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Exclusion of the Locus for Autosomal Recessive Pseudohypoaldosteronism Type 1 from the Mineralocorticoid Receptor Gene Region on Human Chromosome 4q by Linkage Analysis*

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ABSTRACT

Pseudohypoaldosteronism type 1 (PHA1) is an uncommon inherited disorder characterized by salt-wasting in infancy arising from target organ unresponsiveness to mineralocorticoids. Clinical expression of the disease varies from severely affected infants who may die to apparently asymptomatic individuals. Inheritance is Mendelian and may be either autosomal dominant or autosomal recessive. A defect in the mineralocorticoid receptor has been implicated as a likely cause of PHA1. The gene for human mineralocorticoid receptor (*MLR*) has been cloned and physically mapped to human chromosome 4q31.1–31.2. The etiological role of *MLR* in autosomal recessive PHA1 was investigated by performing linkage analysis between PHA1 and three simple sequence length polymorphisms (*D4S192*, *D4S1548*, and *D4S413*) on chromosome 4q in 10 consanguineous families. Linkage analysis was carried out assuming autosomal recessive inheritance with full penetrance and zero phenocopy rate using the MLINK program for two-point analysis and the HOMOZ program for multipoint analysis. Lod scores of less than −2 were obtained over the whole region from *D4S192* to *D4S413* encompassing *MLR*. This provides evidence against *MLR* as the site of mutations causing PHA1 in the majority of autosomal recessive families. (*J Clin Endocrinol Metab* **80:** 3341–3345, 1995)

PSEUDOHYPOALDOSTERONISM type 1 (PHA1) is an uncommon inherited disorder characterized by salt wasting in infancy and associated with failure to thrive, hyponatremia, and hyperkalemia (1, 2). Marked elevation of serum aldosterone levels is present in all cases, and PRA is increased in most (3). Patients are unresponsive to mineralocorticoids, but respond to salt supplementation which can often be discontinued after infancy. Since the first report by Cheek and Perry in 1958 (4), over 100 cases have been reported.

Clinical expression of the disease varies from severely affected infants who may die to apparently asymptomatic individuals (5). This depends in part on whether mineralocorticoid resistance is confined to renal tubules (renal form) or involves multiple target organs, including sweat and salivary glands and colon (generalized form). Inheritance is Mendelian and may be either autosomal dominant or recessive (6, 7). It has been suggested that the renal form is usually milder and is inherited as an autosomal dominant (AD) trait. Families with the generalized form are usually more severely affected and display an autosomal recessive (AR) inheritance with an increased incidence of consanguinity.

The molecular basis of PHA1 is unknown. Cheek and Perry suggested that "the disorder may be due to a refractory state on the part of the renal tubules to endogenous mineralocorticoids," an end-organ hormone insensitivity syndrome. A primary defect in the human mineralocorticoid receptor (hMR) has remained the most plausible hypothesis for the molecular basis of PHA1. Since Albright's description of pseudohypoparathyroidism in 1942 (8), many hormone resistance syndromes have been described. These include resistance to insulin, androgens, vitamin D, cortisol, T_4, and vasopressin. Causative mutations have been described in several members of the steroid receptor superfamily of genes, including those for androgen, calcitriol, and glucocorticoids.

In 1985, Armanini and co-workers demonstrated reduced or absent binding sites for tritiated aldosterone in circulating

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mononuclear lymphocytes in three patients with PHA1 (9, 10). These observations have been confirmed in subsequent studies (11–13) and provide evidence in favor of a defect in hMR in PHA1. However, the possibility exists that they may represent a secondary effect of down-regulation due to high aldosterone levels.

