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Mapping Autosomal Recessive Vitamin D Dependency Type I to Chromosome 12q14 by Linkage Analysis

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Summary

Linkage analysis in French-Canadian families with vitamin D dependency type I (VDD1) demonstrated that the gene responsible for the disease is linked to polymorphic RFLP markers in the 12q14 region. We studied 76 subjects in 14 sibships which included 17 affected individuals and 17 obligate heterozygotes. Significant results for linkage were obtained with the D12S17 locus at the male recombination fraction ($\hat{\theta}_m$) .018 ($Z[\hat{\theta}_m\hat{\theta}_f]$ = 3.20) and with D12S6 at ($\hat{\theta}_m$ = .025 ($Z[\hat{\theta}_m\hat{\theta}_f]$ = 3.07). Multipoint linkage analysis and studies of haplotypes and recombinants strongly suggest the localization of the VDD1 locus between the collagen type II alpha 1 (COL2A1) locus and clustered loci D12S14, D12S17, and D12S6, which segregate as a three-marker haplotype. Linkage disequilibrium between VDD1 and this three-marker haplotype supports the notion of a founder effect in the studied population. The current status of the localization of the disease allows for carrier detection in the families at risk.

Introduction

Since the almost complete disappearance of common rickets in countries where milk is fortified with vitamin D, attention has been attracted to vitamin D refractory syndromes in which rickets and osteomalacia develop despite adequate availability of the vitamin. The most common of these conditions, hypophosphatemic vitamin D–resistant rickets (HYP), is transmitted as a sex-linked dominant trait and has been mapped to the short arm of the X chromosome (Read et al. 1986). In other, more rare instances the phenotype includes early onset of hypocalcemia with secondary hyperparathyroidism and ensuing hypophosphatemia, together with severe rachitic lesions. Before the entity was recognized, early deaths from neurologic complications were common. Its description as "pseudo vitamin D deficiency" in 1961 (Prader et al. 1961) brought about awareness and early attempts at correcting it with large daily amounts of vitamin D. Although somewhat difficult to monitor, the therapy could induce long-term remission, hence the term "vitamin D dependency" (VDD) proposed to characterize it (Scriver 1970).

In most instances, the condition, transmitted as an autosomal recessive trait, is caused by impaired synthesis of 1α,25-dihydroxyvitamin D [1,25$(OH)_2$D], the hormonal form of the vitamin directly active on its target organs (Fraser et al. 1973). Circulating levels of 1,25$(OH)_2$D are low but still detectable (Delvin et al. 1981). This is in contrast with rare cases in which both very high levels of the metabolite and resistance to treatment with 1,25$(OH)_2$D were observed in a similar clinical context. This latter form, also recessively inherited, has been linked, in two cases, to point mutations in the vitamin D receptor gene and has been referred to as "hypocalcemic vitamin D–resistant rickets" or "vitamin D dependency type II" (Hughes et al. 1988). Thus the "classic" pseudo-deficient form is now called VDD type I (VDD1). With minimal adjustment throughout life, daily administration of 1,25$(OH)_2$D, to bring about physiologic circulating concentrations of the metabolite, completely corrects the VDD1 phenotype (Delvin et al. 1981).

Despite this evidence for replacement therapy, the pathophysiology of the disease at the molecular level

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is still uncertain, as the 25(OH)D-1α-hydroxylase is a multicomponent system (Ghazarian and Luca 1974; Ghazarian et al. 1974; Pedersen et al. 1976). Since homozygous individuals have low but detectable levels of $1,25(OH)_2D$, the disease is probably due to inadequate activity of a modified enzyme. It is not known, at present, which one of its components is affected by the mutation.

Although quite rare, the disease is present with unusual frequency in a subset of the French-Canadian population (Bouchard et al. 1984). We thus set out to map the VDD1 locus by using DNA markers and linkage analysis (Botstein et al. 1980; White et al. 1985) to approach the primary defect in the disease. Here we present evidence that the VDD1 gene is located on chromosome 12 at q14. Current information also allowed to detect heterozygous carriers in families at risk; up to now these individuals could not be identified on clinical or biochemical grounds.

Subjects and Methods

Patients

Five French-Canadian families were included in the study. Four families (A, B, C, and E) originate from the same area of Quebec: the Saguenay-Charlevoix area, where conditions of isolation prevailed until recently. The fifth family (D) and the relatives of individual III-14 (not shown) in family A originate from New Brunswick and Nova Scotia. These kindreds include 17 affected individuals currently followed in our Metabolic Clinic for periods ranging from 1 to 12 years. Diagnosis was established clinically and confirmed by measurement of circulating $1,25(OH)_2D$ (performed by Dr. E. E. Delvin, Shriners Hospital). Of the 59 healthy relatives of our patients who accepted the invitation to participate in the study, 17 were obligate heterozygotes. Phlebotomy was performed in all subjects to obtain blood for lymphocyte separation and direct DNA extraction. The procedure, in most cases, took place in the individuals' homes in northeastern Quebec.

