Genetic analysis of diet-induced hypercholesterolemia in exogenously hypercholesterolemic rats

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Abstract **The exogenously hypercholesterolemic (ExHC) rat is an established strain that exhibits a polygenic syndrome of hypercholesterolemia after feeding on a cholesterol-containing diet, and the extent of this differs between male and female rats in the strain. The present study was performed to determine the genetic background of diet-induced hypercholesterolemia in ExHC rats. We used quantitative trait locus (QTL) analyses of the F2 progeny derived from ExHC and Brown-Norway rats. Rats were fed a diet containing 1% cholesterol, and a genome-wide scan was then performed. Significant QTLs for serum total cholesterol levels were revealed on chromosomes 5 and 14 in the vicinity of markers D5Rat95 and D14Rat43, having maximum logarithm of the odds scores of 6.0 and 5.8, respectively. A suggestive QTL for the trait was also detected on chromosome 3 at D3Rat140. In particular, the QTL on chromosome 5 was specific for female rats. These loci were novel QTLs for postdietary serum total cholesterol levels. In addition, cross-mating analysis in F1 generations suggested that the responsiveness to dietary cholesterol in ExHC rats is partly attributable to X-linked inheritance. Identifying such genetic factors may be useful in predicting the risks associated with diet-induced hypercholesterolemia in humans.**—Asahina, M., M. Sato, and K. Imaizumi. **Genetic analysis of diet-induced hypercholesterolemia in exogenously hypercholesterolemic rats.** ***J. Lipid Res.*** **2005.** 46: **2289–2294.**

Supplementary key words dietary cholesterol • quantitative trait locus • serum cholesterol • sex difference • X-linked inheritance

Hypercholesterolemia in humans (1) and animals (2) is provoked by dietary cholesterol, but the extent varies even in the same species because it has a polygenic basis (3, 4). One study showed that the sex of the subject affects responses of serum total cholesterol to dietary cholesterol in human (5), although another study did not supported the sex difference (6). Individual genetic variability makes it difficult to estimate the influence of dietary cholesterol on serum total cholesterol levels. Thus, animal strains that exhibit consistent hypercholesterolemia after feeding on a cholesterol-containing diet are important models for dissecting the components involved in hypercholesterolemia and for identifying underlying gene variants.

Exogenously hypercholesterolemic (ExHC) rats isolated from Sprague-Dawley (SD) strains by Imai and Matsumura (7) easily elicit hypercholesterolemia after feeding on a cholesterol-containing diet without exhibiting hyperthyroidism. Multiple genes are involved in stable hypercholesterolemia in this strain, but the detailed mechanisms responsible for this have not been clarified (8). Our previous results showed that ExHC rats less effectively take up ^{125}I-labeled β-VLDL via the liver and excrete bile acid into the feces to a lesser extent than do SD rats, although the absorption rates of dietary cholesterol do not differ between the strains (9). These results indicate that ExHC rats specifically alter cholesterol metabolism in an undefined manner in response to dietary cholesterol.

To analyze the genetic background of hypercholesterolemia in ExHC rats, we performed genome scanning of F2 progeny derived from ExHC and the genetically distant Brown-Norway (BN) rat inbred strains after feeding on a cholesterol-containing diet. We detected two significant quantitative trait loci (QTLs) on chromosomes 5 and 14 that have an effect on serum total cholesterol concentration in response to dietary cholesterol. In particular, the QTL on chromosome 5 was specific for females.

MATERIALS AND METHODS

Animals and diets

Inbred ExHC/Sea (ExHC) and BN/Sea (BN) rats were purchased from Seac Yoshitomi (Fukuoka, Japan). BN rats were chosen because their genetic background is highly different from that of SD rats, according to the Rat Genome Database Genome

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Abbreviations: BN, Brown-Norway; cM, centimorgan; ExHC, exogenously hypercholesterolemic; LOD, logarithm of the odds; LRS, likelihood ratio statistic; QTL, quantitative trait locus; SD, Sprague-Dawley; WAT, white adipose tissue.

