

Localisation of pseudohypoaldosteronism genes to chromosome 16p12.2–13.11 and 12p13.1-pter by homozygosity mapping

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Pseudohypoaldosteronism type 1 (PHA1, OMIM 264350) is a rare Mendelian disorder characterised by end-organ unresponsiveness to mineralocorticoids. Most steroid hormone insensitivity syndromes arise from mutations in the corresponding receptor, but available genetic evidence is against involvement of the mineralocorticoid receptor gene, *MLR*, in PHA1. A complete genome scan for PHA1 genes was undertaken using homozygosity mapping in 11 consanguineous families. Conclusive evidence of linkage with heterogeneity was obtained with a maximum two-locus admixture lod score of 9.9. The disease locus mapped to chromosome 16p12.2–13.11 in six families and to 12p13.1-pter in the other five families. The two chromosomal regions harbour genes for subunits of the amiloride-sensitive epithelial sodium channel: *SCNN1B* and *SCNN1G* on 16p and *SCNN1A* on 12p. Liddle's syndrome of hypertension and pseudoaldosteronism has been shown to arise from mutations in *SCNN1B* and *SCNN1G*. These results strongly suggest that PHA1 and Liddle's syndrome are allelic variants caused by mutations in genes encoding subunits of this sodium channel. These genes are of broad biological interest both in relation to sodium and water homeostasis in mammals and by virtue of their homology to

the *mec* genes of *Caenorhabditis elegans* involved in mechanosensitivity and neuronal degeneration.

INTRODUCTION

Pseudohypoaldosteronism type 1 (PHA1, OMIM 264350) is an uncommon inherited disorder characterised by target-organ unresponsiveness to mineralocorticoids. Since the first report by Cheek and Perry in 1958 (1) over 100 cases have been reported. Marked elevation of serum aldosterone levels is present in all cases, and is associated with salt-wasting, hyponatraemia, hyperkalaemia and increased plasma renin activity. Clinical expression of the disease varies from severely affected infants who may die to apparently asymptomatic individuals (2).

Familial and sporadic cases have been reported. Inheritance is Mendelian and may be either autosomal dominant or recessive. The inheritance pattern appears to correspond to whether the hormonal insensitivity is renal or multisystem (3). Multisystem PHA1 is more severe with salt loss from all aldosterone sensitive end organs including salivary glands, sweat glands, colon and kidney, and is usually inherited as an autosomal recessive trait. The renal form of PHA1 is characterised by renal salt-wasting only and is usually inherited as an autosomal dominant trait (3). The majority of reported cases falls into one of these categories (2,3). There is a high incidence of consanguinity in autosomal recessive PHA1.

The molecular basis of PHA1 is unknown (4). By analogy with other end-organ hormone insensitivity syndromes a defect at the

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level of the human mineralocorticoid receptor (hMR) has long been suspected, and the observation of reduced or absent binding sites for tritiated aldosterone in lymphocytes of patients with PHA1 (5) supported this hypothesis. The molecular cloning of cDNA encoding hMR (6) and localisation of the gene for hMR (*MLR*) to chromosome 4q31.1–31.2 has allowed direct genetic approaches to evaluating the hypothesis that PHA1 arises from mutations in *MLR*. Both direct sequence analysis of hMR cDNA (7–9), and linkage analysis using three simple sequence length polymorphisms spanning *MLR* (10) have excluded *MLR* as the disease locus in some families including those analysed in this study. A genome scan for regions of homozygosity was therefore undertaken in 11 consanguineous PHA1 families in order to map the disease locus or loci. Homozygosity mapping is a powerful strategy for mapping rare recessive traits in children of consanguineous marriages (11,12), and has been successfully applied to a number of diseases (13–15).

Evidence of genetic heterogeneity was obtained with families mapping either to chromosome 16p or chromosome 12p. Genes encoding subunits of the amiloride-sensitive epithelial sodium channel, *SCNNIG*, *SCNNIB* and *SCNNIA* map to these chromosomal regions (16,17). This strongly suggests that PHA1 is allelic to Liddle's syndrome of hypertension and pseudoaldosteronism, which has recently been shown to arise from mutations in *SCNNIG* and *SCNNIB* (18,19).