DNA Preparation, Probes, and Hybridization

Blood samples were collected in 25 mM EDTA as anticoagulant and were frozen at −70°C until used. After defrosting in a water bath at room temperature, lymphocyte DNA was extracted by the phenol-chloroform method (Gustafson et al. 1987). DNA (5–7 μg) was digested with the appropriate restriction enzyme, followed by electrophoretic fractionation in horizontal 0.6%–0.9% agarose gels at 2–3 V/cm for 16–22 h, depending on the size of the DNA fragments to be separated. The separated DNA was blotted on a nylon membrane (BioTrace RP) by standard procedure (Maniatis et al. 1982).

The DNA probe/enzyme systems used were p640/*Taq*I (locus KRAS2 [Barker et al. 1983; Balaz et al. 1987]), pmcf.2-11/*Ban*I (locus D12S32 [Oberle et al. 1988]), pKEV4/*Pvu*II-*Hin*dIII (locus COL2A1 [collagen type II α1 chain] [Vaisanen et al. 1988]), pEFD33.2/*Msp*I (locus D12S14 [Nakamura et al. 1988*b*]), pYNH15/*Msp*I (locus D12S17 [Nakamura et al. 1988*a*]), p1-7/*Msp*I (locus D12S6 [Buroker et al. 1986]), and p7G11/*Msp*I (locus D12S8 [O'Connell et al. 1987]). The set of randomly selected DNA probes (markers), including chromosome 12 probe pEFD33.2, pYNH15, and p1-7, was provided by Dr. Y. Nakamura (Howard Hughes Medical Institute Research Laboratories, Salt Lake City), p640 and p7G11 by Drs. W. Cavenee and D. James (Ludwig Institute for Cancer Research, Montreal); pmcf. 2-11 by Dr. D. Birnbaum (Hôpital d'Enfants de la Timone, Marseille); and pKEV4 by Drs. L. Peltonen (University of Helsinki) and E. Vuorio (University of Turku, Finland).

Probe inserts were isolated from DNA plasmids in a low-melting-temperature agarose gel and were labeled with [^{32}P]dCTP by using a hexanucleotide-primed reaction (Feinberg and Vogelstein 1984). Prehybridization (2–4 h at 42°C) and hybridization (16–20 h at 42°C) were performed in the same solution: 5 × SSC, 1 × Denhardt's, 0.02 M sodium phosphate (pH 6.7), 500 μg sonicated herring sperm DNA/ml, 10% dextran sulfate, and 50% formamide. The D12S14 marker required prehybridization with an excess of human DNA to prevent sequences homologous to genomic repeats from hybridizing to the blots and obscuring single- and low-copy bands (Sealey et al. 1985). After hybridization, the blots were washed twice for 30 min at 65°C in 0.1 × SSC, 0.1% SDS, except for hybridization with the probe p640, for which 1 × SSC, 0.1% SDS solution was used.

Linkage Analysis

Linkage analysis was carried out using the likelihood method (Ott 1985). The results of analyses are reported in terms of male recombination fraction ($\hat{\theta}_m$) and female recombination fraction ($\hat{\theta}_f$). Maximum-likelihood estimates (MLE) of $\hat{\theta}_m$ and of lod score (Z) were computed using the LINKAGE package of programs (Lathrop et al. 1984, 1985; Lathrop and Lalouel 1988). We as-

sumed a constant ratio of female to male map distance of 3 (O'Connell et al. 1987). The VDD1 allele frequency was assumed to be .02. Allele frequencies for the marker loci were taken from published information.

Multipoint mapping to the region of the chromosome 12 map was carried out by the method of location scores (Lathrop et al. 1984). Log likelihoods were computed using the LINKMAP and MLINK programs of the LINKAGE analysis package (Lathrop et al. 1984, 1985; Lathrop and Lalouel 1988). Both a constant female to male map distance ratio of 3, as in the case of the two-point linkage analysis, and no interference were assumed. The male genetic map distances for the chromosome 12 loci were taken from O'Connell et al. (1987), except that the male map distance between D12S17 and D12S6 was assumed to be 0.33 cM. $\hat{\theta}$ Values were calculated using Haldane's mapping function (Haldane 1919). The KRAS2 locus, assigned to the short arm of chromosome 12 (O'Connell et al. 1987), was arbitrarily taken as the origin of the map. The other reference loci—COL2A1, D12S17, D12S6, and D12S8—are on the long arm of chromosome 12 (O'Connell et al. 1987).