Molecular cloning of a complementary DNA (cDNA) encoding hMR (14) has allowed a direct genetic approach to evaluating the hypothesis that PHA1 arises from mutations in the gene for this receptor. Analysis has been carried out by 3 independent groups on 7 patients, including the original sporadic case of Cheek and Perry. In this patient and his family, Southern blot analysis using 3 probes from the hMR cDNA revealed bands of normal size and intensity (15). In another patient and his family, hMR cDNA was amplified using 11 pairs of oligonucleotide primers, and total ribonucleic acid from peripheral blood leukocytes was amplified by reverse transcriptase-polymerase chain reaction (PCR). Sequencing of the products showed no deviation from the wild-type sequence (16). Similar results were reported in a study of 5 other patients with PHA1 (17, 18). Sequencing of the patient's hMR cDNA identified a nonconservative homozygous base change in 4 of the 5 patients, and 1 of them had an additional conservative heterozygous base change. Both of these base changes were in the immunogenic domain and were also found to be present in a considerable number of normal individuals. It was concluded that these sequence variations did not have any pathological significance. The sequences of the first untranslated exon, 0.9 kilobase of the 5′-regulatory region, and the DNA- and ligand-binding receptor domain in these 5 patients were all normal. Full evaluation of the gene for hMR (*MLR*) by direct mutational analysis awaits characterization of its genomic organization. These results do not, of course, exclude *MLR* as the site of mutations causing PHA1, particularly in the recessive variety, as genetic (locus or allelic) heterogeneity may exist.

Homozygosity mapping provides a powerful approach to testing the role of a candidate gene in a rare autosomal recessive disorder with a high level of parental consanguinity (19). *MLR* has been mapped to human chromosome 4 by somatic cell hybridization and regionally localized to 4q31.1–31.2 by *in situ* hybridization (20, 21). Linkage analysis was, therefore, carried out in 10 inbred families (see Fig. 1) using 3 simple sequence length polymorphisms spanning the *MLR* region.

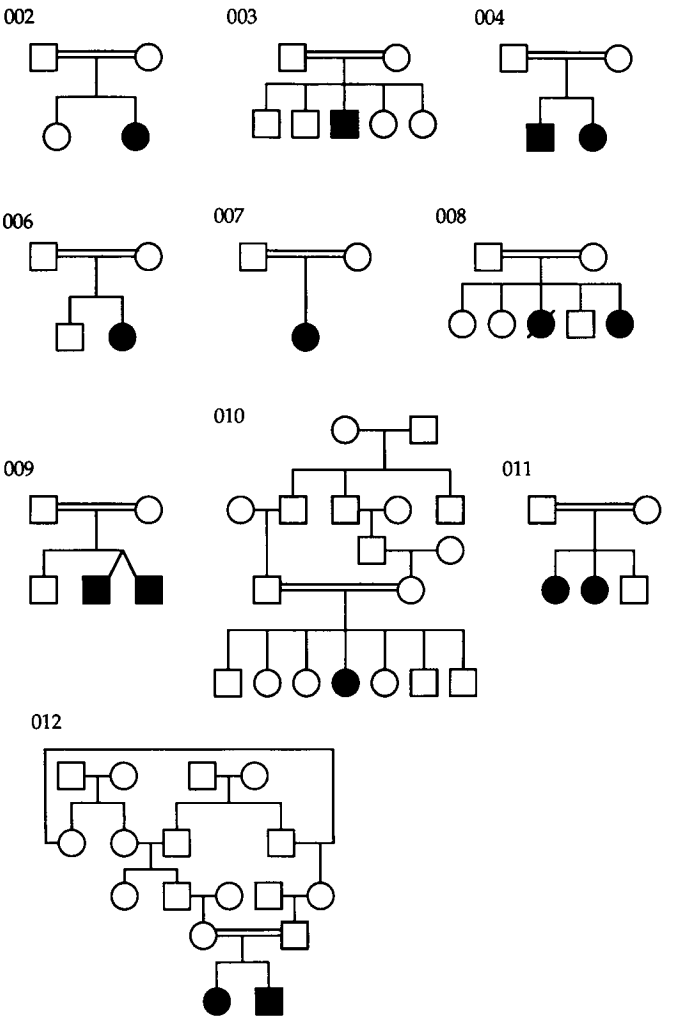

FIG. 1. PHA1 pedigrees used for linkage analysis. All affected individuals are offsprings of marriages between first cousins, except in two families (010 and 012) as shown.

