	+/+	Q/Q192	Q/R192	R/R192
Number (females/males)	93/80	161/174	118/85	200/153	149/164	23/21
Age (years)	38 ± 14	37 ± 15	39 ± 16	38 ± 15	38 ± 14	37 ± 15
BMI (kg/m^2)	28 ± 4	28 ± 5	29 ± 5	28 ± 5	28 ± 5	28 ± 4
LDL cholesterol (mmol/l)	3.33 ± 0.88	3.27 ± 0.92	3.06 ± 0.88	3.18 ± 0.92	3.31 ± 0.88	3.04 ± 0.88
apo B (g/l)	1.25 ± 0.29	1.22 ± 0.31	1.14 ± 0.29	1.17 ± 0.29	1.24 ± 0.31	1.19 ± 0.34

Abbreviations: As in Tables 2 and 3

analysis. The *P* values for the lipoprotein traits in subjects classified by genotype are shown in Table 3. Age, sex, and $\log_e$ BMI were included as covariates. Siblings who shared no alleles identical-by-descent (IBD) at the *LDLR* locus had significantly greater variation in plasma LDL cholesterol, as measured by the squared trait difference, than those siblings that shared two alleles IBD at this locus ($P = 0.026$). With the Bonferonni correction, this *P* value was 0.050. *APOE*, *LPL*, and *PON1* were not linked with variation in plasma LDL-C or apo B concentrations in this subsample of 719 Hutterites.

To reduce the estimation of IBD by SIBPAL from sibships with unknown parental genotypes, a subset of the 199 sibships, in which both parental genotypes were known, was analyzed. This subset included 64 nuclear families comprising a total of 538 sib pairs. In this subset, none of the four genes was significantly linked with variation in plasma LDL cholesterol or apo B concentration (data not shown).

Discussion

We demonstrate both association and linkage between variation in plasma LDL cholesterol concentration and variation in the *LDLR* gene, as marked by a relatively informative silent bi-allelic nucleotide polymorphism in exon 12. The consistency of the results using two independent analytic strategies suggests that *LDLR* gene structural variation influences LDL receptor function and affects plasma lipoprotein concentrations in this sample of Hutterites. Since the *LDLR* nucleotide change that was used as the marker in these studies is silent at the amino acid level, there must be another functional change within or flanking the *LDLR* gene that is in linkage disequilibrium with the exon 12 variation. Furthermore, variation at the three other candidate genes studied was found to be associated with, but not linked to, variation in plasma LDL cholesterol and apo B concentrations. The average allelic effects for each candidate gene were modest for each quantitative trait. This confirms previous suggestions that, when compared with linkage analysis, association analysis may be more sensitive for identifying small allelic effects of candidate genes on variation in quantitative phenotypes (Lander and Schork 1994).

Previously reported associations of *LDLR* gene variation with plasma LDL cholesterol concentration variation in normolipidemic samples are inconsistent. For example, a common nucleotide polymorphism in *LDLR* intron 15 detected by *Pvu*II was associated with variation of plasma

LDL cholesterol in samples of Norwegian (Pedersen and Berg 1988), German (Schuster et al. 1990), Czech (Poledne et al. 1993), and Italian (Humphries et al. 1991) subjects, but not in British (Taylor et al. 1988) or Danish subjects (Klausen et al. 1993). In an Icelandic sample, a common functionally silent amino acid polymorphism (A370T) in *LDLR* exon 8 was associated with variation in plasma lipoproteins in men only (Gudnason et al. 1995). Two other common nucleotide polymorphisms in the *LDLR* gene, one in exon 13 detected by *Ava*II and another in exon 18 detected by *Nco*I, were associated with variation in plasma total and LDL cholesterol in Hispanic and non-Hispanic white women (Ahn et al. 1993).

The inconsistencies among the many association studies of *LDLR* gene variation and variation in plasma LDL cholesterol may relate to confounding attributes such as population admixture, gene-gene interactions or gene-environment interactions (Lander and Schork 1994). Presumably, such confounding factors are diminished in a genetic isolate, whose members share relatively more genetic background and environmental factors than a non-isolated population. The Alberta Hutterites have advantages for the study of genetic determinants of quantitative traits. The population is young and had a small number of founders (Fujiwara et al. 1989). Therefore, functional alleles of quantitative trait loci are likely to be lower in number than in an outbred population. Also, recessive genetic effects on phenotypes are likely to be more easily identified, since homozygotes are more likely to share functional alleles IBD than in outbred populations. Furthermore, linkage disequilibrium between a genetic marker and a functional variation is more likely to exist over longer genetic distances if the population was founded relatively recently, as were the Alberta Hutterites (Fujiwara et al. 1989). In addition, polygenic effects from the genetic background may be reduced. Finally, communal living and shared environment probably lessens the confounding effect of background environment heterogeneity.