Version 3.5 of LINKAGE was provided by Dr. J. Ott (Columbia University), and version 4.8 was provided by Dr. G. M. Lathrop (Centre D'Etude du Polymorphisme Humain, Paris). The programs were recompiled for execution on a Sun 3/260 workstation. Multipoint linkage analysis for six loci was carried out with single precision real variables because of limited computing resources.

Results

Linkage Analysis and Haplotype Associations

Initially the genotypes for RFLPs of individuals in three large related kindreds (pedigree A; fig. 1) were determined using 15 randomly chosen polymorphic DNA markers. In this way an indication of linkage of VDD1 to the D12S14 locus was obtained. In order to gain stronger support for the linkage, we typed additional VDD1 families. Although the D12S14 locus had been assigned to chromosome 12 by multilocus linkage analysis with loci D12S2 (on proximal 12p) and with loci COL2A1, D12S4, and D12S6 (on proximal 12q) (Nakamura et al. 1988*b*), its precise map position was unknown. Therefore we investigated chromosome 12 markers available for this region. This analysis included the loci shown in figure 2, where we indicate their published order and estimated genetic distances (O'Connell et al. 1987).

The studied families include 10 sibships with one or more affected individuals and consist of a total of 76 individuals of whom 17 are affected. In linkage analysis, consanguinity and distant kinship in pedigree A were not taken into account because most grandparents and distant ancestors were not available for typing. As a result, individual IV-10 and her father, as well as the couple (III-37 and III-38) with two unaffected children were uninformative for linkage analysis.

The results of the two-point linkage analysis of VDD1 and chromosome 12 marker loci (table 1) are significant for close linkage of VDD1 to D12S17 ($\hat{\theta}_m$ = .018, $\hat{\theta}_f$ = .053, Z [$\hat{\theta}_m, \hat{\theta}_f$] = 3.20) and to D12S6 ($\hat{\theta}_m$ = .025, $\hat{\theta}_f$ = .070, $Z[\hat{\theta}_m, \hat{\theta}_f]$ = 3.07). In addition, there are marginally significant results for linkage of VDD1 to COL2A1 ($\hat{\theta}_m = \hat{\theta}_f$ = .0, $Z[\theta_m, \theta_f]$ = 2.14) and to D12S14 ($\hat{\theta}_m$ = .036, $\hat{\theta}_f$ = .100, $Z[\theta_m, \theta_f]$ = 2.44). These results suggest that the VDD1 locus is located near COL2A1 and three other chromosome 12q marker loci: D12S14, D12S17, and D12S6. Although no recombinants were observed between COL2A1 and VDD1, the lod score of $Z(\hat{\theta}_m, \hat{\theta}_f)$ = 2.14 did not reach the conventional level of significance of 3. In addition, tight linkage of D12S14 to D12S17 ($\hat{\theta}_m$ = .0, $\hat{\theta}_f$ = .0, Z [$\hat{\theta}_m$, $\hat{\theta}_f$] = 11.23) and to D12S6 ($\hat{\theta}_m$ = .019, $\hat{\theta}_f$ = .056, $Z[\hat{\theta}_m, \hat{\theta}_f]$ = 4.70) was found. Only one recombinant between these three loci was observed (pedigree A, individual III-43; fig. 1). Therefore D12S14 is provisionally mapped to chromosome 12q very close to D12S17 (fig. 2).

To localize more precisely the VDD1 locus, multilocus linkage analysis was also done. Multipoint lod scores (Z) of the location of VDD1 on the map of five chromosome 12 loci—KRAS2, COL2A1, D12S17, D12S6, and D12S8—are graphed in figure 3. The maximum-likelihood location of VDD1 coincides with the COL2A1 locus (Z = 4.8). A lower peak (Z = 3.7) occurs in the interval between D12S6 and D12S8, approximately 17 cM from KRAS2, making this second location less likely.