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Scanner (http://rgd.mcw.edu/). ExHC rats were crossed with BN rats to yield F1 progeny by reciprocal crosses. A total of 217 F2 rats were produced by the intercross of (BN×ExHC)F1 population. These animals were maintained in a temperature-controlled room at 21–23°C with a 12 h light cycle (8:00 AM to 8:00 PM). F1 and F2 rats were weaned after 3 weeks and individually housed with free access to rat chow (NMF; Oriental Yeast Co., Tokyo, Japan) and nonionic water until they reached of 4 weeks of age. Subsequently, they were fed an AIN76-based purified diet containing 1% cholesterol and 10% olive oil for 2 weeks (9). Blood samples were obtained from the tail vein or aorta at 10:00 AM without fasting, and livers were excised from ExHC, BN, and F2 rats. This experiment was carried out under the guidelines for animal experiments of the Faculty of Agriculture and Graduate Course at Kyushu University and Law 105 and Notification 6 of the Government of Japan.

Phenotypic characterizations

Serum total cholesterol, HDL cholesterol, and triacylglycerol concentrations were measured with enzyme assay kits [Cholesterol C-Test from Wako Pure Chemicals (Tokyo, Japan); HDL-C2 Test from Diichi Chemicals (Tokyo, Japan); and Triglyceride E-Test from Wako Pure Chemicals]. Fat pads were dissected from ExHC, BN, and F2 rats. Then, the weights of epididymal white adipose tissue (WAT) and perirenal WAT were determined.

Genotyping and QTL analyses

To map genetic loci associated with the hyperresponse to dietary cholesterol in ExHC rats, we genotyped 217 F2 progeny with 108 microsatellite markers spanning all chromosomes with an average interval of 13 centimorgan (cM) except for chromosome Y. DNA was prepared from the livers of F2 progeny via phenol-chloroform extraction subsequent to proteinase K (Wako Pure Chemicals) digestion and was kept in Tris-EDTA solution. PCR primers for microsatellite markers were obtained from Research Genetics (Huntsville, AL) and the Otsuka GEN Research Institute (Tokushima, Japan). Microsatellites were amplified by PCR with a thermal cycler (Program Temp Control PC800 System; ASTEC, Fukuoka, Japan) according to the instructions for the primer sets (Research Genetics). To determine the microsatellite length, PCR products were electrophoresed on 4% agarose gels and 10% polyacrylamide gels, followed by visualization with ethidium bromide or silver staining. QTL analyses were performed using the interval-mapping method (10) with the program Map Manager QTX (11). QTL analyses according to sex were also separately carried out for male and female F2 rats. The results were expressed as logarithm of the odds (LOD) scores, and statistically significant values for interval mapping were determined by a permutation test (5,000 permutations) using a free regression model. This permutation test revealed the values for likelihood ratio statistic (LRS) as follows: highly significant LRS = 26.1 (LOD score = 5.7), significant LRS = 16.1 (LOD score = 3.5), and suggestive LRS = 9.7 (LOD score = 2.1). LOD scores were calculated by dividing the LRS by 4.605. The map positions for the microsatellite markers were obtained from the Rat Genome Database (http://rgd.mcw.edu/).

Statistical analyses

The results are presented as means ± SEM. Phenotypic values in the parental strains, ExHC and BN rats, were analyzed using the Student's *t*-test ($P < 0.05$), and sex differences in each strain were similarly analyzed. Serum total cholesterol levels in ExHC, BN, and F1 progeny were analyzed by one-way ANOVA with Sheffe's multiple comparison posttest: $P < 0.05$ was considered statistically significant. This statistical analysis was also used to confirm the allele effect at the QTL markers identified in the genome-wide scan in (BN×ExHC)F2 rats.

RESULTS

Phenotypic characterization of ExHC, BN, F1, and F2 rats

The phenotypic values of male ExHC and BN rats are shown in **Table 1**. Compared with BN rats, ExHC rats displayed significantly decreased baseline (before the cholesterol diet) serum total cholesterol concentrations, but the ExHC rats showed significantly increased serum total cholesterol and triacylglycerol concentrations when fed a cholesterol-containing diet for 2 weeks. There was no significant difference in the serum baseline triacylglycerol and postdietary HDL cholesterol levels (data not shown) between the parental strains. Additionally, the ExHC rats showed significantly increased WAT weight per 100 g of body weight compared with the BN rats.

