Quantitative Trait Loci for Hypercalciuria in a Rat Model of Kidney Stone Disease

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Abstract. Hypercalciuria is the most common risk factor for kidney stones and has a recognized familial component. The genetic hypercalciuric stone-forming (GHS) rat is an animal model that closely resembles human idiopathic hypercalciuria, with excessive intestinal calcium absorption, increased bone resorption, and impaired renal calcium reabsorption; overexpression of the vitamin D receptor (VDR) in target tissues; and calcium nephrolithiasis. For identifying genetic loci that contribute to hypercalciuria in the GHS rat, an F2 generation of 156 rats bred from GHS female rats and normocalciuric WKY male rats was studied. The calcium excretion was six- to eightfold higher in the GHS female than in the WKY male progenitors. Selective genotyping of those F2 rats with the highest 30% and lowest 30% rates of calcium excretion was performed, scoring 98 markers with a mean interval of 23 cM across all 20 autosomes and the X chromosome. With the use of strict criteria for significance, significant linkage was found between hypercalciuria and a region of chromosome 1 at D1Rat169 (LOD, 2.91). Suggestive linkage to regions of chromosomes 4, 7, 10, and 14 was found. The proportion of phenotypic variance contributed by the region on chromosome 1, with appropriate adjustments, was estimated to be 7%. Candidate genes encoding the VDR and the calcium-sensing receptor were localized to regions on rat chromosomes 7 and 11, respectively, but the suggestive quantitative trait locus on chromosome 7 was not in the region of the VDR gene locus. Identification of genes that contribute to hypercalciuria in this animal model should prove valuable in understanding idiopathic hypercalciuria and kidney stone disease in humans.
higher for WKY than for F344 relative to GHS (see the Results section), so we chose to breed GHS × WKY. Because the largest phenotypic difference occurred between the GHS female and WKY male rats (Table 1), we performed breeding in one direction, using GHS female rats (generations 42 and 47) and WKY male rats to simplify the gender analysis. From the resulting F1 progeny, we selected randomly and without knowledge of the calcium excretion brother–sister pairs for breeding to produce an F2 generation (Figure 2). In the F0 rats bred for these intercrosses, there was a more than sevenfold difference in calcium excretion (GHS female rats, 7.01 ± 0.21 mg/24 h; WKY male rats, 0.94 ± 0.23 mg/24 h).

**Rat Diet and Measurement of Ca Excretion Levels**

All rats were separated by gender at weaning. At 8 wk of age, rats were placed in individual metabolic cages for measurement of urinary calcium excretion on a normal-calcium diet as described previously (8–10, 12–15). The animals were fed 13 g/d of a diet containing 0.6% calcium, 0.65% phosphorus, 0.24% magnesium, 0.40% sodium, and 0.43% potassium and 2.2 I.U. of vitamin D3/g of food. On days 4 to 10 from the start of this period, complete individual 24-h urine collections were made in 0.25 ml of 12 N HCl, and food and fluid intake were recorded. Collections for each day were analyzed individually, and the individual values were added to obtain the 7-d accumulated totals. Any rat that consumed less than 12 g of food or less than 15 ml of fluid on any day of the study was excluded. Ca levels were determined as previously reported (8–10, 12–15).

**Genotyping**

Genomic DNA was prepared using the Puregene DNA Isolation Kit (Genta Laboratories, Inc., Minneapolis, MN) from frozen livers isolated immediately after death and stored at −70°C. Primers for microsatellite markers were obtained from Research Genetics based on the listing of polymorphisms (www.resgen.com/productsRtMPs.php3). PCR reactions were carried out as recommended and analyzed either on DNA sequencing gels or on 4% agarose gels by standard methods. We identified 255 markers that are polymorphic for the two strains and scored 98 of these markers across all 21 rat chromosomes for a mean interval of approximately 23 cM between scored markers (16). We performed selective genotyping on the F2 generation and completed a whole genome scan of those rats in the top and bottom 30% of the calcium excretion phenotype. In addition, we genotyped all F2 rats at seven markers distributed across chromosome 1: D1Rat196, D1Mgh6, D1Mgh7, D1Rat200, D1Mrt2, D1Mgh11, and D1Rat169. All F2 rats were also genotyped at one additional marker on each of the other chromosomes (2 through X).