The three markers D12S14, D12S17, and D12S6 are tightly linked and segregate as a haplotype. Since each of the contributing markers in this three-marker haplotype reveals four, three, and two alleles, respectively, the maximum number of haplotypes is 24 (4 × 3 × 2). In our study we observed 13 different three-marker haplotypes among the 76 studied individuals. Three crossing-overs were observed between VDD1 and the three-marker haplotype (pedigree A, individuals III-3 and III-13; and pedigree E, individual II-2; fig. 1). Those events place the VDD1 locus outside the three-marker

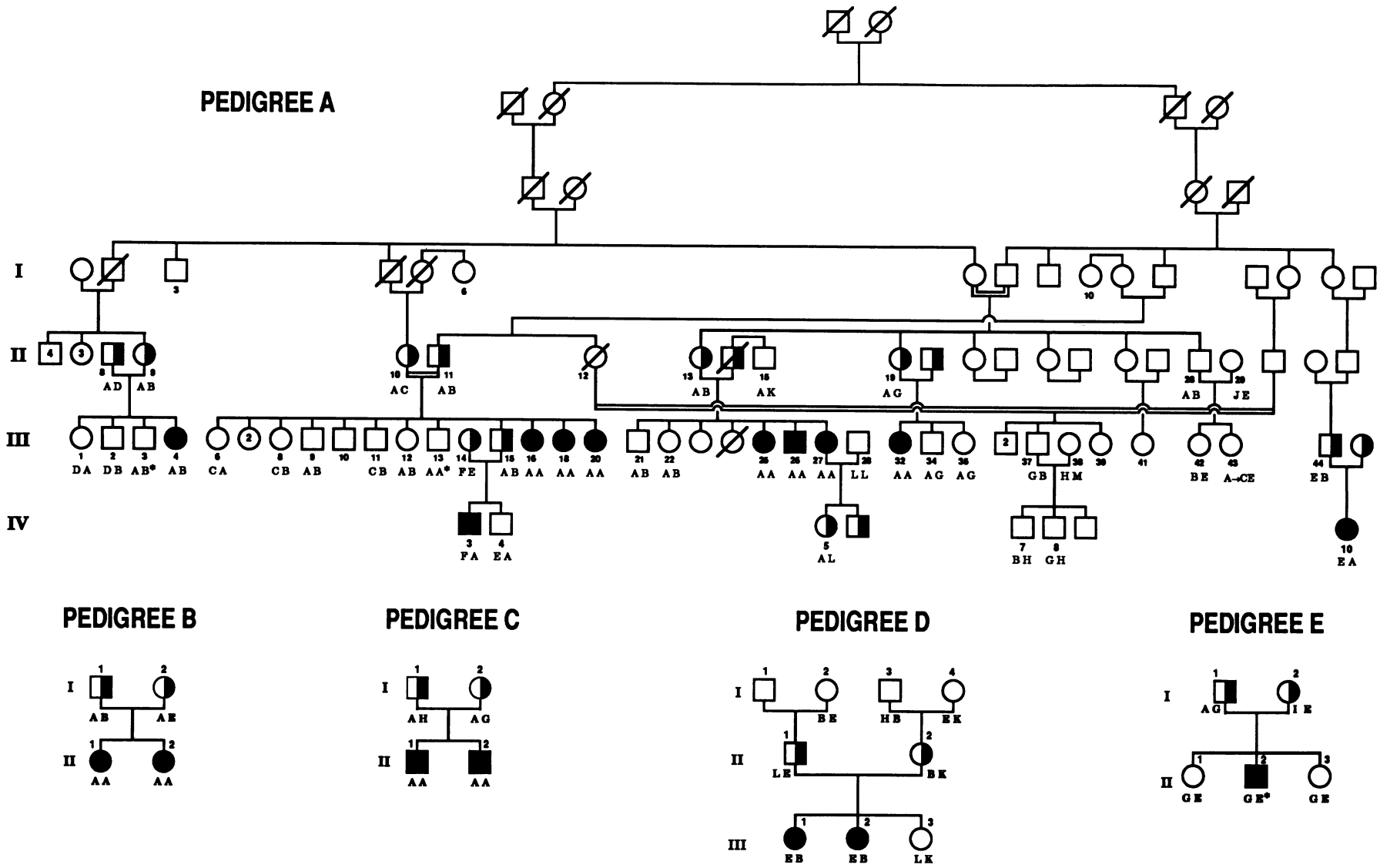

Figure 1 Pedigrees of the families studied by linkage analysis. Shaded circles (females) and squares (males) represent affected individuals; half-shaded symbols represent obligate heterozygotes. Letters below the individuals represent the various forms of the three-marker haplotype localized on chromosome 12q14. The haplotype consists of the marker loci D12S14, D12S17, and D12S6; these haplotypes are as follows: A = 2,1,1; B = 4,2,2; C = 2,1,2; D = 3,3,1; E = 2,2,2; F = 1,1,2; G = 4,2,1; H = 1,3,2; I = 1,2,1; J = 1,2,2; K = 1,1,1; L = 2,2,1; M = 4,3,2, where 1–4 correspond to the alleles of the respective markers. Asterisks mark recombinants between the three-marker haplotype and the VDD1 locus. A→C indicates a recombinant within the haplotype (pedigree A, individual III-43).

