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Genetic Linkage Analysis in Familial Benign (Hypocalciuric) Hypercalcemia: Evidence for Locus Heterogeneity

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Summary

Familial benign hypercalcemia (FBH, or hypocalciuric hypercalcemia) is characterized by inheritance, in an autosomal dominant pattern, of lifelong hypercalcemia without hypercalciuria, which is often mistaken for classical primary hyperparathyroidism. Recently, the FBH trait was linked, in four families, to chromosome 3q. We report genetic linkage analysis in 140 persons from five additional families having FBH (65 affected, 67 unaffected, and 8 unclassifiable). In four families, FBH mapped to chromosome 3q, between D3S1215 and D3S20, maximum multipoint lod score 12.9. By contrast, in the fifth kindred FBH mapped to chromosome 19p13.3, tightly linked to the marker loci D19S20 and D19S266 (two-point lod score at recombination fraction = .001 is 3.44 and 3.70, respectively). Thus, the FBH phenotype results from mutations at two separate loci on chromosomes 3q and 19p.

Introduction

Persons affected with familial benign hypercalcemia (FBH; also termed "hypocalciuric hypercalcemia"; Foley et al. 1972; Marx et al. 1977) have hypercalcemia, mildly reduced serum phosphate, generally normal levels of parathyroid hormone (PTH) in plasma, and normal to low urinary calcium excretion (reviewed in Marx et al. 1981*a;* Law and Heath 1985; DA Heath 1989; H Heath 1989). FBH is inherited in an autosomal dominant pattern, is nearly fully penetrant, and is manifest at or shortly after birth. Despite lifelong hypercalcemia, there are few if any symptoms attributable to the disorder (Foley et al. 1972; Law and Heath 1985; DA Heath 1989; H Heath 1989). Despite its benignity, FBH is worthy of study, because the disorder is often mistaken for primary hyperparathyroidism, leading to inappropriate surgical treatment (Marx et al. 1981*a;* Law and Heath 1985; DA Heath 1989; H Heath 1989). FBH is a biological puzzle, moreover, because the responsible mutation simultaneously causes increased renal tubular reabsorption of calcium, parathyroid resistance to calcium, and absence of the usual adverse effects of hypercalcemia (Marx et al. 1981*a,* 1981*b;* Law and Heath 1985). Furthermore, homozygosity for the FBH gene may cause severe neonatal hyperparathyroidism with parathyroid hyperplasia (Marx et al. 1982; Steinmann et al. 1984; Cooper et al. 1986), so the mutated gene could also be involved in causation of parathyroid neoplasia.

Recent technical advances have awakened interest in a positional cloning approach to finding the FBH gene. Several groups attempted earlier to identify the FBH locus by genetic linkage analysis, using blood groups, HLA typing, and selected RFLPs (Menko et al. 1984; Sopwith et al. 1984; Paterson et al. 1985; Almahroos et al. 1987; Kowalska et al. 1987). Previously, we examined a number of candidate genes, without success (Heath and Leppert 1992), but Chou et al. (1992) reported, in four families, linkage of the FBH locus to markers on the long arm of chromosome 3. In the present report, we confirm and refine localization of the FBH locus to 3q in four families but demonstrate that in a fifth family having the FBH phenotype the disease gene maps to the short arm of chromosome 19. This evidence for genetic heterogeneity in the face of clinical

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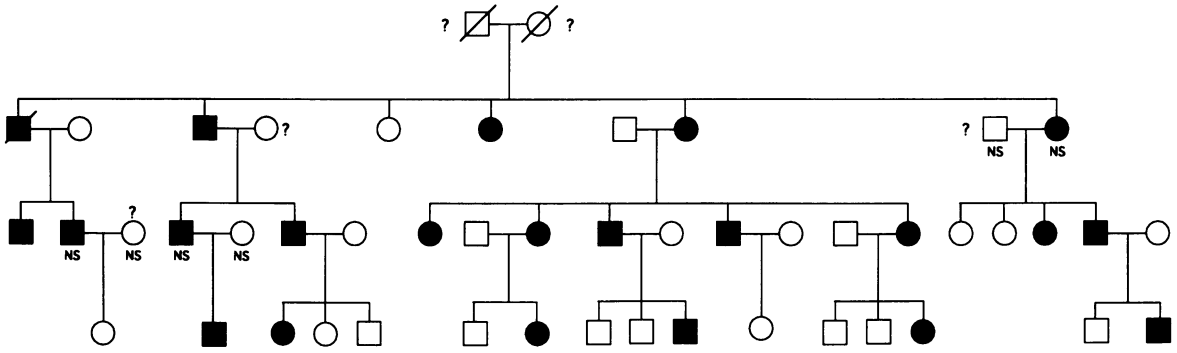