**Statistical Analyses**

The number of genes segregating in an F2 generation were estimated using the Sewall Wright formula, based on the phenotypic difference between the F0 progenitors and the phenotypic variances in the F1 and F2 populations (17). The MapManager QTX software package (18) was used as a database for the marker data as it was produced. Thereafter, the analysis was conducted using the Pseudomarker programs (19), written in the Matlab programming language (www.mathworks.com) and the statistical package R (www.r-project.org). The F2 calcium excretions were log-transformed before statistical analysis as a variance-stabilizing transformation. Gender and cross-identity were used as covariates in QTL mapping.

**Genome Scans**

Genome scans were performed by the multiple imputation method of Sen and Churchill (19) using a 5-cM pseudomarker grid and 64 imputations. All scans included gender and cross-identity as additive covariates. In addition to regular one-dimensional and two-dimensional scans, a one-dimensional scan with gender and cross as “interacting” covariates was performed. To assess the significance of the one-dimensional scans, we used permutation tests (20) with 1000 shuffles. To determine whether there were linked or interacting QTL in the cross, we followed a two-stage model selection procedure. In the first stage, we selected locus pairs (21). In the second stage, we pulled together all of the locus pairs selected in the first stage and dropped terms by backward elimination (modification of Broman and Speed (22)).

**Locus Pair Selection**

All pairs that cleared the permutation test threshold (5% level) for the full two-locus model (LOD score approximately 7 based on 400 permutations) were subjected to secondary tests. For the coat-tail effects, the secondary test was conducted at a nominal P value of 0.002 (corresponding to the one-dimensional genome scan 5% threshold LOD score of 2.97, based on 1000 permutations). For the interactions, we used a nominal P value of 0.0001 (corresponding to a 5% permutation test threshold for the interaction test only; this amounted to a LOD score of 2.75, based on 400 permutations) (21).

**Backward Elimination**

All locus pairs selected in the previous stage were put together in a multiple-QTL model. The multiple-QTL model was formed by taking a typed marker closest to the locus selected. When there was no typed marker within 10 cM of the locus, a pseudomarker was used. Then each term in the model was dropped and the change in the LOD score was noted. (This corresponds to a type III ANOVA). When the nominal P value of the change in the LOD score was > 0.002 for the main effects and > 0.0001 for the interactions, that term was dropped. This procedure was continued until no term could be dropped (22).

**Candidate Gene Localization**

**Chromosome Localization of the Casr and Vdr Genes.** The rat radiation hybrid (RH) cell panel available for mapping rat genes (23, 24) was used; the DNA samples were genotyped by PCR. The primers used were as follows: for Casr, 5'-GATTGGAGGCTG-GAGACC-3' and 5'-TGGCCTGCTACCGTCGAA-3' (position 51 to 69 and 182 to 200 of the Genbank sequence U10354, respectively);
for \textit{Vdr}, 5'-CCATCCGGCTTTCCAACC-3' and 5'-TCCTGGGATCATCTTGGCA-3' (position 583 to 601 and 720 to 738 of the Genbank sequence E14584, respectively). The position of the genes was determined using the RH mapping server at the Medical College of Wisconsin (rgd.mcw.edu/RHMAPSERVER/) (23). Fluorescence \textit{in situ} hybridization (FISH) analysis was performed to identify the chromosomal regions of the CaSR and VDR genes. The FISH experiment was done as described previously (25) except that the \textit{Vdr} probe was labeled by nick-translation. The \textit{Vdr} probe was the pVDR1784 plasmid, containing a 1784-bp EcoRI insert (26).

**Results**

**Rat F2 Intercross**

Urinary calcium excretion rose progressively through the first 30 generations of breeding and has been at maximal levels for more than 20 generations (Figure 1). This progressive increase in calcium excretion through generations suggests that multiple genes contribute to hypercalciuria in this model. (Because this involved selective breeding of the most hypercalciuric rats from among several litters in each generation, it would be expected to have taken longer to reach homozygosity at all loci than with brother–sister matings.) Microsatellite marker data for generation 42 indicate full allele homozygosity (not shown).