The serum total cholesterol concentrations of the parental strains and F1 and F2 rats that fed on a cholesterol-containing diet for each sex are shown in **Fig. 1**. ExHC and (BN×ExHC)F1 rats after feeding on the cholesterol-containing diet exhibited a sex difference in serum total cholesterol that was higher in females than in males. However, such a sex difference was not observed in BN, (ExHC×BN)F1, and F2 rats. (BN×ExHC)F1 males showed significantly lower serum total cholesterol levels than did (BN×ExHC)F1 females, (ExHC×BN)F1 males, and (ExHC×BN)F1 females. The F2 populations compared with the parents and F1 rats exhibited a wider distribution of serum total cholesterol of between 129 and 944 mg/dl.

QTL analyses

A genome-wide scan of the F2 populations revealed two regions on chromosomes 5 and 14 with significant linkages to the serum total cholesterol concentrations after feeding on the cholesterol-containing diet (**Fig. 2A**). These regions exhibited maximum LOD scores of 6.0 and 5.8 in the vicinity of markers D5Rat95 and D14Rat43, respectively (Fig. 2B, C). In particular, the QTL on chromosome 5 was likely to be a female-specific QTL (nearest marker,

TABLE 1. Phenotypic values in the parental strains

Variable	ExHC (n = 7)	BN (n = 11)
Baseline		
Serum total cholesterol (mg/dl)	84 ± 2.7[a]	104 ± 3.1
Serum triacylglycerol (mg/dl)	98 ± 7.3	108 ± 5.9
Postdietary (1% cholesterol diet)		
Serum total cholesterol (mg/dl)	453 ± 23.8[a]	211 ± 8.9
Serum triacylglycerol (mg/dl)	216 ± 15.5[a]	79 ± 13.3
WAT (g/100 g body weight)	1.7 ± 0.1[a]	0.5 ± 0.0

BN, Brown-Norway; ExHC, exogenously hypercholesterolemic; WAT, white adipose tissue. The values are expressed as means ± SEM. The number of rats is shown in parentheses.

[a] Values that are significantly different ($P < 0.01$) between ExHC and BN rats.

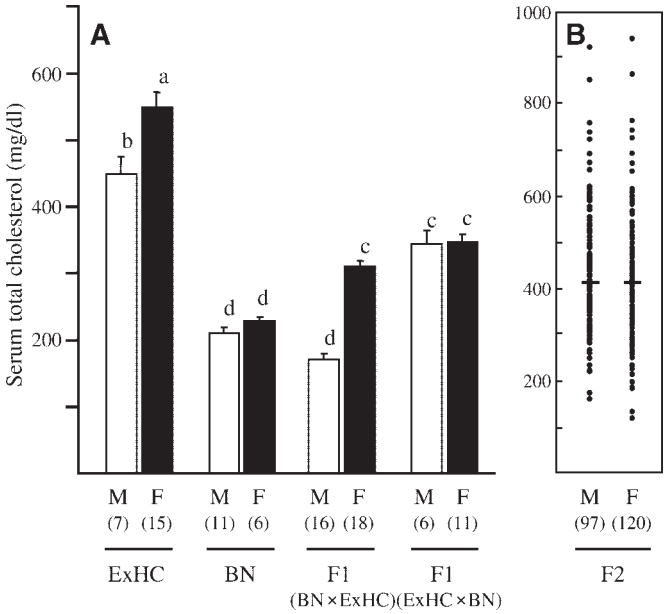

Fig. 1. Serum total cholesterol concentrations in male (M) and female (F) parental, F1, and F2 populations. The rats were fed a cholesterol-containing diet. The number of rats is shown in parentheses. A: For the exogenously hypercholesterolemic (ExHC), Brown-Norway (BN), and F1 rats, the values are means ± SEM. Different letters above the bars indicate significant differences at $P < 0.05$. B: In the F2 population, the distribution of serum total cholesterol levels is shown. Mean values are indicated by the horizontal bars.

