Exclusion of candidate loci and cholesterol biosynthetic abnormalities in familial Pallister-Hall syndrome

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Abstract

Pallister-Hall syndrome (PHS) was originally described in 1980 in six sporadic cases of children with structural anomalies including hypothalamic hamartoma, polydactyly, imperforate anus, and renal and pulmonary anomalies. In 1993, the first familial cases were reported, including affected sibs and vertical transmission. Three of these families are sufficiently large to allow initial evaluation by linkage studies to candidate genes or loci. We have evaluated candidate loci for PHS based on three clinical observations. The first is a patient with PHS-like malformations, including a hypothalamic hamartoma, and an unbalanced translocation involving 7q and 3p. The second is a family with familial PHS where the founder's father had an autosomal dominant hand malformation previously mapped to 17q. The third is the phenotypic overlap of PHS and Smith-Lemli-Opitz syndrome. In this report, we exclude these loci as candidates for linkage to the PHS phenotype on the basis of lod scores of less than -2.0 . We conclude that hypothalamic hamartoma is not specific to PHS and that the dominant hand malformation in one of the families was a coincidence. To evaluate the relationship of PHS to Smith-Lemli-Opitz syndrome, we analysed levels of cholesterol and intermediate metabolites of the later stages of cholesterol biosynthesis. There is no evidence of a generalised disorder of cholesterol biosynthesis in patients with familial PHS. On genetic and biochemical grounds, we conclude that PHS and Smith-Lemli-Opitz syndrome are not allelic variants of a single locus.

(J Med Genet 1996;33:947-951)

Key words: hypothalamic hamartoma; polydactyly; autosomal dominant inheritance; linkage analysis.

Pallister-Hall syndrome (PHS) is a rare disorder of morphogenesis that includes malformations of the central nervous system, limbs, anus, and viscera.^{1,2} Although it was originally reported in six sporadic neonatal cases who died of their anomalies, the disorder has been expanded to include much less severe anomalies and familial transmission.³ The malformations and inheritance of PHS and the

morphogenetically related conditions of oral-facial-digital syndromes, Smith-Lemli-Opitz syndrome (SLOS), short rib-polydactyly syndromes, hydroletharus, and Kaufmann-McKusick syndrome have recently been reviewed.^{4,5} Pallister-Hall syndrome has been categorised into two different schemata by recent authors. Verloes *et al*⁶ coined the term CAVE (cerebro-acro-visceral-early lethality) complex. Neri *et al*⁷ have developed a different classification scheme that has been termed oral-facial-skeletal syndromes. These conceptual constructs illustrate the nature of the malformations and acknowledge the overlap and variable severity of the disorders. The existence of mildly affected and large families with long survival permits dissection of the aetiology of PHS by linkage analysis. We analysed several families for linkage to candidate loci that were chosen on the basis of clinical and laboratory evaluation of families seen by us or published cases.

The four families used in this study include two previously reported families and two additional families. The diagnostic criteria are described in the methods section below. The first family was recently described by Grebe and Clericuzio⁷ and includes seven affected subjects who segregate PHS in an autosomal dominant pattern (fig 1, family 1). As reported by those authors, the father of the PHS founder in the pedigree had proximal symphalangism, although none of the persons affected with PHS in this family also had proximal symphalangism. Proximal symphalangism is a mild hand malformation inherited in an autosomal dominant pattern.^{8,9} This disorder has recently been mapped to chromosome 17q, in an 8 cM region between D17S790 and D17S808.¹⁰ Interestingly, the father of the PHS founder in the report by Grebe and Clericuzio⁷ is a descendant of the family that was analysed in the proximal symphalangism linkage study. We elected to determine if PHS was linked to the proximal symphalangism locus on the basis of these observations. The second family is a large kindred identified by one of the authors (JCA)¹¹ (fig 1, family 2). This family includes 22 affected members and shows segregation of PHS in an autosomal dominant pattern. Pedigree analysis of this family shows that the disorder appears to be highly penetrant (seven of seven obligate heterozygotes affected). In addition, 11 members of this family (selected by the presence of polydactyly) have undergone cranial MRI examination and nine of these showed

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Received 13 February 1996
Revised version accepted for publication 17 June 1996

Figure 1 Pedigrees of the families studied in this report. Family 1 is the family reported by Grebe and Clericuzio,⁷ family 2 is the family identified by Biesecker et al,¹¹ family 3 is the family identified by Olney, and family 4 is the family reported by Topf et al.¹² The pedigrees show that the disorder is inherited in an autosomal dominant pattern and is fully penetrant as evidenced by an abnormal phenotype in all obligate heterozygotes. The expressivity is variable as evidenced by the absence of hypothalamic hamartoma in several obligate heterozygotes.

a lesion compatible with a hypothalamic hamartoma. In addition, three of the people in the family have had airway endoscopy that showed clefting of the epiglottis, another malformation typical of sporadic cases of PHS.² The third family was identified by one of the authors (AHO) (fig 1, family 3). It contains a newborn proband with PHS and an affected father, paternal uncle, and paternal grandfather. The fourth family is the father-son pair described by Topf et al.¹² (fig 1, family 4).