Figure 1 Pedigree for kindred 11677, in which the FBH trait mapped to chromosome 3q. Squares denote males; and circles denote females. Blackened symbols denote individuals affected with FBH; and a diagonal line denotes that individual is deceased. ? = Affection status unknown; and NS = not sampled.

homogeneity adds both complexity and interest to the effort to identify specific mutations associated with the FBH syndrome.

Subjects and Methods

Study Families

All studies were conducted in accordance with standards set by the Institutional Review Boards of the Mayo Foundation, Rochester, MN (where H.H. initially contacted the families), and the University of Utah. Informed consent was obtained before collection of clinical information or blood samples. Members of the study families have been in contact with the principal investigator for up to 14 years. The five families (11672, 11673, 11675, 11676, and 11677) have been described earlier (Law and Heath 1985); complete pedigrees for families 11672, 11673, 11675, and 11676 appear in our recent linkage paper (Heath and Leppert 1992). Family 11677 (fig. 1), almost all members of which reside in the U.S. upper Midwest, included 46 persons (21 affected with FBH, 22 unaffected, and 3 of unknown status). Portions of this family have also been reported by Marx et al. (1981*a*, 1982). All five families reside in the United States, are descended from U.K., western European, or Scandinavian immigrants, and are believed to be unrelated. All families have well-established FBH, meeting our published criteria (Law and Heath 1985; Heath 1989). In brief, FBH was diagnosed in families showing autosomal dominant inheritance of uncomplicated hypercalcemia, no evidence for primary hyperparathyroidism or multiple endocrine neoplasia, and mean urinary calcium:creatinine clearance ratios <0.01 (Marx et al. 1981*a*, 1982). Members of kindred 11677 were 5–81 years of age; mean serum total calcium ± SE was 11.0 ± 0.09 mg/dl (normal adult range 8.9–10.1 mg/dl; Keating et al. 1969); and serum ionized calcium was 5.78 ± 0.05 mg/dl (normal adult range 4.75–5.20 mg/dl; Burritt et al. 1990). We have published biochemical data for the other families (Heath and Leppert 1992). There were no phenotypic distinctions among the families, except that average serum calcium values in affected members of kindreds 11672 and 11675 were lower (10.7 and 10.4 mg/dl, respectively) than in the other three kindreds (11.0–11.9 mg/dl) and that kindred 11677 was the only one in which

Table 1

Markers Used in the Present Studies

Chromosome	Locus	Marker	Heterozygosity[a] (%)
3q21-qter	ACPP	ACPP	61
3	D3S20	CRI-1169	74
3p14-p13	D3530	pYNZ86.1	49
3	D3S47	CRI-C17	69
3	D3S196	Mfd17	NA
3p13	D3S693	cCI3-570	28
3q23	D3S706	cCI3-607	49
3q21-qter	D3S1206	LA153	62
3cen-q21	D3S1215	MIT-MS207	80
3	D3S1238	Mfd125	78
3q21-q24	RHO	Mfd2	NA
19p13.3	D19S20	pJCZ3.1	79
19	D19S21	cMCOB5	63
19	D19S24	MCT6	67
19	D19S266	pYNZ21	NA

NOTE.—Physical map locations are given where available (NIH/CEPH Collaborative Mapping Group 1992).

[a] NA = not available.