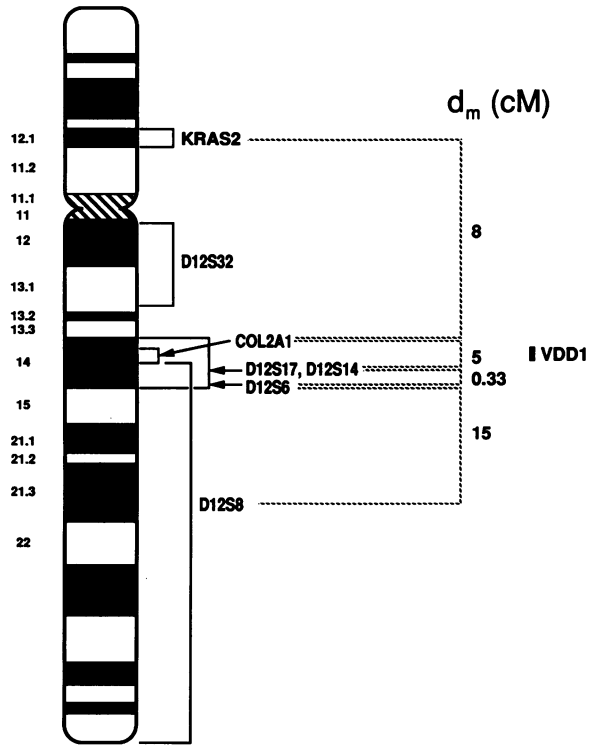

Figure 2 Chromosome 12. Male genetic map distances (d_m) are given in centimorgans (cM) between the selected DNA marker loci KRAS2, COL2A1, D12S17, D12S6, and D12S8, as reported by O'Connell et al. (1987), except that the distance between D12S17 and D12S6 was adjusted to be 0.33 cM. Female genetic map distances are assumed to be three times the male map distances. D12S32 was assigned by in situ hybridization (Oberle et al. 1988), D12S14 is assigned in the present study by linkage analysis, and the proposed localization of VDD1 is 12q14.

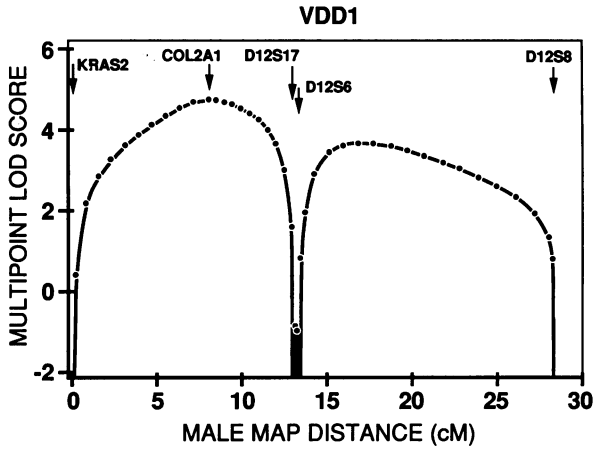

Figure 3 Multipoint linkage analysis of the location of VDD1 relative to a genetic map of five loci on chromosome 12 (see fig. 2 legend and Subjects and Methods for details). The location of VDD1 is varied across the map from KRAS2 to D12S8. Male genetic map distances are measured cumulatively in centimorgans from KRAS2. No interference is assumed. Values of the multipoint lod score < -2 were excluded from the graph.

haplotype. Furthermore, because COL2A1 reveals two different two-allelic polymorphorisms related to *Pvu*II and *Hin*dIII restriction sites, they can be presented in the form of a COL2A1 haplotype. In pedigree E (fig. 4) crossing-over was observed between the three-marker haplotype and the VDD1 locus, with, at the same time, segregation of the disease allele with the COL2A1 haplotype 1,1 (individual II-2). The same situation occurred in individuals III-3 and III-13 in pedigree A (fig. 4). However, individual III-13 is indistinguishable from his affected sibs, because their father is homozygous for the COL2A1 haplotype. Therefore we conclude that the VDD1 locus is located above the three-marker haplotype and near the COL2A1 locus, most likely between the latter and the three-marker haplotype. This indicates the following order of loci: cen–COL2A1–VDD1–(D12S14, D12S17)–D12S6–D12S8–qter. Since COL2A1 was physically assigned to the 12q14.3 region by using a panel of somatic cell hybrids containing various por-