These favorable attributes of the Alberta Hutterites allowed us to previously estimate that variation in the *LDLR* gene accounted for about 2.5% of the variation in plasma LDL cholesterol, using partial regression coefficients (Hegele et al. 1995). In the present analysis, the *LDLR* 1773T and 1773C alleles raise and lower the plasma LDL cholesterol by 0.10 and 0.16 mmol/l, respectively. The range of this variation is about 8% of the mean plasma LDL cholesterol. Therefore, using either estimate, the influence of the *LDLR* variation on plasma LDL cholesterol is small, but significant, in this study sample.

The ability to detect such small effects on a quantitative trait in an association study has been suggested to be enhanced in consanguineous populations (Génin and Clerget-Darpoux 1996a). An inbreeding coefficient of about 0.01 was suggested to be associated with an increase in the power to detect the effect of a candidate gene (Génin and Clerget-Darpoux 1996b). The high inbreeding coefficient of the current generation of the Alberta Hutterites of about 0.05 (Hegele et al. 1994, 1995), may thus partly explain the higher sensitivity of the association analysis in this sample. Furthermore, consanguinity has been suggested to increase the type I error rate in sib-pair linkage analysis, leading to falsely positive conclusions of linkage (Génin and Clerget-Darpoux 1996b). Thus, the absence of significant linkages between the candidate genes and quantitative traits studied is remarkable, given that false-positive linkages would be expected to be more likely in the Alberta Hutterites.

Other attributes of the study design could have affected the analysis and interpretation of results. Both the proportion of the variance, also called heritability, of the locus and the information content of the genetic marker are important in sib-pair linkage analysis of quantitative traits (Blackwelder and Elston 1982; Risch 1990; Risch and Zhang 1996). This may account for the presence of association but the absence of linkage between the *APOE* genotype and plasma LDL cholesterol in the Hutterites. A consensus from association studies is that *APOE* isoforms account for about 10% of the total population variance in LDL cholesterol (Walden and Hegele 1994). Thus, the *APOE* alleles can be considered to be identical-by-state (IBS) functional variants that affect LDL cholesterol across various study samples. Our results suggest that while functional IBS alleles may be relatively common in a population, they may be unsatisfactory as markers for estimating IBD for the locus for the purpose of linkage analysis. Assuming that, in the Alberta Hutterites, the heritability of LDL cholesterol that is due to *APOE* is no greater than 20%, and that the information content for *APOE* using restriction isotyping is no greater than 30%, and that it is mainly the extremely discordant pairs that contribute meaningfully to the linkage analysis, then the number of sib pairs necessary to be screened to be reasonably confident of detecting a significant dominant or additive effect of *APOE* restriction isotype on the LDL cholesterol phenotype is more than 2500 (Risch and Zhang 1996). This is more than twice as many sib pairs than we have screened. For smaller effects on phenotype and for less informative marker systems, the sample size screened must be even larger. Such large samples will likely need to be screened in spite of strategic modifications to quantitative trait linkage analysis, such as interval mapping (Cardon and Fulker 1994), applying IBD distribution rather than estimation (Kruglyak and Lander 1995; Gessler and Xu 1996), or strategies to select specific types of sib pairs (Risch and Zhang 1996; Zhao et al 1997). Our results demonstrate that, compared to association analysis, linkage analysis with IBS alleles lacks the sensitivity to detect a modest genetic effect on phenotype if the information content of the marker system is low, even when the marker is identical with the mechanistic basis for the phenotypic difference.

In summary, we have shown that a common variation of *LDLR* for which the codon 570 variation is a marker, is both associated and linked with modest but significant variation in plasma LDL cholesterol in normolipidemic subjects. The consistency of the results from two different methods suggests a functional basis for this relationship in the Alberta Hutterites. The findings also highlight the importance of the choice of both the markers and the analytic strategy to identify the genetic determinants of com-

plex disease. Many of the positive results from genetic association studies of quantitative traits are related to linkage disequilibrium with functional variants and may not be reproducible in different populations. Also, in instances of a consistent association and a plausible mechanistic basis, as in the case of *APOE*, sib-pair linkage analysis is less sensitive than association to identify a significant relationship. A functional IBS allele may be common in the population and thus an important source of variation in the trait of interest, but as a marker for estimating IBD for the gene, it may be inadequate for linkage analysis.

Acknowledgments The sib-pair linkage analysis reported herein was run using the SIBPAL 2.8 subroutine of the SAGE (Statistical Analysis for Genetic Epidemiology) 2.2 statistical software package for genetic epidemiology, from the Department of Epidemiology and Biostatistics, Case Western Reserve University.

Dr. Boright is a fellow of the Heart and Stroke Foundation of Canada. Dr. Brunt is a Career Investigator of the NHRDP. Dr. Hegele is a Career Investigator of the Heart and Stroke Foundation of Ontario. This project was supported by grants from the MRC (Canada) (No. MA-13430) to Dr. Hegele and from the Canadian Genetic Diseases Network (to Dr. Morgan).

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