Original Article

Genetic Association of Low-Density Lipoprotein Receptor-Related Protein 2 (*LRP2*) with Plasma Lipid Levels

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Aim: Not all genetic factors predisposing phenotypic features of dyslipidemia have been identified. We studied the association between the low density lipoprotein-related protein 2 gene (*LRP2*) and levels of plasma total cholesterol (T-Cho) and LDL-cholesterol (LDL-C) among 352 adults in Japan.

Methods: Subjects were obtained from among participants in a cohort study that was carried out with health-check screening in an area of east-central Japan. We selected 352 individuals whose LDL-C levels were higher than 140 mg/dL from the initially screened 22,228 people. We assessed the relation between plasma cholesterol levels and single-nucleotide polymorphisms (SNPs) in the *LRP2* gene.

Results: We identified significant correlations between plasma cholesterol levels and two of 19 examined SNPs in *LRP2*, c. +193826T/C and IVS55-147A/G. In particular, the association of c. +193826T/C with the T-Cho level was prominent ($p=0.003$), showing a co-dominant effect of the minor C-allele on lowering T-Cho and LDL-C levels: for 24 homozygous C-allele carriers, T-Cho = 240.7 ± 24.2 mg/dL and LDL-C = 166.1 ± 21.0 mg/dL; for 130 heterozygous carriers, 248.5 ± 23.5 mg/dL and 166.6 ± 19.3 mg/dL; and for 196 homozygous T-allele carriers, 253.9 ± 23.5 mg/dL and 172.0 ± 21.0 mg/dL. Linkage disequilibrium (LD) analyses based on 19 selected SNPs showed that c. +193826T/C and IVS55-147A/G were in tight LD and that both were located in an LD block covering the genomic sequence from exon 55 to exon 61.

Conclusion: We confirm the association between *LRP2* and levels of T-Cho and LDL-C in human plasma. The results suggest that genetic variations in *LRP2* are important factors affecting lipoprotein phenotypes of patients with hypercholesterolemia.

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Key words; Association study, Single-nucleotide polymorphism (SNP), Linkage disequilibrium (LD), haplotype

Introduction

Hyperlipidemia has become a major health problem in many countries because of its high prevalence and

a causal relationship with serious medical conditions such as coronary heart disease (CHD) and stroke¹⁾. Common forms of hyperlipidemia arise from interactions among multiple gene products, environmental factors, and behavior. Although environmental factors are important, considerable evidence indicates that genetic factors have significant roles. Rare mutations in certain genes, for example, the coding elements for LDL receptor (*LDLR*) and apolipoprotein B (*APOB*), are responsible for certain monogenic lipoprotein dis-

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orders^{2, 3)}. However, despite recent advances toward an understanding of genetic influences that might bring about hyperlipidemia, the precise genetic etiologies of non-monogenic lipid anomalies remain obscure.

A whole-genome association study would be the best method for identifying genetic variants responsible for common diseases, in which no assumptions would be made about the genomic location of potentially causal variants. The International HapMap Project⁴⁾ defined the haplotype structure of the human genome, and eventually suggested a sufficiently comprehensive set of SNPs for whole-genome association studies; however, even with the results of this project, a minimum of 300,000-1,000,000 SNPs will have to be assessed in order to scan the entire human genome. Until that difficulty of scale can be overcome, the candidate-gene approach is a practical alternative.

LRP2 was originally identified as Megalin, a primary antigen in Heyman nephritis^{5, 6)}. This member of the LDLR-related protein (LRP) family is a multi-ligand receptor that is expressed in a number of different tissues but mainly in glomeruli and proximal tubule cells of the kidney. Members of this family are cell-surface receptors that transport macromolecules into cells. LRP2 is a glycoprotein of approximately 600 kD containing a large amino-terminal extracellular domain, a single transmembrane domain, and a short carboxy-terminal cytoplasmic tail. The extracellular domain contains four clusters (I-IV) of complement-type/LDLR class A repeats that constitute ligand-binding regions. Its ligands include apolipoproteins B and E, and lipoprotein lipase^{7, 8)}. The actual mechanisms that deliver these lipid-soluble signaling molecules to target cells have not been clarified.