RESULTS

Genome search

Affected individuals only were typed initially using about 200 microsatellite loci covering the autosomes at intervals of approximately 15 cM. Loci displaying apparent excess homozygosity (homozygous in four or more unrelated affected individuals) were further investigated by analysis of parents and subsequently by analysis of additional adjacent loci if indicated. A total of 341 marker loci were analysed. Multipoint analysis using the MAPMAKER/HOMOZ program revealed no evidence of linkage assuming genetic homogeneity. However direct inspection of raw allele data and the admixture test for locus heterogeneity applied using the HOMOZ program revealed evidence of linkage to two chromosomal regions: 16p12.2–13.11 and 12p13.1-pter.

Linkage to 16p and 12p

Six families showed evidence of linkage to 16p12.2–13.11. Using HOMOZ analysis, a maximum admixture lod score of 4.4 was obtained with data from 15 loci in this region. Haplotypes and pedigrees of the six linked families are shown in Figure 1a. Genetic and physical map data for these loci are shown in Figure 2a. Regions of homozygosity ranged from less than 7 cM to 73 cM in affected individuals. The common region of homozygosity in these six families encompassed loci *D16S412*, *D16S403* and *D16S417*.

HOMOZ analysis using data from 13 loci on 12p also provided highly significant statistical support in favour of linkage with heterogeneity. A maximum admixture lod score of 3.4 was obtained. Four families showed clear evidence of linkage to this region. In addition, family 009 has a short length of homozygosity and had a conditional probability of linkage of only 0.47. Haplotypes and pedigrees of these five families are shown in Figure 1b. Relevant map data for these loci are shown in Figure

2b. The observed region of homozygosity ranged from less than 4 cM to 30 cM. The common region of homozygosity shared by these five families encompassed loci *CD4*, *D12S889* and *D12S374*. These analyses on 16p and 12p identified two discrete groups of families.

Combined analysis of individual family lod scores across these two chromosomal regions obtained from MAPMAKER/HOMOZ was undertaken using HOMOZ3R. A two-locus maximum admixture lod score of 9.9 (likelihood ratio = 8.2×10^9) was obtained in favour of six families linked to 16p and five families (including family 009) to 12p against no linkage. The conditional probability of linkage of each family to the two regions is shown in Table 1.

Table 1. Results of HOMOZ3R analysis showing conditional probability of linkage of each family to 16p and 12p

Family	Conditional probability of linkage to	
	16p	12p
002	0.999	0.001
003	0.979	0.022
006	0.999	0.001
013	0.999	0.001
014	1.000	0.000
015	0.999	0.001
004	0.001	0.999
008	0.000	1.000
009	0.019	0.981
011	0.001	0.999
012	0.006	0.994

DISCUSSION

These observations demonstrate that autosomal recessive pseudohypoaldosteronism type 1 is genetically heterogeneous and provide conclusive evidence for the existence of loci causing this disease on chromosome 16p12.2–13.11 and 12p13.1-pter. They represent the first application of homozygosity mapping to the direct elucidation of locus heterogeneity within such a limited resource of inbred pedigrees, and the first extension of the 'positional candidate' approach to the implication of a multimeric candidate channel on account of the mapping of the corresponding disease loci to chromosomal regions encoding its separate subunits.

The genome screen for shared regions of homozygosity proved sufficiently powerful to detect unequivocal linkage despite the presence of locus heterogeneity. Highly significant statistical support for this conclusion was obtained. HOMOZ3R analysis produced a likelihood ratio of 8.2×10^9 in favour of locus heterogeneity with all families accounted for by linkage to either 16p or 12p. It is recognised that linkage analysis in consanguineous families provides results which are highly sensitive to both disease and marker allele frequencies (20). Although allele frequencies for the loci analysed have been determined for particular family groups such as the CEPH family collection, the relevant frequencies are of course those for the population from