Table 2

Total Lod Scores for Markers on Chromosome 3, for Kindreds 11672, 11673, 11676, and 11677 and Excluding Kindred 11675

Locus[a]/Marker	Total Lod Score at θ =						
	.001	.01	.05	.10	.20	.30	.40
D3S30/pYNZ86.1	−4.66	−3.35	−1.90	−1.19	−.53	−.24	−.11
D3S693/CI3-570	−1.74	−.13	1.18	1.61	1.58	1.08	.43
D3S1215/MIT-MS207	10.26	11.00	10.83	9.97	7.69	5.01	2.18
D3S20/CRI-1169	3.03	3.26	3.05	2.71	1.88	.99	.26
RHO/Mfd2	2.85	3.71	4.08	3.94	3.23	2.26	1.10
D3S1238/Mfd125	.28	2.18	3.71	4.16	3.87	2.91	1.57
D3S47/CRI-C17	−3.05	.85	4.92	6.17	6.04	4.53	2.32
D3S1206/LA153	−6.29	−3.43	.00	1.31	2.02	1.70	.82
D3S706/CI3-607	−3.07	−1.79	−.46	.07	.35	.29	.14
D3S196/Mfd17	−2.70	.23	2.57	3.43	3.46	2.57	1.26

[a] Loci are shown in genetic map order from centromeric to telomeric.

severe neonatal hypercalcemia had been recognized (in two brothers).

For these studies, we confirmed affection status for each member, drawing blood with minimal venous stasis and measuring both total and ionized calcium and interpreting the values against age- and sex-specific norms (Keating et al. 1969; Burritt et al. 1990). Classifications were determined a priori and before determination of genotypes. Because FBH is essentially fully penetrant, and because serum calcium values are bimodally distributed in FBH families (Rajala and Heath 1987), we were able to assign, unambiguously, affection status in 94% of individuals. To minimize misclassification, we took into account both total and ionized calcium values, as well as past data from clinical and research records. Individuals whose total or ionized calcium values either fell within a zone of ambiguity (±0.025 mmol/liter of the appropriate upper limit of normal) or were uninterpretable because of other medical conditions were classified as "unknown" (Heath and Leppert 1992). Of the total 140 members, only 8 (5.7%) were classified as unknown (3 at risk of FBH and 5 not at risk). We classified 65 as affected and 67 as unaffected.

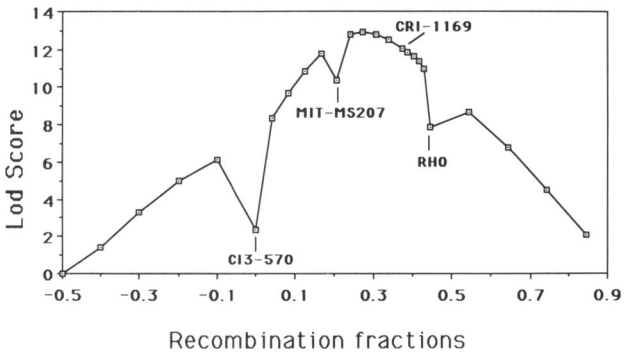

Figure 2 Multilocus analysis of chromosome 3q linkage data for kindreds 11672, 11673, 11676, and 11677, with markers cCI3-570, MIT-MS207, CRI-1169, and Mfd 2. The highest likelihood for location of the FBH gene is between loci defined by markers MIT-MS207 and CRI-1169 (maximum lod score 12.9). For all four markers used in this specific multilocus analysis, no allele number reduction was necessary.

Biochemical Methods

Total serum and urine calcium concentrations were determined in the clinical laboratories of the Mayo Clinic, either by automated clinical chemistry analyzer or atomic absorption spectroscopy using lanthanum-containing diluent, with calibration against a standard provided by the U.S. National Bureau of Standards (Keating et al. 1969; Burritt et al. 1990). Serum ionized calcium was measured at the Mayo Clinic by using an ion-selective electrode (Radiometer ICA-1; Radiometer, Copenhagen) (Burritt et al. 1990). Plasma intact PTH was quantified by a two-site immunochemiluminometric assay at the Mayo Clinic (Kao et al. 1992).