In this study we found the significant association of two *LRP2* polymorphisms with age- and gender-adjusted levels of T-Chol and LDL-Chol in human plasma. We also evaluated LD and the haplotype structure within this gene. Our results indicated that certain genetic variations of the *LRP2* gene can influence lipid metabolism and give rise to dyslipidemia.

Materials and Methods

Subjects

Subjects were chosen from among participants in a cohort study that was originally carried out concurrently with health-check screening in an area of east-central Japan, as described previously in detail^{9, 10)}. From the initially screened 22,228 individuals, we selected 352 whose LDL-C concentrations were higher than 140 mg/dL, following the multi-step selection described previously¹⁰⁾. None of the selected participants

had medical complications or was undergoing treatment for conditions known to affect plasma lipoprotein levels, such as pituitary disease, hypo- or hyperthyroidism, diabetes mellitus, liver disease, or renal disease. None was receiving anti-hyperlipidemic therapy. All provided written informed consent prior to the study, which was approved by the Institutional Review Boards of the Research Consortium.

Physical and clinical profiles of these subjects, i.e., age, gender, body-mass index, and plasma lipid levels, were described in our previous report¹⁰⁾. Those data were obtained through a special outpatient clinic for dyslipidemic patients at the University of Chiba, where lipid levels were measured in plasma collected from each participant after 12-16 hours of fasting, as previously described^{10, 11)}. Genomic DNA was extracted from peripheral blood samples as previously described⁹⁾.

SNP Selection and Genotyping

We extracted 19 SNPs covering the entire *LRP2* gene from the dbSNP database of the NCBI (<http://www.ncbi.nlm.nih.gov/SNP/>) and from the SNP Browser Software of Applied Biosystems (<http://www.appliedbiosystems.com/>). Genotyping of five of those variations (c.+193826T/C, c.+119436A/G, c.+103321A/G, S83N, c.-972G/A) was performed by Invader assay (Third Wave Technologies, Madison, WI) according to the manufacturer's protocol^{12, 13)}. Genotypes for the other 14 selected SNPs were determined by TaqMan[®] Assays, using specifically designed probes, primers, and reagents of TaqMan[®] Assays-on-Demand[™] (Applied Biosystems, Foster City, CA). The manufacturer provided designed probe sets along with the required reagents. Genotypes were determined according to the manufacturer's protocol, using the ABI prism 7900 HT (Applied Biosystems)¹⁴⁾.

Statistical Analysis

Plasma concentrations of lipids were adjusted for each patient's age and gender, using standard data obtained from 11,994 individuals in a 2001 cohort study of the general Japanese population. Coefficients of skewness and kurtosis were calculated to test deviation from a normal distribution. Because clinical and biochemical traits in each genotypic group did not always distribute normally, we applied a nonparametric Mann-Whitney test or analysis of variance (ANOVA) with linear regression analysis as a post hoc test ($p < 0.05$) to compare those traits among groups divided by a SNP¹⁵⁾. Chi-square test was used to confirm Hardy-Weinberg equilibrium among genotypes ($p < 0.05$). LD for all possible two-way combinations of variations was tested by D' and r^2 ^{16, 17)}. Haplotypes were in-