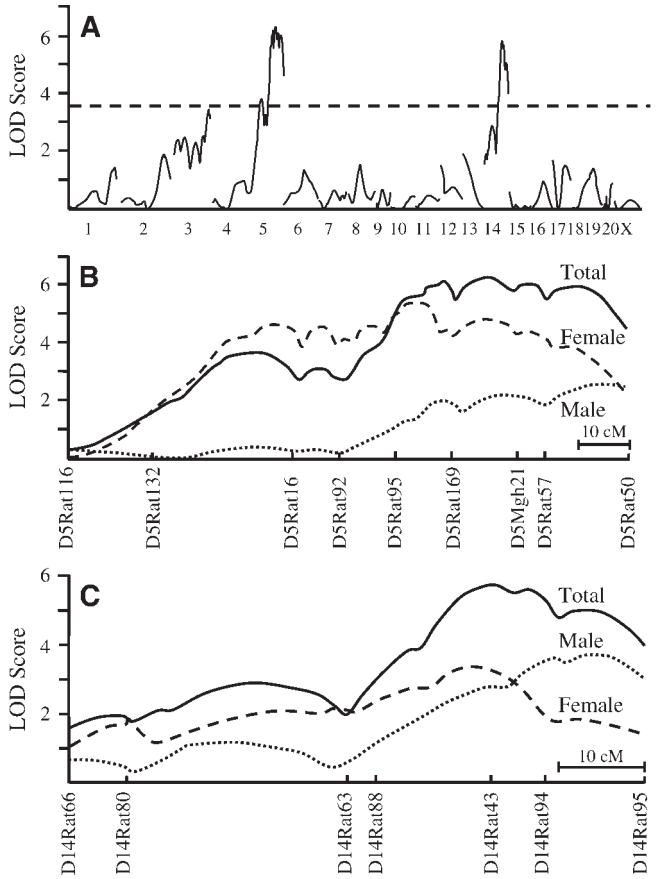

Fig. 2. Quantitative trait locus (QTL) analyses for diet-induced hypercholesterolemia in rats. A genome-wide scan for serum total cholesterol (A) and QTL analyses for the trait according to sex on chromosomes 5 (B) and 14 (C) in F2 rats fed a cholesterol-containing diet are shown. Chromosomes 1 through X and microsatellite markers are represented in the genome-wide scans and on chromosomes 5 and 14, respectively, on the horizontal axis. The dashed horizontal line in A represents a significant ($P < 0.05$) logarithm of odds (LOD) score of 3.5, determined by a permutation test. cM, centimorgan.

D5rat95; LOD score = 5.3 only in females). Such a female-specific QTL was not found in the vicinity of D14Rat43 on chromosome 14. A suggestive QTL for the trait was also detected in the vicinity of genetic marker D3Rat140 on chromosome 3 (maximum LOD score of 3.4; Fig. 2A). At the most closely linked marker, each QTL on chromosomes 3, 5, and 14 was responsible for 7, 12, and 11%, respectively, of the variance of serum total cholesterol concentrations in the F2 population when fed the cholesterol-containing diet (**Table 2**). No statistical epistatic interaction among these loci was detected.

In addition to QTLs for postdietary serum total cholesterol, QTLs related to baseline serum total cholesterol and WAT weight are shown in Table 2. In chow-fed F2 rats, significant QTLs involved in baseline serum total cholesterol levels were found on chromosomes 4, 10, and 12 .We also found a suggestive QTL for perirenal WAT weight on chromosome 1.

The allele effect at each microsatellite marker linked to a significant QTL for diet-induced hypercholesterolemia is presented in **Fig. 3**. At the sex-specific QTL for the trait on chromosome 5 linked to D5Rat95, the ExHC allele increased serum total cholesterol levels in female rats in a recessive manner (Fig. 3A), but such a significant influence was not seen in male rats fed a cholesterol-containing diet (Fig. 3B). The microsatellite marker D14Rat43 exhibited a recessive inheritance pattern associated with increased cholesterol levels. However, there was no sex difference in allelic effects at this marker.