Table 1

Two-Point Linkage Analysis of VDD1 versus Chromosome 12 Loci

	$Z(\theta_m,\theta_f)$ AT (θ_m) OF						MLE OF	
CHROMOSOME 12 LOCI	.0	.01	.05	.10	.20	.30	$\hat{\theta}_m$	$Z(\hat{\theta}_m,\hat{\theta}_f)$
KRAS2	$-\infty$	−.99	−.08	.03	.00	−.01	.104	.03
D12S32	$-\infty$	−.61	−.07	−.03	−.06	−.04	.500	.00
COL2A1	2.14	1.98	1.41	.86	.27	.08	.000	2.14
D12S14	$-\infty$	1.98	2.39	1.92	.97	.42	.036	2.44
D12S17	$-\infty$	3.14	2.91	2.21	1.09	.47	.018	3.20
D12S6	$-\infty$	2.93	2.92	2.31	1.15	.49	.025	3.07
D12S8	$-\infty$	−.41	.14	.25	.22	.12	.124	.26

NOTE.—Significant results for linkage between VDD1 and D12S17 and between VDD1 and D12S6 are shown. Two other loci, D12S14 and COL2A1, show marginally significant results for linkage.

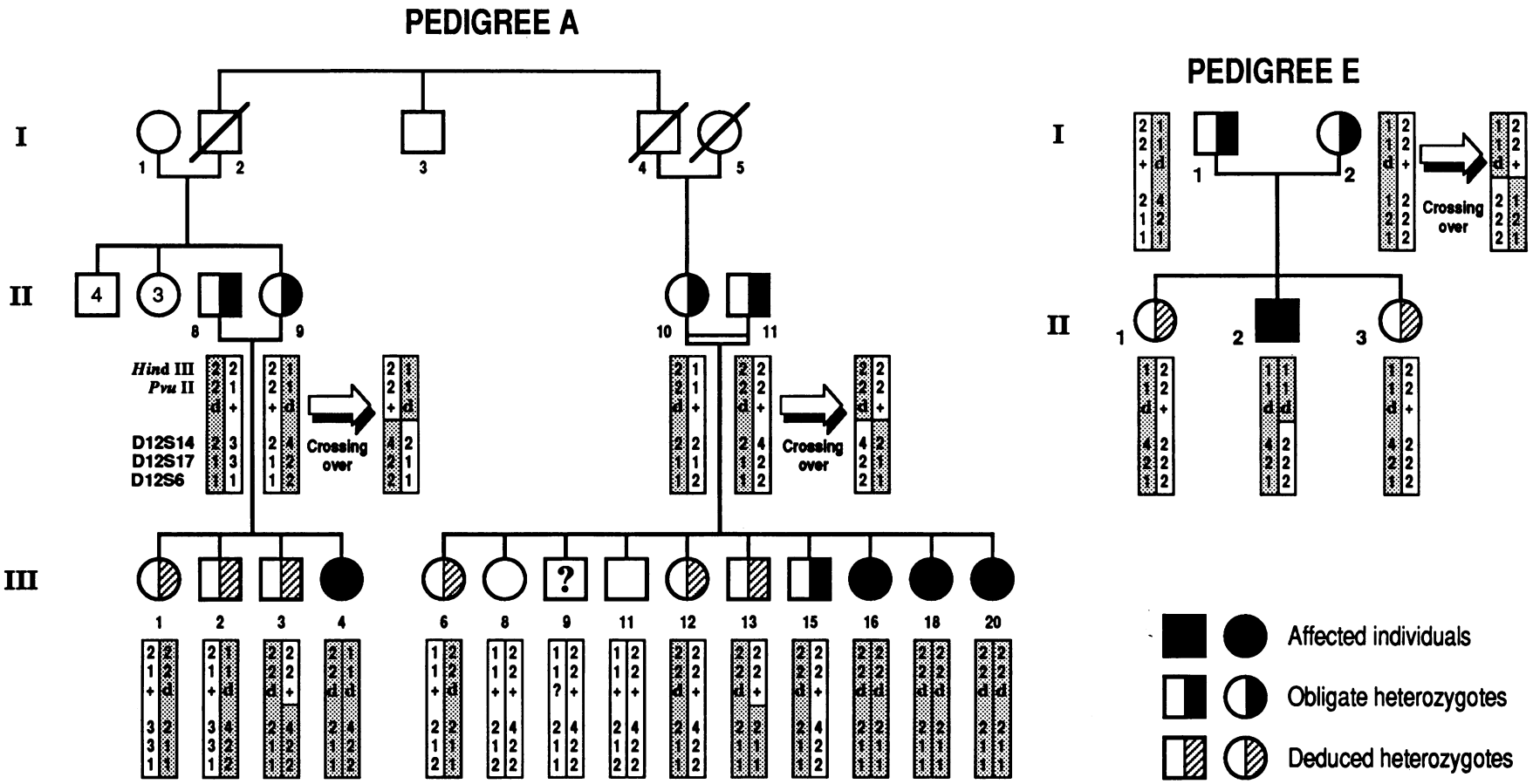

Figure 4 Selected families with recombinant individuals. Under each genotyped subject, alleles are listed for the loci COL2A1 (*Pvu*II, and *Hind*III—upper part of the box) and D12S14, D12S17, and D12S6 (lower part of the box). *d* = VDD1 allele; + = normal allele. Shaded boxes designate chromosomes with the mutation. The carrier status of individual III-9 is ambiguous (?) because his maternal haplotype is the result of a recombination with no precise point of crossing-over (the mother is not informative for D12S14 and D12S17). Recombination could have occurred anywhere between the COL2A1 and D12S6 loci. The crossover in pedigree E was assumed to be maternal, on the basis of the higher recombination fraction in females.

tions of chromosome 12 (Law et al. 1986), the implication is that the VDD1 locus is located in the 12q14 region.

The total number of observed three-marker and COL2A1 haplotypes on VDD1 and normal chromosomes is given in table 2. Eleven (69%) of 16 of the VDD1 chromosomes were bearing the same 3-marker haplotype A. Among 15 normal chromosomes bearing nine different three-marker haplotypes, only one haplotype A was found. The association of the disease with the three-marker haplotype A, and of the normal allele with haplotypes other than A, is significant by Fisher's exact test (two-tailed test, $P = .0006$). This indicates a linkage disequilibrium (allelic association) between the VDD1 locus and the three-marker haplotype, a