In addition to the 17q candidate locus suggested by the family of Grebe and Clericuzio,⁷ other authors have reported findings that point to candidate loci for PHS. A patient with hypothalamic hamartoma, microphthalmia, and visceral anomalies was reported by Kuller et al.¹³ This child was found to have an unbalanced chromosomal rearrangement specified as 46,XY,-7,+der7,t(3;7)(p25.4;q36). We elected to exclude the 7q and 3p regions of the genome on the hypothesis that dosage imbalance of a PHS gene in these regions may have caused the phenotype in this child. Finally, Donnai et al.¹⁴ and Verloes et al.¹⁵ have commented on the overlap between PHS and Smith-Lemli-Opitz syndrome (SLOS). Several groups have described a block in the conversion of 7-dehydrocholesterol to cholesterol in patients with SLOS.^{15,16} In addition, a patient with SLOS has been found to have a balanced translocation involving 7q32.1.¹⁷ On the basis

of these observations we elected to assess the cholesterol metabolism and linkage to 7q32.1 in families with PHS.

Methods

LINKAGE ANALYSIS

Clinical information and blood samples were collected from the four families after informed consent was obtained. The diagnostic criteria for PHS used for this study were those determined at a recent international workshop on PHS that was held at the National Institutes of Health. The criteria are as follows: the index case in a family must have (1) central or insertional polydactyly and (2) a hypothalamic hamartoma diagnosed by cranial MRI or biopsy. Other cases in the family may be diagnosed by the presence of either (1) or (2) if they are related to the index case in an autosomal dominant pattern. Blood was processed according to standard techniques for the isolation of genomic DNA and transformation of lymphocytes. PCR amplification of genomic DNA was performed with *Taq* (Cetus, Inc) in standard conditions using primers (Research Genetics, Inc) for microsatellite markers on 7q, 3p, and 17q. The PCR products were separated in 6% denaturing polyacrylamide gels (Kimberly Clark diagnostics) and exposed to autoradiography film (Kodak XAR) at -80°C. Genotypes were read manually and entered into

Figure 2 This plot of LINKMAP data shows the results of a four point analysis of the disease locus, D7S483, D7S550, and D7S559. The x axis has small hatch marks at 4 cM intervals and larger hatch marks at 20 cM intervals. A horizontal line is drawn through the lod score value of -2.0 to show the linkage exclusion threshold. The vertical lines dropped from the intersections of this line and the lod data line shows the region of exclusion on the genetic map. This exclusion region is wider than an overestimate of the genetic map distance from the 7q translocation breakpoint to 7qter. The lod scores extend below -20 but were truncated from all figures for clarity.

Figure 3 This plot is similar to fig 2 but shows the data for the 3pter region. The vertical lines that intersect the lod data line show that the region of exclusion is larger than the genetic distance from the 3p breakpoint of Kuller *et al*¹³ and 3pter. The x axis has small hatch marks at 5 cM intervals and larger hatch marks at 20 cM intervals.

the Linkage Manager database.¹⁸ The Linkage Manager files were sequentially converted into UNIX format and transferred to a Sun SPARCstation 5 computer. The linkage analysis calculations were done with FASTLINK

(version 3.0P),^{19,20} which is a faster version of LINKAGE 21, using an autosomal dominant model with 99% penetrance. Genetic intervals with a lod score of ≤ -2.0 were considered to be excluded as candidates.