Subjects and Methods

Patients and families

Ascertainment of families has been achieved through collaboration with the physicians who had reported cases of PHA1 in the literature and British pediatric endocrinologists or nephrologists who care for children with PHA1. The 10 families included in this study are shown in Fig. 1. All parents are consanguineous (all were first cousins except in 2 families), and autosomal recessive inheritance is assumed. There were 2 affected individuals in 6 of the families. The study includes a total of 39 individuals, 14 of whom were affected (5 males and 9 females).

Diagnosis was made according to standard and clinical biochemical criteria. All affected individuals had documented elevated urinary sodium in the presence of hyponatremia, hyperkalemia, increased PRA, elevated serum aldosterone level, and normal renal and adrenal function. Probands in 7 of the 10 families (families 002, 003, 008, 009, 010, 011, and 012) have documented evidence of generalized involvement, *i.e.* elevated sodium in sweat, saliva, or stool. In the other 3 families, the spectrum of organ involvement other than renal has not been fully evaluated. Details of individuals 003 IV-3 (2), 008 IV-5 (7), 009 IV-2 (22), 009 IV-3 (22), 010-IV (23), 012 V-1 (6), and 012 V-2 (6) have been reported previously.

DNA analysis / marker typing

DNA was extracted from white cells by standard protocols (24). The primer sequences and conditions for the three markers (*D4S192*, *D4S1548*, and *D4S413*) have been described previously (25, 26). DNA was amplified by PCR and labeled by incorporating [α-^{32}P]deoxynucleotide. PCR was performed in 36-well microtiter plates (Hybaid). Each well contained 50–100 ng genomic DNA; 1.5 mmol/L $MgCl_2$; 1 × reaction buffer [16 mM $(NH_4)_2SO_4$, 67 mM Tris-HCl (pH 8.8 at 25 C), 0.01% Tween-20; Bioline, UK]; 200 μmol/L each of deoxy (d)-GTP, dATP, and dTTP; 20 μmol/L dCTP; 0.05 μL [^{32}P]dCTP (3000 Ci/mmol); 50 ng of each primer; and 0.2 U BIOTAG polymerase (Bioline) in a total volume of 20 μl. Thirty cycles were performed in a thermocycler (Hybaid Omnigene, Hybaid Ltd, UK). Alleles were separated by 6% polyacrylamide gel electrophoresis and detected by autoradiography.

Linkage analysis

The maps used in the chromosome 4q analysis were from the reports of NIH/CEPH Collaborative Mapping Group (25), Gyapay *et al.* (26), and Mills *et al.* (27). The relative positions of *MLR* and the three simple sequence length polymorphisms used (*D4S192*, *D4S1548*, and *D4S413*) are shown in Fig. 2. Linkage analysis was carried out assuming a fully penetrant autosomal recessive inheritance with a disease allele frequency of 0.0002 and a zero phenocopy rate. Lod scores (logarithms to the base 10 of odds that two loci are linked) were calculated using the MLINK (v5.1) program (28) for two-point analysis and the HOMOZ program (29) for multipoint analysis. The markers were assumed to have five alleles of equal frequencies. Apparently normal siblings were not included in the analysis.

Results

The alleles of the affected individuals at the three loci studied are shown in Table 1. Loci were homozygous on only 10 of 41 occasions among the 14 affected individuals typed at the 3 markers (2 for *D4S192*, 4 for *D4S1548*, and 4 for *D4S413* were homozygous). The 2-point lod scores are shown in Table 2. The lod score was less than −2 for at least 10 centimorgans (cM) around *D4S192* and *D4S413* and for 5 cM around *D4S1548*. The results of the multipoint analysis are shown in Fig. 3. A lod score of less than −2 was obtained over the whole region from *D4S192* to *D4S413*.

Discussion

These results provide strong genetic evidence against *MLR* as the site of mutations causing pseudohypoaldosteronism in these families. Linkage analysis in inbred families provides a powerful strategy for examining the role of a candidate gene and for identifying the true location of the gene(s) causing this disease. Aspects of the interpretation of the linkage data and consideration of the possible nonreceptor molecular defects underlying AR PHA1 and strategies for future investigations are discussed below.