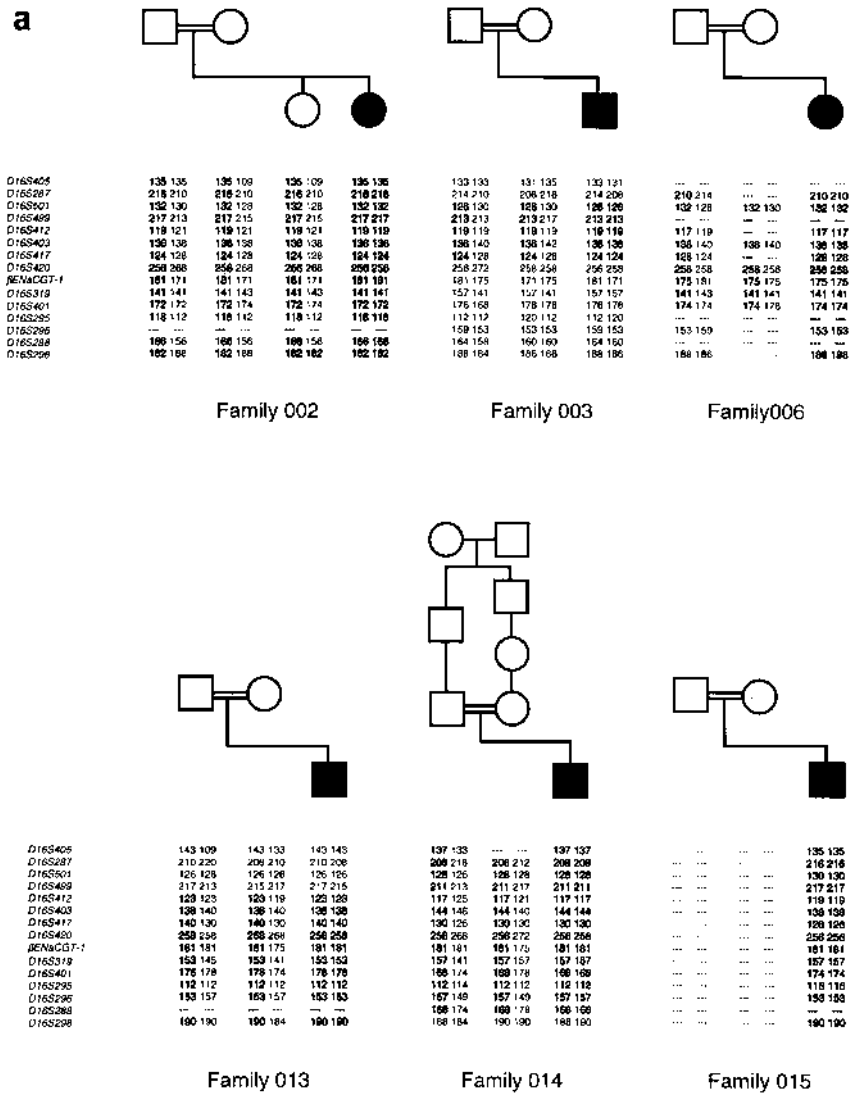


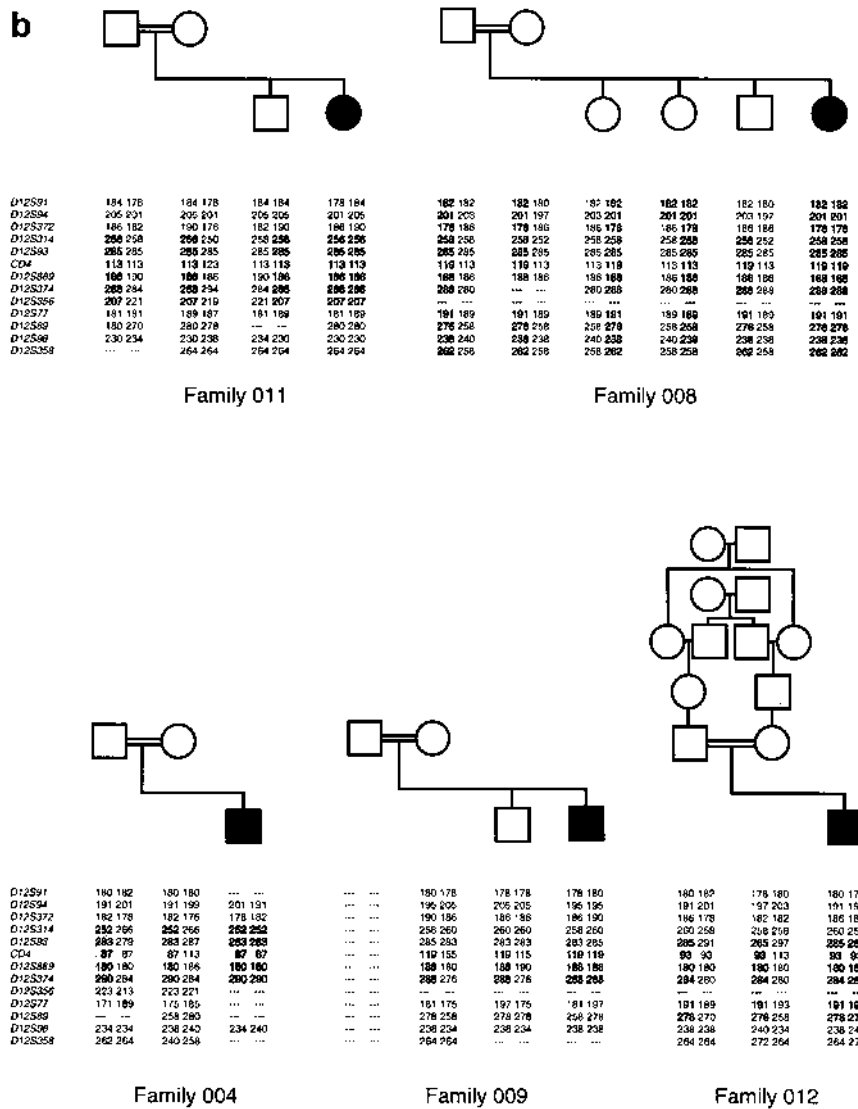
Figure 1. Pedigrees and allele data of PHA1 families. Consanguineous marriages are all between first cousins unless indicated. Below each parent and child are the alleles at the loci covering the chromosomal region to which that family is linked. The numbers are allele sizes, and are based on PCR product sizes measured in mobility units. Genotypes unavailable due to lack of DNA are given as (--- ---). The region of homozygosity in the affected individuals is shaded. The corresponding chromosomal regions in the parents and siblings are similarly marked. (a) families linked to 16p; (b) families linked to 12p.