DNA Analyses

We obtained blood samples, prepared DNA, transformed lymphoblasts with Epstein-Barr virus, and carried out Southern analyses by standard methods detailed elsewhere (Heath and Leppert 1992). Analysis of sequence-tagged repeat sequences (STRs) was also conducted, by widely utilized PCR methods (Weber and

Table 3

Two-Point Lod Scores for Four Markers from Chromosome 3, Applied to Kindred 11675

Locus/Marker	Two-Point Lod Score at θ =						
	.001	.01	.05	.10	.20	.30	.40
D3S1215/MIT-MS207	−6.63	−4.14	−2.09	−1.21	−.42	−.08	.04
D3S196/cCRI-1169	−7.18	−5.29	−3.79	−2.82	−1.50	−.74	−.27
RHO/Mfd2	−4.06	−3.16	−2.04	−1.27	−.49	−.13	.01
D3S47/cCRI-C17	−7.13	−5.21	−3.72	−2.94	−1.66	−.79	−.27

Note.—The data do not support a chromosome 3 localization for the FBH locus in this family.

May 1989; Chou et al. 1992). In brief, oligonucleotide primers were end-labeled for 30 min at 37°C in a 20-μl reaction volume (14 μl [^{32}P] ATP [3,000 Ci/mmol]; New England Nuclear, Boston), 10 μmol primer DNA, 1 × kinase buffer (50 mM Tris pH 7.5, 10 mM $MgCl_2$, and 5 mM DTT), and 30 U of T4 polynucleotide kinase (Molecular Biology Resources, Madison). The reaction was stopped by heating at 90°C for 2 min. Labeled primers were stored overnight at 0°C or were used directly.

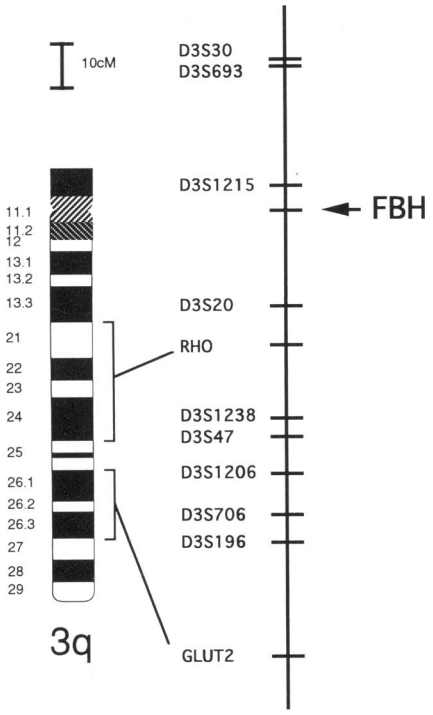

Figure 3 Schematic diagram of chromosome 3, and genetic map showing relative locations of markers linked to the FBH phenotype in kindreds 11672, 11673, 11676, and 11677, and calculated placement of the FBH locus. Note that the bulk of the short arm is omitted from the diagram. Distances between markers are in centimorgans.

We amplified genomic DNA sequences by PCR (Techne Thermal Cycler PHC-3; Princeton, NJ) in a 25-μl final volume, using 200 ng DNA template, 200 μmol each of dATP, dCTP, dGTP, and dTTP, 1 × PCR buffer (10 mM Tris pH 8.4, 40 mM NaCl, and 1.5 mM $MgCl_2$), 0.25 mM spermidine (Sigma Chemicals, St. Louis), 0.25 pmol of end-labeled primer, 10 pmol of unlabeled A and B primers, 1.25 μl of dimethylsulfoxide, and 2 U of *Taq* DNA polymerase (Boehringer Mannheim, Indianapolis). Samples were overlaid with one drop of mineral oil and were processed through 30 cycles at temperatures and times as indicated for specific primers. Ten microliters of sample buffer (50 ml of formamide, 50 mg of xylene cyanol FF, and 50 mg of bromophenol blue, in 10 mM NaOH) was added to each sample. Five microliters of sample was subjected to electrophoresis in 7% polyacrylamide DNA sequencing gels. An M13 dideoxy sequencing ladder was included for size comparisons. Gels were placed immediately on film (Amersham Hyper-film MP; Amersham, Arlington Heights, IL) at −70°C.