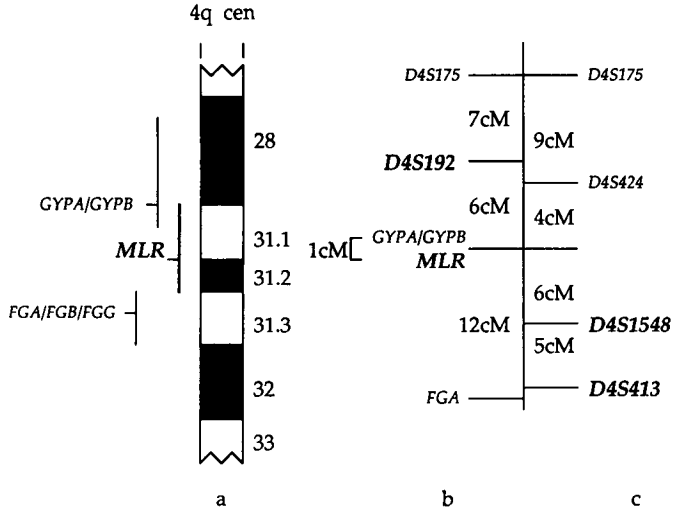

FIG. 2. Physical and genetic maps of human chromosome 4q indicating relative positions of *MLR* and marker loci *D4S192*, *D4S1548*, and *D4S413*. The map information shown is a composite of published data: a, Refs. 20, 21, and 27; b, Refs. 25 and 27; and c, Ref. 26. Genetic distance is sex averaged and calculated using the Kosambi function.

TABLE 1. Alleles at marker loci D4S192, D4S1548, and D4S413 in affected individuals

Family	Person	D4S192	D4S1548	D4S413
002	II-2	12	13	55
003	II-3	45	12	11
004	II-1	44	22	13
004	II-2	44	22	23
006	II-2	14	14	35
007	II-1	23	44	15
008	II-5	34	22	13
009	II-2	24	34	24
009	II-3	24	34	24
010	V-4	35	—[a]	24
011	II-1	23	34	33
011	II-2	23	34	33
012	V-1	12	13	23
012	V-2	12	13	23

[a] Unavailable because of failure of amplification.

TABLE 2. Two-point lod scores between PHA1 and marker loci on 4q

	Lod score at recombination fraction (θ)					
	0.00	0.01	0.05	0.10	0.20	0.30
PHA1-D4S192	−21.16	−8.38	−2.89	−1.04	0.03	0.12
PHA1-D4S1548	−12.08	−4.21	−0.71	0.33	0.71	0.49
PHA1-D4S413	−∞	−7.25	−2.40	−0.78	0.13	0.21

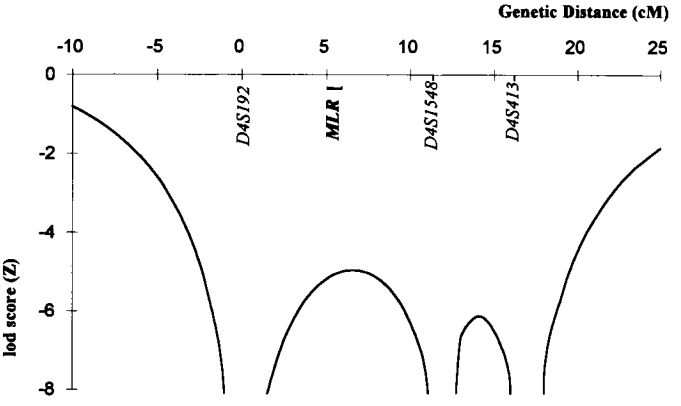

FIG. 3. Multipoint analysis around the *MLR* locus region.

The increased incidence of rare recessive diseases in the offspring of consanguineous matings arises in general from affected individual inheriting disease alleles at a particular locus from a common ancestor. The affected individual is, therefore, said to be homozygous by descent (rather than by state) for the disease allele. This increases the power of linkage data from consanguineous families, a factor exploited in the strategy of homozygosity mapping of disease loci in rare recessive disorders (see below) (19). However, the possibility of a disease chromosome being introduced into a consanguineous family by a married-in unrelated member cannot be totally excluded. This could produce an affected individual who may be heterozygous at the disease locus. This is extremely unlikely, especially if the disease is rare, as is PHA1. Exclusion of a candidate gene is definitively achieved by following the segregation of an intragenic polymorphism, which allows identification of obligate recombinants within families with at least two affected children. As an informative

intragenic polymorphism for *MLR* is not currently available, microsatellite loci spanning the region harboring *MLR* were used.