To select another rat strain that was appropriate for these intercrosses, we screened Fisher 344 rats and WKY rats for calcium excretion and allele polymorphism rates relative to the GHS rats. Calcium excretion, measured at 8 to 10 wk of age under controlled metabolic conditions as described (8–10,12–15), is shown in Table 1. For both male and female rats, there are large differences in calcium excretion between GHS and both other rat strains. We screened these three rat strains using 50 simple sequence length polymorphisms (SSLP) and found that the polymorphism rate for WKY \textit{versus} GHS (72.0%) was higher than for F344 \textit{versus} GHS (43.8%). Thus, we chose to use the WKY strain for the intercrosses. Upon completion of the 255 markers used in this study, we have accumulated a polymorphism rate between WKY and GHS over this larger sample of 53%.

**Calcium Excretion Phenotypes**

**GHS × WKY F1 Phenotypes.** The F1 rats generated from the parental GHS and WKY strains showed a normal distribution of the calcium excretion phenotype, with a mean of excretion of 4.6 mg/24 h, very close to the midpoint between the calcium excretions of the parents. Figure 2 shows this distribution on a log scale for F1 and F2 rats separated by gender. In the F1, we observed no difference in mean calcium excretion between male (4.37 mg/24 h) and female rats (4.51 mg/24 h; unpaired \textit{t} test, \(P = 0.629\)).

**F2 Phenotypes.** The mean calcium excretion in the 156 F2 rats was 2.31 ± 0.10 mg, which fell between the parental means. There was no significant difference in calcium excretion between female (2.45 ± 0.14 mg/d) and male (2.16 ± 0.13 mg/d) F2 rats (\(P = 0.14\)). The distribution of excretion rates in the F2 generation was consistent with the effects of several QTL (Figure 2). The distribution was skewed toward the central tendency for the WKY rats, suggesting that WKY alleles are dominant in activity over GHS alleles. Applying the Sewall Wright formula to these data yields a value of 6.24, which predicts the presence of at least six QTL in this rat model. However, it should be kept in mind that this formula makes a number of assumptions that limit its usefulness, such as that the effects of individual QTL are equal, that independent assortment of QTL occurs, that there is little or no epistasis among the QTL, and that all of the alleles that contribute to hypercalciuria are those of the GHS strain.
QTL Mapping

QTL Affecting Calcium Excretion in the F2 Progeny. We have identified a significant QTL for calcium excretion (HC1) near the locus D1Rat169 and have data suggestive for additional QTL on chromosomes 4, 7, 10, and 14 (Figure 3), using one- and two-dimensional scans with the multiple imputation method of Sen and Churchill (19), as described (see the Materials and Methods section).

To assess the significance of the peaks of the genome scan, we performed a permutation test (20) with 1000 permutations. The 5% threshold was 2.97. The peak of the genome scan was at D1Rat169 on chromosome 1 with a LOD score of 2.91. The P value of the peak corrected for multiple testing with the permutation test was 0.07. The genome-wide thresholds for P = 0.37 (suggestive) and P = 0.10 (significant) are shown in Figure 3. The 95% confidence interval for the QTL HC1 is from 120 cM to 160 cM, which is in the marker interval D1Rat193 to D1Rat142. The proportion of variance contributed by the chromosome 1 QTL, adjusting for the variance accounted for gender and cross, is approximately 7%.

The suggestive QTL (genome-wide P < 0.368; LOD = 2.15) are on chromosome 4, near marker D4Rat222, with a LOD of 2.1; on chromosome 7 near D7Rat164, with a LOD of 2.1; on chromosome 10 near D10Rat47, with a LOD of 2.4; and on chromosome 14, near D14Mgh1, with a LOD of 2.3. In the genome scan conducted using gender and cross as interacting covariates, none of these peaks cleared the genome-wide 0.1 threshold for significance, but they do meet criteria suggestive of significant linkage. By two-dimensional scan, locus pair selection, and backward elimination (see the Materials and Methods section), we did not find any evidence for two-locus interactions in this study.