Genetic Linkage Analysis

This study began with a candidate gene strategy in which we tested seven loci (Heath and Leppert 1992) and then proceeded to a general linkage search. In the latter, we tested 49 DNA probes or PCR-based markers on 20 chromosomes before achieving linkage on chromosome 19p for kindred 11675. The sites on chromosome 3 for which we initially achieved linkage in the remaining families were chosen on the basis of the report by Chou et al. (1992).

All films were read independently by at least three people, and the senior investigators (H.H. and M.F.L.) reached agreement on all genotypes. Data were entered into the local clinical data base (Sybase) in the Howard

Table 4

Two-Point Lod Scores for Markers From Chromosome 19p for Kindred 11675

Locus/Marker	Two-Point Lod Score at θ =						
	.001	.01	.05	.10	.20	.30	.40
D19S21/cMCOB5	1.20	1.18	1.09	.97	.72	.44	.17
D19S20/pJCZ3.1	3.44	3.38	3.14	2.83	2.14	1.38	.59
D19S266/pYNZ21	3.70	3.64	3.40	3.08	2.40	1.64	.82
D19S24/MCT6	−.38	.46	.98	1.06	.91	.60	.26

Note.—Map distances between the four 19p markers are as follows: D19S21–.07–D19S20–.06–D19S266–.51–D19S24.

Hughes Medical Research Institute Laboratories computers, for analysis with the LINKAGE program. In over 30 families with FBH, we have never seen evidence for nonpenetrance in obligate carriers, but we made a conservative estimate of penetrance, .90, and assumed the gene frequency to be .001. We chose this conservative approach to minimize effects of misclassifications due to artifacts affecting serum calcium values (e.g., altered serum proteins), possible incomplete penetrance, and rare phenocopies (e.g., unrecognized coincidental primary hyperparathyroidism). Allele frequencies of marker loci used in linkage calculations were determined from genotypes of individuals (n = 26–30) marrying into the five FBH families. We calculated logarithm of odds (lod) scores at various recombination fractions (θ). Multilocus linkage analyses were performed by using LINKMAP, a subroutine of LINKAGE. We analyzed adjacent markers jointly in triplets, carrying out sequential three-point linkage runs across the region and using each marker as a starting point. Because four marker loci (D3S1215, D3S1238, D3S47, and D3S196) were highly polyallelic, it was necessary for these calculations to reduce alleles as described elsewhere (Lange and Weeks 1989). Allele reductions were carried out in a manner that did not significantly alter lod scores (data not shown).

All of the RFLPs, VNTR polymorphisms, and PCR-based polymorphisms used in the genetic linkage analysis have been mapped and published (Nakamura et al. 1989; NIH/CEPH Collaborative Mapping Group 1992). The markers used (11 for chromosome 3 and 4 for chromosome 19), their map locations, and percent heterozygosity are presented in table 1. Initially, all families were analyzed together, until statistical evidence for heterogeneity emerged.

Results

Chromosome 3 Families

Because localization to chromosome 3q had been recently described elsewhere (Chou et al. 1992), we applied 10 markers from chromosome 3, finding significant linkage with 6 of them. Two-point lod scores at various θ values are given in table 2. The highest total lod score, 11.0, was obtained with marker MIT-MS207 for D3S1215 (3cen-q21). Two markers mapped to 3p

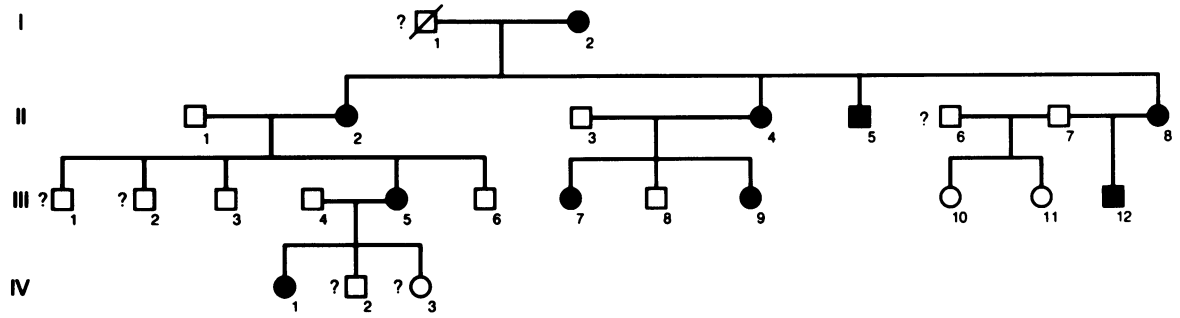

Figure 4 Pedigree of kindred 11675, which showed linkage of FBH to genetic markers on chromosome 19p (genotypes shown in table 5). Symbols are as in fig. 1. II-6 was the first husband of II-8 (III-10 and III-11 are their offspring), and II-7 is the second husband of II-8 (III-12 is their offspring).