linkage disequilibrium which provides additional evidence for the mapping of the VDD1 locus. The observed linkage disequilibrium is consistent with the suggested founder effect for VDD1 in the French–Canadian population of the Saguenay-Charlevoix region of eastern Quebec (Bouchard et al. 1984; De Braekeleer et al. 1988). There is no significant association of the mutation with any of COL2A1 haplotypes (table 2).

Discussion

We have assigned the gene for autosomal recessive VDD1 to chromosome 12 by linkage analysis. Its most likely location is in the region 12q14 near the COL2A1 locus and a three-marker haplotype. The maximum-likelihood location of VDD1, on the basis of multipoint linkage analysis (fig. 3), coincides with the position of the COL2A1 locus. If a 10:1 support limit (White and Lalouel 1987) is used as an approximate confidence interval for the location of VDD1 around COL2A1, this interval would extend about 4 cM on either side of COL2A1 on the male genetic map. However, on the basis of the analysis of the extended haplotypes (COL2A1 and three-marker haplotypes) in family B (fig. 5), in which both parents (I-1 and I-2) originate from two neighboring hamlets in the Saguenay-Charlevoix region, we suggest that VDD1 is located between COL2A1 and the three-marker haplotype. On both parental chromosomes the whole region encompassing VDD1 and the three-marker haplotype is very likely to be identical by descent, because the mutation is associated with haplotype A. Because the occurrence of haplotype A in the studied families is rare if not associated with the VDD1 mutation (see, e.g., the normal chromosomes in table 2), its random association with the mutation is very unlikely. Thus, if COL2A1 were localized between VDD1 and the three-marker haplotype, then homozygosity of the three-marker haplotype would imply homozygosity of the COL2A1 haplotype. Therefore, if a common ancestor for the VDD1 mutation on both parental chromosomes in family B is assumed (and if double recombination, very unlikely in such a small region, is excluded), the data can be explained by recombination between the COL2A1 haplotype and VDD1, if VDD1 is placed internally between the COL2A1 locus and the three-marker haplotype.