DISCUSSION

ExHC rats easily elicit hypercholesterolemia, and female ExHC rats showed higher serum total cholesterol levels than did males when fed a cholesterol-containing diet. In the genome-wide scan, we located three loci on rat chromosomes 3, 5, and 14 that were responsible for diet-induced hypercholesterolemia. In particular, the QTL on chromosome 5 was specific for females. At this locus, the ExHC allele increased serum total cholesterol concentrations only in females. Thus, this female-specific QTL likely accounts for the sex difference in the responsiveness to dietary cholesterol between female and male ExHC rats. Postdietary serum total cholesterol levels in the F1 populations produced by reciprocal crosses suggest that the chromosome X derived from the ExHC rats had some effect on the responsiveness to dietary cholesterol, although QTLs for diet-induced hypercholesterolemia were not detected on chromosome X. Therefore, the female-specific QTL on chromosome 5 detected in (BN×ExHC)F2 rats

TABLE 2. Summary of quantitative trait loci identified in the BN×ExHC intercross when fed a chow diet (baseline) or a cholesterol-containing diet (post dietary)

Trait	Chromosome	Nearest Marker (Centimorgan)	Logarithm of the Odds Score	Significance	Variance
					%
Serum total cholesterol					
Baseline	4	D4Arb15 (76)	3.5	Significant	7
	10	D10Rat25 (51)	3.6	Significant	7
	12	D12Rat36 (42)	5.0	Significant	10
Postdietary	3	D3Rat140 (79)	3.4	Suggestive	7
	5	D5Rat95 (65)	6.0	Significant	12
	14	D14Rat43 (41)	5.8	Significant	11
WAT					
Postdietary	1	D1Rat49 (78)	3.1	Suggestive	6

and the effects of chromosome X derived from ExHC rats seem to be genetic factors associated with the sex difference in responsiveness to dietary cholesterol in ExHC rats.

Several QTLs for postdietary serum total cholesterol levels have been reported in mice (12–19) and rats (20) fed cholesterol-containing diets. Among these loci, genetic analysis of the F2 population derived from LEW/OlaHsd and BC/Cpbv rats revealed a female-specific QTL for postdietary serum total cholesterol on rat chromosome 5 in the vicinity of genetic marker D5Rat209 (20). However, this QTL region is located near the centromere of rat chromosome 5 and does not overlap the QTL, as was seen in the present study. The sex-specific locus detected in F2 rats between SHRSP and WKY rats appeared to overlap the region identified on rat chromosome 5 in the present study, although the F2 rats were fed a diet without cholesterol (21). QTLs for diet-induced hypercholesterolemia on chromosomes 3 and 14 identified in rats fed a cholesterol-containing diet are unlikely to overlap the loci reported previously in rats. There exist some reports on QTLs influencing serum total cholesterol concentrations in mice fed a cholesterol-containing diet (12–19). However the syntenic regions in mice to QTLs on rat chromosomes 3, 5, and 14 in the present study are not reported as QTLs for the responsiveness to dietary cholesterol in mice. Syntenic regions were evaluated using the Mouse Genome Informatics database (http://www.informatics.jax.org/). Therefore, QTLs for postdietary serum total cholesterol levels in (BN×ExHC)F2 rats that were fed a cholesterol-containing diet on rat chromosomes 3, 5, and 14 are novel.

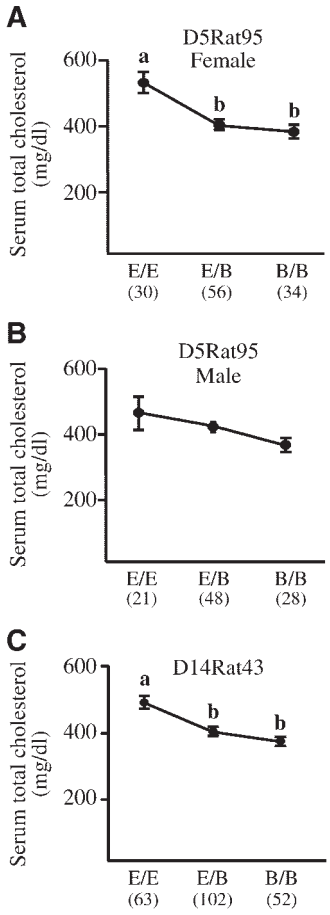

Fig. 3. Genotypic effects on postdietary serum total cholesterol. Allelic contributions to the QTLs detected on chromosomes 5 (A, B) and 14 (C) for diet-induced hypercholesterolemia are shown. Homozygous ExHC alleles are represented by E/E, homozygous BN alleles by B/B, and heterozygous alleles by E/B. Values shown are means ± SEM. Different letters above the symbols indicate significant differences at $P < 0.05$. The number of rats is shown in parentheses.