Chromosome Localization of the Rat Casr and Vdr Genes. The Casr and Vdr genes were localized using the panel of rat RH (23,24) and primers designed on the rat sequence available in Genbank (U10354 and E14584, respectively). The Casr gene was placed on chromosome 11, between D11Got57 (distance, 5.3 cR) and D11Got53 (distance, 28.8 cR). It should be noted that the chromosome 11 RH map shown by the MCW server (RGD, rgd.mcw.edu/maps/) was upside down. Figure 4A shows a map that is correctly oriented (11pter at the top) and includes markers at the two ends of the map, as well as markers in the vicinity of the Casr gene. The RH map position of Casr corresponds to the cytogenetic bands 11q21-q22. This region is known to be homologous to the human chromosome region 3q11-q24 and mouse chromosome 16, which also include the CASR/Casr gene (24,27) (GDB, gdbww.gdb.org/; MGD, www.informatics.jax.org/). The Vdr gene was previously assigned to rat chromosome 7 (28). RH mapping placed it at the 7q telomere end, between D7Rat2 (dis-
tance, 1.0 cR) and D7Mit26 (distance, 5.0 cR), as shown in Figure 4B. A Vdr probe being available (26), we also mapped the Vdr gene by FISH and obtained specific signals at 7q36, the telomeric band of chromosome 7, as expected (data not shown). The telomeric end of rat chromosome 7 is homologous to the human chromosome region 12q12-q14, which also contains the VDR gene (28) (GDB, gdbwww.gdb.org/) and to the mouse chromosome 15, but the mouse Vdr gene resides on chromosome 5 (MGD, www.informatics.jax.org/).

The Casr and Vdr genes were considered to be two very strong candidate genes for this study. However, none of our QTL map to chromosome 11, and our suggestive QTL mapping to chromosome 7 is centered near D7Rat164, in the middle of the chromosome, not near the VDR locus. D7Rat164 maps to approximately 420 on the RH map, whereas VDR is at 785.8 on the RH map (Figure 4), very close to the telomere. With size estimates of 153 Mb for chromosome 7, our suggestive QTL map to approximately 420 on the RH map, whereas VDR is at 785.8 on the RH map (Figure 4), very close to the telomere. With size estimates of 153 Mb for chromosome 7, our suggestive QTL map to approximately 75 Mb away from the VDR gene.

**Discussion**

In this rat model of genetic idiopathic hypercalciuria, physiological data indicated that hypercalciuria was a polygenic trait (8–11,14,29). We found evidence of significant linkage for hypercalciuria to the region of D1Rat193 to D1Rat142 on chromosome 1 and evidence suggestive of linkage on four other chromosomes. Our analyses estimate that 7% of the phenotypic variance is attributable to the locus on chromosome 1.

The region on rat chromosome 1 that links with calcium excretion spans more than 50 Mb and encompasses more than 1000 known or potential genes. Those closest to the peak of the LOD score curve include a calcium-transporting ATPase (Atp2b3), a calcium/calmodulin-dependent protein kinase (Camk1b), subunit 1 of the lysosomal proton-ATPase (Atp6s1), renin-binding protein (Renbp), and insulin-like 6 (Ins6), as well as sequences similar to those encoding a modulator of NfkB, a calcium-binding protein centrin 2 (centractin), s-adenosylmethionine decarboxylase, and a polycystin-2 homolog. Also within the QTL, although not as close to the peak, are sequences resembling those of the two-pore calcium channel protein 2 and of CLCN3 and genes encoding several potassium channels (voltage-gated Kcnq1, inwardly rectifying Kcnk4), several renal organic anion transporters (Out5, Slc22a6, and others), ornithine decarboxylase, uromodulin (Tamm-Horsfall protein), and coflin. It is possible to speculate on a potential role in calcium metabolism for a number of these, but any speculation as to which may contribute to hypercalciuria in the GHS rat is premature at this stage.