which the disease families were drawn. The families analysed are from several quite different population backgrounds making it unlikely that a single frequency for any allele is applicable with any degree of certainty. A stringent approach was therefore adopted by setting a lower bound of 0.10 for all marker allele frequencies when multipoint analysis was carried out using MAPMAKER/HOMOZ.

High resolution mapping by this approach involves the identification of a common region of homozygosity amongst the unrelated affected individuals. On chromosome 16p this is defined by the telomeric locus *D16S499* (Family 013) and the centromeric locus *D16S420* (Family 003), separated by a genetic distance of about 8 cM. The latter boundary is of particular interest, as it excludes the candidate gene *SCNNIB* in family 003 if the assumption of a common ancestral disease allele is correct. If however this assumption is incorrect, the heterozygosity at *βENACGT-1* and *D16S420* does not of course exclude this candidate gene. With either

assumption *SCNNIG* remains a candidate, although no recombinations have previously been reported between *SCNNIB* and *SCNNIG* which are known to be physically within a 400 kb region. On chromosome 12p the common region of homozygosity is defined by the telomeric locus *D12S93* (Family 009) and the centromeric locus *D12S77* (Family 009 and 011), a genetic distance of 10 cM. Clearly, this region might have been substantially smaller had allele data at *D12S356* been available in Family 009 or Family 004.

Linkage disequilibrium mapping may also allow refinement of the genetic localisation in a rare recessive disease if a founder effect is apparent. Unfortunately, the number of disease chromosomes available in this family resource is too small to allow allelic association to be identified unless association happens to be with a very rare marker allele. It is noteworthy that four out of six affected individuals are homozygous for the 181 allele at *βENACGT-1*, but this allele is not significantly less frequent on normal chromosomes in this sample.



Following definitive exclusion of *MLR*, the strongest candidate gene for this disorder, a systematic genome search was undertaken without high priority being assigned to regions harbouring the several additional existing candidate genes. It was therefore interesting to find that *PHA1* could be assigned to two quite separate chromosomal regions each of which harbour genes for sub-units of the amiloride-sensitive epithelial sodium channel. The recent identification of mutations in the genes encoding the β and γ subunits of this channel as the cause of Liddle's syndrome of pseudoaldosteronism (18,19), confirmed its identity as the functional mineralocorticoid-sensitive epithelial sodium channel in humans, and prompted the suggestion that mutations rendering this channel non-functional or unresponsive to aldosterone could cause *PHA1*.