Candidate genes related to lipoprotein or cholesterol metabolism and within the QTL regions on rat chromosomes 5 and 14 were selected using the database LocusLink (http://www.ncbi.nih.gov/Genomes/). A potential candidate gene, Abca1, is located within the QTL region on rat chromosome 5. However, there was no significant difference in Abca1 mRNA expression between ExHC and SD rats fed a 1% cholesterol diet for 2 weeks (unpublished observation). Furthermore, Cyp7a1 (cytochrome P450, family 7, subfamily a, polypeptide 1) and Scp2 (sterol carrier protein 2), which also map to rat chromosome 5, are not located within the QTL region on rat chromosome 5 detected in the present study. By contrast, adequate candidate genes within the QTL region on rat chromosome 14 are not listed in the database described above. The confidential interval for QTLs on chromosomes 5 and 14 was determined by bootstrap analysis with the program Map Manager QTX, and there are ~700 and 250

genes within the QTL region on chromosomes 5 and 14, according to the database Map Viewer in Rat Genome Resources (http://www.ncbi.nlm.nih.gov/genome/guide/rat/index.html).

The physiological mechanism leading to this type of loci-dependent hypercholesterolemia remains to be elucidated. Our previous results showed that the phenotype does not arise from variations in intestinal cholesterol absorption (9). Instead, these loci-dependent phenotypes appear to result from an alteration in cholesterol processing or clearance, because ExHC rats compared with SD rats are partly defective for the hepatic removal of cholesteryl ester-rich lipoproteins in primary cultured hepatocytes as well as in vivo (9). The removal of cholesteryl ester-rich lipoproteins from the circulation is facilitated by molecules such as the LDL receptor, lipoprotein receptor-related protein, heparan sulfate proteoglycan, apolipoprotein E, apolipoprotein Cs, apolipoprotein B, lipoprotein lipase, and hepatic lipase and their interactions (22). Thus, although these are likely to be potential candidate genes, they are not located in the loci detected on rat chromosomes 3, 5, and 14 in (BN×ExHC)F2 rats.

In chow-fed F2 rats, three significant QTLs for baseline serum total cholesterol were detected on rat chromosomes 4, 10, and 12. Syntenic regions in mice to QTL regions for serum total cholesterol in the chow-fed (BN×ExHC)F2 rats on chromosome 4 overlap the QTL for the trait in mice (23, 24). QTL regions on rat chromosomes 10 and 12 overlap no regions previously reported in rats and mice. Compared with BN, ExHC rats show significantly increased WAT weight per 100 g of body weight. We also identified a suggestive QTL for perirenal WAT weight per 100 g of body weight on rat chromosome 1 (D1Rat49). York, Lei, and West (25) previously reported on the syntenic QTL region in mice to this QTL for WAT weight. Watanabe et al. (26) also reported QTLs related to fat level and body weight on rat chromosome 1 (Dmo1) in a genetic analysis of OLETF rats. The D1Rat90 marker that showed the peak LOD score at the Dmo1 locus was mapped near the telomere (148 cM). However, the D1Rat49 marker within the QTL observed in the present study did not coincide with the Dmo1 locus.

In summary, the present study indicates that rat chromosomes 3, 5, and 14 contain novel QTLs that are involved in diet-induced hypercholesterolemia and that the genetic factor on chromosome X is associated with the phenotype in ExHC rats. The production of congenic strains that contain the two significant QTLs revealed in the present study can be useful for reducing the verified and implicated chromosomal region to a size applicable to positional cloning. These genetic factors can be used to reveal genetic backgrounds that contribute to the variation of individual susceptibility to dietary cholesterol, including the sex differences in humans.

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