The high incidence of VDD1 in the French–Canadian population, especially in the Saguenay–Charlevoix region, is likely to be the result of a founder effect in

Table 2

Number of Observed Haplotypes on VDD1 and Normal Chromosomes in Obligate Heterozygotes

	VDD1	Normal
Three-marker haplotype:		
A	11	1
Non-A	5	14
COL2A1 haplotype:		
2,2	12	9
1,1	4	5
2,1	...	1

NOTE.—In the tabulation of the three-marker haplotypes (D12S14, D12S17, and D12S6) on VDD1 and normal chromosomes, only chromosomes of obligate heterozygous parents were counted; copies identical by descent of the same haplotype segregating in the 3-generation pedigree were not counted. The normal paternal chromosome of subject IV-5 in pedigree A was included. Family E was excluded from calculation of the three-marker haplotype, since the affected child was a recombinant.

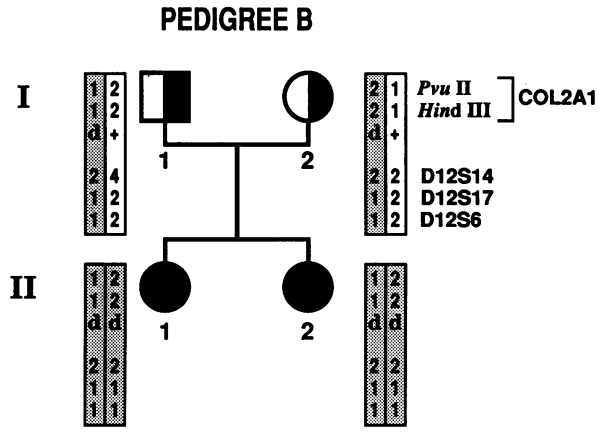

Figure 5 Analysis of extended haplotypes in family B that lead to the proposed localization of VDD1 vs. the COL2A1 locus (see Discussion for interpretation). The three-marker haplotype (D12S14, D12S17, and D12S6 [2,1,1]) corresponds to haplotype A in fig. 1.

a geographically isolated population (Bouchard et al. 1984; De Braekeleer et al. 1988). Thus homozygosity due to identity by descent around the disease locus in affected subjects is expected (Lander and Botstein 1986). Indeed, we have found the mutation to be linked with the same three-marker haplotype A in 69% of the cases. Among the VDD1 chromosomes of obligate heterozygotes from the Saguenay-Charlevoix region, the linkage disequilibrium was even stronger, with 11 (85%) of 13 being associated with the three-marker haplotype A. The non-A haplotypes on VDD1 chromosomes may be the result of crossing-over if there was only one original founder, but more than one independent mutation or more than one founding chromosome cannot be excluded. In the COL2A1 locus, we observed affected individuals with two different *Pvu*II-*Hin*dIII haplotypes: 2,2 and 1,1 (the only two haplotypes found in the studied sample, with the exception of one haplotype 2,1). This would be evidence for recombination between VDD1 and COL2A1, if it is assumed that there was one original VDD1 mutation (although we did not observe any recombination in this study).

The localization of VDD1 allows prediction of carrier status in individuals at risk in the analyzed families, as well as early diagnosis of newborns. If, indeed, the COL2A1 and the three-marker haplotype do flank the VDD1 locus, the accuracy of carrier detection could exceed 99% in families informative for those markers. Using haplotype informations, we deduced carrier status for several individuals in the studied families, as shown in figure 4.

Although it is accepted that VDD1 is due to a deficiency of the renal mitochondrial 1α-OHase, leading to insufficient synthesis of $1,25(OH)_2D_3$ (Fraser et al. 1973), the exact target of the mutation is not known. The mutation could affect either modulators of the enzyme activity expression or the structure of the enzyme itself. The 1α-OHase is composed of three subunits, cytochrome $P\text{-}450_{D1\alpha}$, ferredoxin, and ferredoxin reductase (Ghazarian et al. 1974; Ghazarian and DeLuca 1974; Pedersen et al. 1976) (ferrodoxin is known also as "adrenodoxin," as it was first isolated from adrenal glands). Thus, a priori, each of these could be affected by the mutation. However, it has recently been shown that the two components of the electron-transfer system which are shared with other cytochrome P-450s are encoded by single-copy genes expressed in all steroidogenic tissues (Morel et al. 1988; Solish et al. 1988). Since VDD1 patients express no evident deficiencies in the activity of other cytochrome P-450 systems, the cytochrome P-450–specific component of 1α-OHase remains as the most likely target of the VDD1 mutation. Nevertheless, heterogeneity (in the sense of different loci being involved) in this condition cannot be excluded until further studies are done. One crucial step is the recent production of monoclonal antibodies against cytochrome $P\text{-}450_{D1\alpha}$, since these can be used to identify and isolate the cDNA encoding the protein (Bort and Crivello 1988). If physically assigned to 12q14, such a clone could be used to search for the VDD1 mutation in our patient population, as well as in other ethnic groups.

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