These mapping data substantially strengthen what is already a quite plausible case for dysfunction at this channel as the cause of mineralocorticoid insensitivity in *PHA1*. Although receptor gene mutations have been identified in the majority of steroid hormone insensitivity syndromes, most notably those involving thyroxine, cortisol, androgens and Vitamin D, non-receptor defects are well recognised (21-23). Abundant evidence from physiological studies

indicate that the effects of mineralocorticoids on sodium exchange, especially in the kidneys, are exerted via a pathway that includes the channel encoded by *SCNNIB*, *SCNNIG* and *SCNNIA* (24-26). The clinical features of Liddle's syndrome comprise in many respects a 'mirror-image' of *PHA1*, indeed it is also designated pseudoaldosteronism. The underlying mutations which have been described in β ENaC and γ ENaC all cause truncation of the cytoplasmic carboxyl terminus of these subunits and cause constitutive activation of the channel. It is not unreasonable to propose that mutations elsewhere in these two genes, and in the α ENaC gene on chromosome 12p, may render the channel non-functional or unresponsive to mineralocorticoids. Although the localisation of *PHA1* to two quite separate chromosomal regions each of which harbours genes for subunits of this channel could be coincidental, the mapping data in combination with the existing functional evidence make the suggestion that Liddle's syndrome and *PHA1* are allelic variants compelling.

Direct proof of this hypothesis requires of course the demonstration of mutations correlating with the disease state and work is in progress to screen these genes for mutations in affected individuals in these families. As discussed above, the close

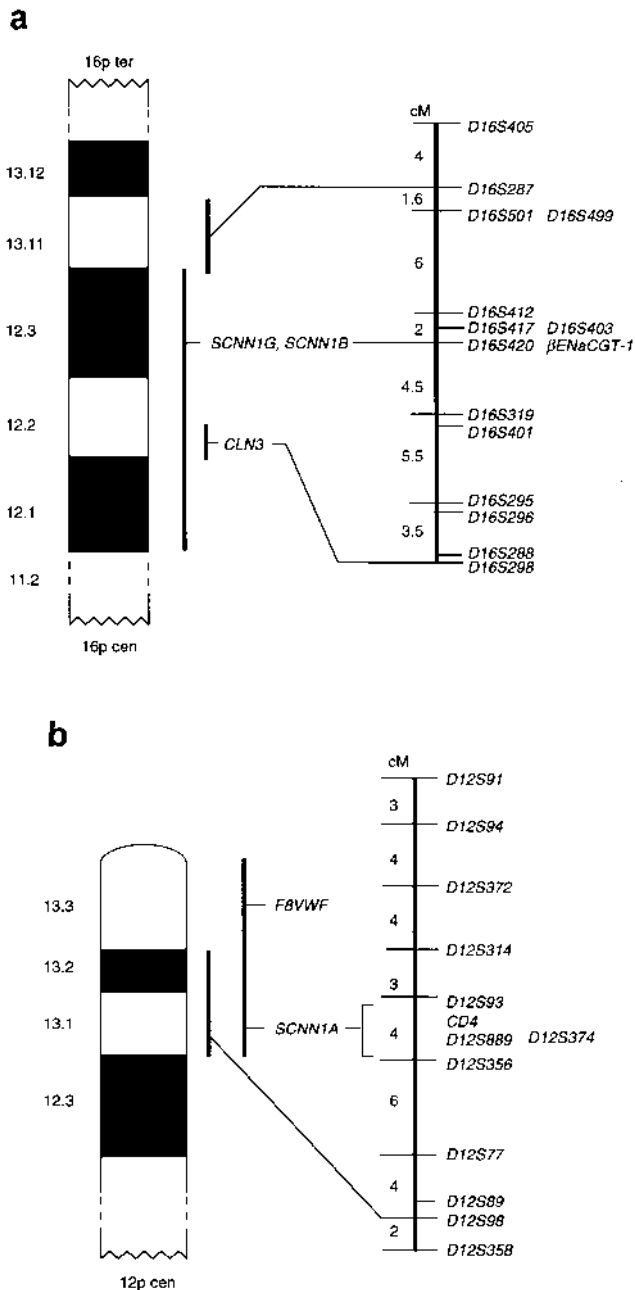


Figure 2. Physical and genetic maps, indicating genetic distances (sex-averaged) between marker loci used. The map information shown is a composite of published data. (a) chromosome 16p (30,31); (b) chromosome 12p (29,31).

proximity of *SCNN1B* and *SCNN1G* render both plausible candidate genes in all chromosome 16p families with the possible exception of family 003. It is also conceivable that different alleles at these loci may account for sporadic cases of PHA1 and for cases in families displaying a dominant pattern of inheritance.