Table 1. Summary of selected polymorphisms in the *LRP2* gene

SNP No.	contig position	SNP name	Location	dbSNP [†]	Allele frequency (Heterozygosity)	N [§]	r	p-value*
1	20429298	c.-972G/A	promoter	rs3755166	0.53 : 0.47 (48%)	351	0.00	NS
2	20403499	IVS1-16688A/G	intron 1	—	0.69 : 0.31 (38%)	351	0.02	NS
3	20384751	S83N	exon 3	rs2229263	0.69 : 0.31 (37%)	350	0.01	NS
4	20369778	IVS4+3430A/C	intron 4	—	0.58 : 0.42 (53%)	352	0.07	NS
5	20354690	IVS9+263G/A	intron 9	rs2161039	0.58 : 0.42 (48%)	351	0.04	NS
6	20345159	IVS12+140T/C	intron 12	rs831004	0.86 : 0.14 (23%)	351	0.05	NS
7	20325005	c.+103321A/G	exon 17	rs2241190	0.56 : 0.44 (48%)	350	0.05	NS
8	20313712	IVS19-270T/A	intron 19	rs831044	0.57 : 0.43 (47%)	351	0.05	NS
9	20308890	c.+119436A/G	exon 24	rs831042	0.57 : 0.43 (47%)	344	0.03	NS
10	20305435	IVS26+19G/A	intron 26	rs830973	0.58 : 0.42 (47%)	349	0.06	NS
11	20283406	IVS34-1049A/G	intron 34	rs2300446	0.57 : 0.43 (48%)	349	0.06	NS
12	20273916	IVS38-739G/C	intron 38	rs2268373	0.72 : 0.28 (40%)	351	0.05	NS
13	20262922	A2872T	exon 46	rs2228171	0.55 : 0.45 (48%)	351	0.06	NS
14	20240334	IVS55-243G/T	intron 55	rs2024481	0.87 : 0.13 (23%)	352	0.06	NS
15	20240238	IVS55-147A/G	intron 55	rs2075250	0.74 : 0.26 (38%)	349	0.15	0.004
16	20234500	c.+193826T/C	exon 61	rs2229268	0.75 : 0.25 (37%)	350	0.16	0.003
17	20220402	K4049E	exon 66	rs2075252	0.57 : 0.43 (47%)	351	0.09	NS
18	20208223	IVS71+378T/C	intron 71	rs2075253	0.60 : 0.40 (48%)	351	0.10	NS
19	20185932		3' flanking	rs1990702	0.71 : 0.29 (37%)	350	0.00	NS

SNP, single-nucleotide polymorphism

NS, not significant

[†]: number from dbSNP database of NCBI (<http://www.nih.gov/SNP/>)[§]: number of the subjects whose genotype was determined.

*: p-value was calculated for regression analysis with ANOVA F-test.

ferred, and haplotype frequencies were estimated using the expectation-maximization (EM) method of haplotype inference included in the Arlequin computer program¹⁸⁾.

Results

Association of Polymorphisms at *LRP2* with Plasma Cholesterol Levels

We focused on genetic variations in *LRP2*. To carry out the correlation analysis, we first clarified allelic frequencies and heterozygosities for all 19 selected SNPs in *LRP2* by genotyping 352 individuals (**Table 1**). When the subjects were genotypically categorized into three groups for each of the 19 SNPs (for instance, 196 homozygous major T-allele carriers, 130 heterozygous carriers and 24 homozygous minor C-allele carriers, for c.+193826T/C), we detected no deviation of genotype frequencies from Hardy-Weinberg equilibrium SNPs.

Analysis of variance (ANOVA) with linear regression analysis detected significant correlation between genotypes of the c.+193826T/C variation and the ad-

justed levels of T-Chol ($|r|=0.16$, $p=0.003$) and LDL-C ($|r|=0.12$, $p=0.02$) (**Table 1**). T-Chol and LDL-C levels among the three genotypic categories, i.e., 24 homozygous C-allele carriers (T-Chol 240.7 ± 24.2 mg/dL and LDL-C 166.1 ± 21.0 mg/dL); 130 heterozygous carriers (248.5 ± 23.5 mg/dL and 166.6 ± 19.3 mg/dL) and 196 homozygous T-allele carriers (253.9 ± 23.5 mg/dL and 172.0 ± 21.0 mg/dL) indicated a co-dominant effect of the minor C-allele on lowering T-Chol and LDL-C (**Fig. 1**). A similar effect was detected for IVS55-147G/A (T-Chol, $|r|=0.15$; $p=0.004$, LDL-C, $|r|=0.12$; $p=0.03$) (**Table 1**). On the other hand, no such association was detected for plasma HDL-C and TG level when subjects were genotyped by these two SNPs.