The genetic localization of *MLR* has not been well established. Little genetic data for *MLR* are available, and therefore, some uncertainty exists concerning the genetic distance between *MLR* and the loci analyzed. *MLR* has been physically mapped to 4q31.1–31.2 by fluorescent *in situ* hybridization. Composite data from existing genetic linkage maps provide good evidence that *D4S192* and *D4S413* flank the region to which *MLR* maps, and *D4S1548* is close to the *MLR* locus. The genetic distance between *D4S192* and *D4S413* is about 18 cM. Analysis of allele data at the 3 loci by 3 parallel approaches provides significant evidence against a locus for PHA1 in this region. Direct inspection reveals homozygosity of affected individuals on 10 of 41 occasions only. The average region of homozygosity expected around the disease locus in affected offspring of a first cousin marriage is at least 15 cM (19). Pairwise linkage analysis generates lod scores of −2 (the threshold accepted for exclusion assuming locus homogeneity) for regions around each locus totalling 25 cM. This includes the putative location of *MLR*. Finally, linkage analysis incorporating data from the 3 loci simultaneously using the program HOMOZ generates lod scores in the exclusion range for a total of at least 35 cM encompassing *MLR*.

These results demonstrate that *MLR* is not a common site of mutations causing PHA1 in this group of families. They do not exclude the possibility that *MLR* is the disease locus in a minority of these families or that a set of linked families might be identified if a much larger resource of AR PHA1 families was studied. Moreover, they do not exclude *MLR* as the locus accounting for PHA1 in those families that appear to display AD inheritance, although it is well recognized that allelic heterogeneity can account for variations in the mode of inheritance of diseases arising from a single locus. The evidence arising from cDNA analyses in sporadic patients does not, however, support *MLR* as the site of mutation in patients who may fall into the AD category, although the possibility that mutations exist in noncoding regulatory regions has not been excluded.

These observations indicate that *MLR* is not the site of mutations causing PHA1 in at least some patients. This is consistent with the observation that nonreceptor defects account for a proportion of cases in several hormone insensitivity syndromes, including androgen insensitivity (30), familial glucocorticoid deficiency (31), and pseudohypoparathyroidism (32). The likely molecular complexity underlying aldosterone function does, of course, provide numerous possible sites at which hormone insensitivity might arise. There is now good evidence that aldosterone exerts an immediate effect on its target cells via a membrane-bound receptor (now known as nongenomic action) (33). This is followed by the classical genomic action of aldosterone, which is thought to act by binding to the cytoplasmic mineralocorticoid receptor, forming an active complex. This complex initiates protein synthesis (aldosterone-induced proteins) by binding to a number of hormone response element of the nuclear DNA.

Many different hypotheses for nonreceptor defects in PHA1 have been proposed. The nongenomic actions of mineralocorticoids allow for dysfunction in pathways that do not include the cytoplasmic receptor. At the prereceptor level, there could be interference with aldosterone binding, by competition for the binding site or by cleavage of the receptor. At the postreceptor level, the most obvious candidates include the aldosterone-induced proteins, which include subunits of Na/K-adenosine triphosphatase and the amiloride-sensitive epithelial Na channels. Liddle's syndrome, or pseudoaldosteronism, has recently been shown to arise from mutations in the β-subunit of the epithelial Na channel gene causing excessive sodium reabsorption and hypertension at normal or low levels of circulating aldosterone (34). In so far as these features represent a mirror image of PHA1, it is conceivable that mutations involving these sodium channels may render them nonfunctional or unresponsive to aldosterone.

Homozygosity mapping represents a powerful strategy for examining the role of additional candidate genes in PHA1. This is being pursued using the present family resource in parallel with a genome search using anonymous microsatellite loci in case the causative locus is an as yet unidentified gene. Identification of the gene for PHA1 will allow improved diagnosis and treatment in affected individuals. It should also provide new insights into the biology of mineralocorticoid function at a molecular level, information of potential value in understanding more common disease states such as hypertension.

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