Elucidation of the molecular basis of pseudohypoaldosteronism is likely to be of broad biological interest. If, as anticipated, mutations are identified in these genes, analysis of their functional consequences will provide insights into the molecular mechanisms involved in the control of the amiloride-sensitive epithelial

sodium channel by mineralocorticoids which is central to sodium and water homeostasis in mammals. Moreover, the close homology of the epithelial sodium channel to members of a family of *Caenorhabditis elegans* genes involved in mechanosensitivity and, when mutated, neuronal degeneration suggests that important processes related to cell volume control may also be illuminated (27).

MATERIALS AND METHODS

Patients and families

Ascertainment of families was carried out through collaboration with the physicians who had reported cases of PHA1 in the literature and British paediatric endocrinologists. The 11 pedigrees included in this study are shown in Figure 1. All parents are consanguineous being first cousins in nine of the 11 families.

Diagnosis was made according to standard clinical and biochemical criteria. All affected individuals had documented raised urinary sodium in the presence of hyponatraemia, hyperkalaemia, increased plasma renin activities, elevated serum aldosterone levels, and normal renal and adrenal function. Proband in seven of the 11 families (family 002, 003, 008, 009, 011, 012 and 013) have documented evidence of generalised involvement, i.e. raised sodium in sweat, saliva, or stool. In the other four families, the spectrum of organ involvement other than renal has not been fully evaluated. Details of families 003, 008, 009, and 012 have been reported previously (see 10 for references).

DNA analysis/marker typing

Genomic DNA was extracted from white cells by standard methods. DNA was amplified by the polymerase chain reaction (PCR) using fluorescently labelled primers or dUTPs (Applied Biosystems). The marker loci were selected from published genetic maps. Primers for a set of 200 polymorphic marker loci were supplied by the HGMP UK (28). Primer sequences are available from GDB. Additional loci on 16p and 12p were identified from published genetic and consortium maps (28–31) except for *betaENaCGT-1*, an intragenic polymorphism in *SCNN1B* (18), and *D12S889* (32). The genes *SCNN1B* and *SCNN1G* lie within 400 kb of each other in the same YAC clone which also contains marker *D16S420* (17). The same cosmid clone contained both *D12S889* and *SCNN1A* which are within 40 kb of each other (32).

PCR was performed in 96 well microtitre plates (Hybaid). Each well contained 20–50 ng of genomic DNA; 1.5 mM $MgCl_2$; 1 × reaction buffer (Advances Biotechnologies, UK); 200 μM each of dGTP, dATP, dTTP and dCTP; 50 ng of each primer and 0.2 U of Red Hot DNA polymerase (Advance Biotechnologies, UK), in a total volume of 15 μl. Thirty cycles were performed in a thermocycler (Hybaid OmnigeneTM). Alleles were separated on a 6% polyacrylamide electrophoresis gel for 3–4 h at a rate limiting voltage of 1000 V using a model 373A DNA sequencer (Applied Biosystems). Analysis of the alleles sizes was carried out by GENESCANTM672 (Ver 1.2) and Genotyper softwares (Applied Biosystems) using GENESCANTM-500 TAMRA or GENESCANTM-2500 ROX as size standards.

Linkage analysis

Linkage analysis was carried out assuming fully penetrant autosomal recessive inheritance with a disease allele frequency of

0.001. Multipoint analysis was performed using the MAP-MAKER/HOMOZ program (20), which uses an algorithm based on Hidden Markov Models. This allows very rapid calculation of multipoint lod scores in small inbred families. This has previously been impractical because of the huge computational time required. The analysis on 16p was performed from *D16S405* to *D16S298* using the marker loci and genetic distances shown in Figure 2a. The analysis on 12p was performed over the region shown in Figure 2 using the marker loci and genetic distances indicated. Allele frequencies were obtained from GDB with frequencies restricted to not less than 0.10. Homogeneity testing was carried out using the HOMOG and HOMOG3R programs (33,34). The HOMOG3R program calculates log likelihoods under the assumption that in a proportion (α_1) of families a trait is linked to locus 1 and in a proportion of other families (α_2) it is linked to locus 2, and the two loci are located in different regions of the genome such that the trait is never linked to both loci.

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