When the subjects were separated into two groups, those who bore at least one C-allele (T/C and C/C) and those with none (T/T), the former group showed significantly lower plasma T-Chol and LDL-C levels (T-Chol, 247.3 ± 23.7 mg/dL versus 253.9 ± 23.5 mg/dL, $p=0.003$) and (LDL-C, 166.6 ± 19.5 mg/dL versus 172.0 ± 21.0 mg/dL, $p=0.01$) (**Fig. 2**). IVS55-147G/A showed similar effects (data not shown).

Fig. 1. Association of c. +193826T/C with plasma lipoprotein levels.

(A), adjusted plasma T-Cho levels; (B), adjusted plasma LDL-Cho levels.

Fig. 2. Comparisons of plasma lipoprotein levels among genotypically determined groups according to one *LRP2* variation, SNP c. +193826.

(A), adjusted plasma T-Cho levels between genotypic groups with or without the C-allele ($n = 154$ or 196 , respectively); (B), adjusted plasma LDL-Cho levels in the same groups of subjects.

LD and Haplotype Structure within *LRP2*

We investigated LD for all possible two-way comparisons among the 19 selected SNPs in *LRP2*. Analysis of D' and r^2 revealed two highly structured LD blocks ($|D'| > 0.7$), a major LD block of about 42 kb extending from exon 17 to exon 34, and a minor LD block (32 kb, exons 55-71) (**Fig. 3**). SNPs c. +193826T/C and IVS55-147A/G both lie within the minor block, and they are in tight LD. Haplotypes were constructed on the basis of genotypes for five sequence variations spanning the minor LD block, and haplotype frequencies in each of the three genotypic populations were estimated using the EM algorithm with phase-unknown samples. Three common haplotypes (frequency more than 5%) were observed within the minor LD block (**Table 2**). A haplotype-2 carrying c. +193826C and IVS55-147G might be the allele responsible for the lowering effect on T-Cho and LDL-C.

Discussion

This study was designed to evaluate a possible association of polymorphisms within the *LRP2* gene with age- and gender-adjusted levels of T-Cho and LDL-C in plasma. The LRP family of molecules is comprised of structurally related endocytic receptors, including the LDLR, apoE receptor 2 (ApoER2), LRP1, and the very low density lipoprotein (VLDL) receptor. Members of this family share common structural motifs, such as ligand-binding domains containing cysteine-rich LDLR class A repeats, acid-regulated epidermal growth factor (EGF) precursor domains, a single

transmembrane domain, and cytoplasmic NPxY sequences. These membrane molecules can transfer macromolecules in large amounts into cells by endocytosis. Subsequently, bound ligands are released in the low-pH milieu of the endosome, and the receptors then return to the cell surface in a process called receptor recycling¹⁹⁻²¹. Because members of the LRP family are functionally similar, and LRP2 has been suggested to mediate endocytosis of LDL and HDL, *LRP2* appeared to be a good candidate for involvement in dyslipidemia.

We found significant correlation between each of two SNPs in the gene encoding LRP2, c. +193826T/C and IVS55-147A/G, and plasma levels of T-Cho and LDL-C. The c. +193826T/C polymorphism, located in exon 61, is a synonymous sequence variation; IVS55-147A/G lies in intron 55. Because neither would be expected to have any functional impact, it seemed likely that a functional variation other than those two SNPs might exist in *LRP2*. To obtain an insight into detecting possibly rare causative variation(s) in the locus, we analyzed the LD structure of this region. The structure of LD in *LRP2* showed that the two variations in question were in tight LD and that both fell within an LD block that covered the region from exon 56 to exon 71; i.e., from the fourth cluster (IV) of the ligand-binding domain to the adjacent EGF precursor domain. Haplotype analysis in this LD block suggested that the variation responsible for dyslipidemia might be linked to a common haplotype-2, which was linked to both c. +193826T/C and IVS55-147A/G.

Fig. 3. Structure of LD in *LRP2*.