Our data also support the likelihood (at the “suggestive” level of significance) of QTL for hypercalciuria on rat chromosomes 4, 7, 10, and 14. Candidates on these chromosomes include, for example, genes encoding two isoforms of the epithelial calcium channel, ECaC1 and ECaC2, that are located on chromosome 4 in the rat. Evaluation of any such candidate genes must await further breeding and mapping studies to confirm the significance of linkage and define the QTL regions more precisely.

In human hypercalciuria as well as the GHS rat, several lines of evidence indicate that multiple genes participate in the genetic component of hypercalciuria. A variety of physiological mechanisms are found, in differing degrees, among the spectrum of patients with IH. These include excessive intestinal absorption of calcium, excessive bone resorption, and a renal calcium leak (30), as well as overexpression of the VDR (10,11,31), all of which also contribute to hypercalciuria in the GHS rat (8–10,12–15). It thus should be instructive to correlate the linkage observations in the GHS rat with the current state of knowledge of the genetics of human hypercalciuria.

There are several conditions in which hypercalciuria occurs in the setting of a recognized symptom complex. These include Bartter syndrome (32–34); inherited hypercalciuria with hypomagnesemia (35); and Dent disease (36). Each of these is associated with other clinical features that clearly are not found in idiopathic hypercalciuria. We have reported that the sequence for the rat CLCN5 gene associated with Dent disease is normal in the GHS rat (37). Our current data do not support linkage to the rat X chromosome that harbors the CLCN5 gene. However, reciprocal mating of (WKY × GHS) could reveal epigenetic influences, such as parent-of-origin regulation of QTL expression in F2 progeny.

Evidence is emerging to associate sequence variations in specific genes with IH. Reed et al. (4) described a locus linked to hypercalciuria on human chromosome 1q34, in a region that contains a gene encoding a product homologous to a rat soluble adenyly cyclase, and polymorphisms in this gene sequence are correlated with calcium excretion and bone density in humans with IH. This region of human chromosome 1 shares synteny with chromosome 13 in the rat, to which we have no evidence of linkage. Prie et al. (38) recently reported a mutation in the NPT2a gene, encoding the renal epithelial sodium-dependent phosphate transporter, in a patient with hypercalciuria and kidney stones. Expression work demonstrated that this mutation reduced the function of the phosphate transporter. The chromosomal location of the NPT2a gene in the rat is unknown. However, Prie’s observations were made in patients with hypophosphatemia, which is not present in our rat model. Finally, in a preliminary report, a gain-of-function mutation in the epithelial calcium-sensing receptor was described in a family with IH (39). We have localized the gene for the calcium-sensing receptor to chromosome 11 in the rat, and our data do not support linkage of this region to hypercalciuria in the GHS rat.

Physiological data implicate the VDR as important in the mechanism of hypercalciuria in the GHS rat. In French-Canadian sibships, linkage at the “suggestive” level of significance has been reported to hypercalciuria for the human locus on chromosome 1q12–14 in the region that harbors the gene for the VDR (40). Thus, the locus for the VDR is an important candidate gene in this model. Our data suggest linkage of hypercalciuria to the region on chromosome 7 near D7Rat164, in the middle of the chromosome, well removed from the VDR locus at the telomere.

Calcium excretion is higher in female WKY, F344, and GHS rats than in the male rats for those strains (Table 1), and this is true for other strains as well (41). In this study, we bred female
GHS with male WKY rats because it maximized the phenotypic difference between progenitors. Because we did not perform reciprocal matings, we cannot rule out potential epigenetic effects.

Application of the Sewall Wright formula to the phenotypic variation in the F2 generation compared with its progenitors predicted that approximately six genes contribute to hypercalciuria in this animal model. Our mapping studies have located potentially five of these loci. More precise localization of the regions of linkage will require further mapping analysis in a larger population of F2 rats and use of congenic strains. Identification of the genes that contribute to the complex genetic trait of hypercalciuria in the rat should prove valuable in identifying the causes of IH and kidney stone disease in humans.

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