Table 5

Genotypes Obtained with Four Markers on Chromosome 19p in Kindred 11675

Pedigree and Individual[a]	D19S21/ cMCOB5	D19S20/ pJCZ3.1	D19S266/ pYNZ21	D19S24/ MCT6
I:				
1?, d	...	...	...	...
2[b]	3,4	3,6	7,12	1,2
1	3,5	...	15,15	1,1
2[b]	3,3	6,9	4,7	1,2
3	2,3	9,9	9,15	1,1
4[b]	3,3	3,6	5,7	1,2
5[b]	3,3	3,6	5,7	1,2
6?, ns	...	...	...	...
7	3,3	8,9	4,8	1,2
8[b]	3,3	3,6	5,7	1,2
II:				
1?	...	...	...	...
2?	3,3	6,9	7,15	1,2
3	3,5	8,9	4,15	1,1
4	2,3	9,9	4,14	1,1
5[b]	3,5	6,8	7,15	1,2
6	3,3	9,9	4,15	1,1
7[b]	3,3	6,9	7,15	1,2
8	2,3	3,9	5,9	1,1
9[b]	2,3	6,9	7,9	1,2
10	3,5	3,7	4,5	1,2
11	3,3	3,5	5,15	1,2
12[b]	3,3	6,8	7,8	1,1
III:				
1[b]	3,3	6,9	4,7	...
2?, ns	...	...	...	...
3?, ns	...	...	...	...

[a] Case codes are taken from the pedigree of the family shown in fig. 4.

[b] Affected with FBH.

(pYNZ86.1 and cCI3-570) were unlinked with FBH. We carried out multipoint linkage analysis using all 10 markers in combinations of up to 4 markers at a time, and we localized the FBH gene between D3S1215 and D3S20, with a maximum location score of 12.9 (fig. 2). Kindred 11675 was analyzed with four markers for 3q, and we confidently excluded linkage in all cases, with lod scores at $\theta = .001$ ranging from -4.06 to -7.18 (table 3). Figure 3 shows schematically the relationships among linked markers on chromosome 3, as well as our estimation of the region containing the FBH locus.

Chromosome 19 Families

The FBH trait in family 11675 mapped to chromosome 19p, as shown by the two-point linkage data for four markers shown in table 4. We found a maximum lod score of 3.70 at $\theta = .001$ with the VNTR marker pYNZ21. In this family, the data were fully informative without recombinants; the pedigree is given for kindred 11675 in figure 4, and genotypes for all four markers are given in table 5. The other four families, when typed with the markers from chromosome 19, showed no evidence of linkage, with total lod scores ranging from -5.49 to -18.19 at $\theta = .001$ (table 6).

Analysis of the lod score data by the HOMOG program, version 3R (data not given), which tests linkage of families to two unlinked marker loci, showed significant evidence for locus heterogeneity among all five kindreds ($\chi^2 = 8.39$, 1 df, $P < .002$). The four kindreds 11672, 11673, 11676, and 11677 gave high conditional probabilities (.996–1.000) of linkage to a locus on chromosome 3q, while one family (11675) gave a conditional probability of 1.000 for linkage to a locus on chromosome 19p.