The index of LD (D') was calculated for every possible pair among 19 variations, shown as a schematic block. Shaded boxes show pair-wise LD of $D' > 0.9$ (black), $0.8 < D' < 0.9$ (medium gray), and $0.7 < D' < 0.8$ (light gray). The index of LD (r^2) was also calculated for every possible pair. Shaded boxes in that section indicate pair-wise LD of $r^2 > 0.7$ (black), $0.6 < r^2 < 0.7$ (medium gray), and $0.5 < r^2 < 0.6$ (light gray).

Table 2. Analysis of haplotypes for five variations within minor LD block

SNP no.	SNP-14	SNP-15	SNP-16	SNP-17	SNP-18	Frequency
SNP name	IVS55-243G/T	IVS55-147A/G	c.+193826T/C	K4049E	IVS71+378T/C	
haplotype-1	1	0	0	0	1	0.522
haplotype-2	1	1	1	1	0	0.244
haplotype-3	0	0	0	1	0	0.088

Number 0 shows the major allele of each variation.

Number 1 shows the minor allele of each variation.

The molecular mechanisms by which sequence variations in *LRP2* might affect plasma LDL-C levels should be clarified. As has been shown by functional analyses of *LDLR* mutations²², sequence variations in *LRP2* might affect the function of its product during the process from ligand binding to the release of LRP2 into the cytoplasm, resulting in dyslipidemia. LRP2 is likely to have a crucial role in transferring macromole-

cules into cells by endocytosis. LRP2 binds to lipoprotein lipase, apolipoprotein E-enriched beta-VLDL, apoB-100, and apolipoprotein J/clusterin *in vitro*⁸, evidence that this receptor has several discrete roles in lipoprotein metabolism. *In vitro* studies using cultured cells have suggested that LRP2 participates in LDL catabolism by binding to apoB-100²³, and that LRP2 also cooperates with cubilin to mediate HDL endocy-

tosis²⁴⁾. Thus, the predominant function of *LRP2* variation in lipid metabolism could be assumed to be lipid catabolism; however, because no association was detected between HDL-C and the genetic variations in our study, the selective effect of *LRP2* variations on LDL-C catabolism has to be explained. Alternatively, since our study was conducted with a limited number of subjects, a lack of statistical power might have failed to detect the association between HDL-C and the genotypes. This possibility should be tested in future analyses.

Alternative mechanisms of the influences of *LRP2* variations on lipid homeostasis may be related to renal uptake of molecules involved in lipid metabolisms. *LRP2* is predominantly expressed in the renal proximal tubules, and many ligands filtered by glomeruli are taken up through *LRP2* in proximal tubular cells. For example, *LRP2* can bind to liver-type fatty acid-binding protein-1 (FABP1), an abundant constituent of the cytoplasm that regulates lipid transport and metabolism²⁵⁾. FABP1 binds free fatty acids, their CoA derivatives, bilirubin, organic anions, and other small molecules, and is required for cholesterol synthesis and metabolism. As another example, leptin is a circulating factor secreted by adipocytes and was recently found to be a ligand for *LRP2*²⁶⁾. Leptin is primarily metabolized by the kidneys, and *LRP2* appears to have a crucial role in its metabolism; circulating leptin is filtered by glomeruli and taken up by proximal convoluted tubules, where *LRP2* likely mediates its binding and uptake. Thus, sequence variations in *LRP2* might affect the regulation of food intake and energy expenditure by changing the serum leptin level. Unfortunately, in our current study, the implicated function of *LRP2* on serum leptin levels and basic renal function could not be tested because of the limitation of the initial study design and the informed consent. The possibilities, including the influences of *LRP2* variation on serum FABP1 and leptin levels, as well as influences on basic renal functions, should be tested in future analyses.

In conclusion, we identified the association between *LRP2* and levels of T-Cho and LDL-C in human plasma. Even though further investigation will be necessary to clarify the effect of sequence variations in *LRP2* on lipoprotein metabolism, our results provide some insight into the complex mechanisms determining hypercholesterolemia, and may lead to a better understanding of the pathogenesis of dyslipidemias.

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