Discussion

Our linkage analyses confirm that, in most families manifesting the FBH phenotype, the disease gene maps to chromosome 3q (in four of our five study families and eight of the nine families studied so far; Chou et al. 1992) but reveal that an indistinguishable clinical phenotype is also linked to a region on the short arm of chromosome 19. However, whereas Chou et al. (1992) found the FBH locus to be most closely linked to the RHO and D3S47 loci in a region spanning about 9 cM, that finding was based on linkage analyses using only three markers, including D3S196. Our linkage analysis is based on calculations using 10 markers, and it places the FBH locus more centromeric on chromosome 3q, with recombination frequencies approximately 10% centromeric to D3S20, and about 7% telomeric to D3S1215. No genes known to be involved in calcium transport have been localized thus far to this region of chromosome 3q.

There are several possible explanations as to why our localization on 3q differs from that reported by Chou et al. (1992). First, they did not apply markers centromeric to ACPP, as we did. However, when we performed location-score calculations using the same markers (Mfd2 [RHO], CRI-C17 [D3S47], and Mfd17 [D3S196]) with the same map order and distances as reported by Chou et al., our localization of FBH was still centromeric to RHO. Furthermore, we rigorously defined affection status, and we classified as unknown those individuals whose calcium values lay in the zone of ambiguity between the normal and FBH distribu-

Table 6

Two-Point Lod Scores for Four Markers from Chromosome 19p, Applied to the Kindreds (11672, 11673, 11676, and 11677) Showing Linkage to Markers on Chromosome 3q

	Two-Point Lod Score at θ =						
Locus/Marker	.001	.01	.05	.10	.20	.30	.40
D19S21/cMCOB5	−7.04	−4.99	−2.88	−1.65	−.48	.00	.12
D19S20/pJCZ3.1	−18.19	−13.33	−7.51	−4.47	−1.61	−.41	.01
D19S266/pYNZ21	−14.98	−9.59	−4.46	−2.28	−.55	.01	.10
D19S24/MCT6	−5.49	−4.47	−2.70	−1.80	−.90	−.42	−.16

Note.—The data clearly exclude a 19p localization of the FBH gene for these four families

tions (Rajala and Heath 1987). Chou et al. (1992), on the other hand, appear to have classified members only as affected or not, on the basis of serum calcium values above or below +3 SD from the normal mean. Nonetheless, only further studies with additional families, using a common set of markers and the same diagnostic criteria, will determine the correct map location.

For family 11675, the FBH gene appears to lie on chromosome 19p, with a peak lod score of 3.70 at θ = .001 for pYNZ21. Lack of recombinants or of additional family material prevents finer mapping. Similarly to the 3q FBH locus, there are no known calcium-regulatory genes localized to this region of 19p. Nonetheless, the data strongly suggest that there are at least two FBH genes. The FBH phenotype is bland and stereotypical (Foley et al. 1972; Marx et al. 1977, 1981*a;* Law and Heath 1985; DA Heath 1989; H Heath 1989), and we are unable to detect any systematic difference between kindred 11675 and the others, with one possible exception: the mean serum calcium level of affected persons in kindred 11675 is the lowest of the five families we have studied, although it is not significantly different from the mean value for kindred 11672, which has the next highest mean. Only study of additional families will tell both the frequency of the 19p variant of FBH and whether it is generally characterized by the mildest hypercalcemia.

Our work and that of other groups has firmly excluded a number of loci and candidate genes for FBH, including genes encoding parathyroid hormone, Ca, Mg-ATPase isoform 4, a sodium:calcium exchanger, the loci mapped for multiple endocrine neoplasia types 1 and 2, calbindin D_{28K}, and basic fibroblast growth factor (Menko et al. 1984; Sopwith et al. 1984; Paterson et al. 1985; Almahroos et al. 1987; Kowalska et al. 1987; Chou et al. 1992; Heath and Leppert 1992). Therefore, we believe that the FBH phenotype results from mutations of as-yet-unknown genes on chromosomes 3q and 19p.

Successful positional cloning of the FBH genes may reveal hitherto unrecognized substances that play major roles in cellular and systemic calcium homeostasis. We suspect that the responsible gene(s) may also be involved in parathyroid gland growth and development, since homozygosity for the gene appears to cause severe parathyroid hyperplasia in infants (Marx et al. 1981*b;* Steinmann et al. 1984; Cooper et al. 1986). More important for affected persons, determining the FBH gene(s) will be of practical value in testing to distinguish between hypercalcemic states that do or do not need surgical correction.

Acknowledgments

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