CHOLESTEROL METABOLISM ANALYSIS

Lymphocytes from people affected with PHS were transformed with EBV by standard techniques. Additional EBV transformed lymphoblasts were obtained from the Johns Hopkins Kennedy Krieger Institute Mental Retardation Research Center Cell Bank. These cell lines included samples from normal subjects, from patients with SLOS and their parents, and from sibs and parents of patients with known genetic syndromes without defects in cholesterol metabolism. Lymphoblast cultures were expanded in sealed 25 cm² flasks in bicarbonate buffered RPMI 1640 (1 mmol/l glucose) with 10% (v/v) Serumax (Sigma Corp, St Louis, MO). To induce cholesterol biosynthesis, lymphoblasts in stationary phase were harvested by centrifugation at 600 *g* and 40 μ l of packed cells were resuspended in 10 ml of medium. This medium consisted of RPMI 1640 with 10% (v/v) fetal calf serum depleted of cholesterol by treatment with Cab-o-sil (Eastman Kodak Corp, Syracuse, NY) as previously described.¹⁶ The final cholesterol concentration was less than 1 μ g/ml as determined by gas chromatography. After three days in this medium, the cells were harvested by pelleting at 600 *g*, resuspended in 10 ml of Dulbecco's PBS (Ca and Mg free, pH 7.4), and repelleted. The washed cells were resuspended in PBS at a concentration of 250 μ l of PBS per 1 μ l of pellet. The protein concentration was determined by a Lowry method using bovine serum albumin as the standard. A 500 μ l portion of the cell suspension (containing typically 400–500 μ g of protein) was measured into an extraction tube and pelleted at 2000 *g* for five minutes. The supernatant was aspirated and the cell pellet was processed for sterol quantification as described elsewhere.¹⁶ Sterols were quantified by ion ratio electron impact gas chromatography and mass spectrometry. The quantified sterols included cholesterol, dihydrocholesterol, cholest-8(14)-en-3 β -ol, zymosterol, desmosterol, 7-dehydrodesmosterol, 7-dehydrocholesterol (cholesta-5,7-dien-3 β -ol), lathosterol, methosterol, lanosterol, and dihydrolanosterol. Gas chromatography with electron impact mass spectrometry in the scanning mode was used to screen for increased levels of other natural sterols not quantified as part of the ion ratio protocol.

Results

MAPPING DATA

The genetic exclusion analysis was performed by estimating the genetic size of the candidate locus or region and then comparing the region of exclusion (≥ -2.0 lod) for markers in the test region. We first estimated the genetic interval of the translocation breakpoints (from the patient described by Kuller *et al*¹³ to deter-

Figure 4 This graph shows the MLINK data for the lod score of marker D7S635 in the Smith-Lemli-Opitz syndrome critical region and the PHS phenotype. The -2.0 linkage exclusion range is shown similarly to fig 2 and is larger than the SLOS critical region.

Figure 5 Lod score for the D17S809 marker and the PHS phenotype. The lod score is less than -2.0 for a region that is greater than the interval into which proximal symphalangism has been mapped (between D17S790 and D17S808).

mine if those regions were excluded in this analysis. We estimated the 7q breakpoint as being telomeric to marker TCRB and the 3p breakpoint as telomeric to D3S1537. These are conservative estimates that assume the most telomeric marker that has been mapped in the nearest chromosomal band centromeric to the reported breakpoints are the actual breakpoints. For example, Kuller *et al*¹³ reported that the breakpoint was 7q36.¹³ We therefore selected the next band on the centromeric side of 7q36, which is 7q35.3. The most distal marker in 7q35.3 is TCRB. The mapping data used for these estimates is from the Southampton summary maps.²² The lod scores from

the multipoint analyses yield scores of ≥ -2.0 for both the 7q (fig 2) and 3p (fig 3) regions. For the 7q32.1 SLOS candidate locus, we have shown lod scores of ≥ -2.0 in a range of ± 19 cM (fig 4). This translocation breakpoint has been localised to a region that is approximately 300 kb in length and lies less than 900 kb from D7S635. We overestimated the genetic distance from D7S635 by assuming it is as far as the nearest genetic marker that flanks the translocation breakpoint, which is D7S686. These two markers, D7S635 and D7S486, are spaced at approximately 3.2 cM (sex averaged distance) on the Southampton summary map. Since the exclusion limit of ≥ -2 lod score holds in a region extending further than this distance, we concluded that the translocation breakpoint was not linked to the PHS phenotype.

Proximal symphalangism has been mapped to a region between D17S808 and D17S790.¹⁰ These loci are tightly linked to D17S809. The Southampton summary map orders the loci as: cen-D17S809-3.1 cM-D17S790-6.6 cM-D17S808-tel.²² Because the -2.0 lod score exclusion holds in a region greater than ± 10 cM from D17S809, we conclude that the proximal symphalangism locus is not linked to PHS (fig 5).

BIOCHEMICAL TESTING RESULTS

Six lymphoblastoid lines from PHS patients were analysed for their cholesterol and 7-dehydrocholesterol levels by one of us (RIK). These six lines showed no consistent deviation from normal controls in the levels of cholesterol, 7-dehydrocholesterol, or the chol/7-dhc ratios (fig 6), nor any other consistent abnormality in the levels of other sterol precursors of cholesterol. From these experiments we conclude that there is no abnormality of the terminal stages of cholesterol metabolism in patients with PHS.

Discussion

When initiating genetic analysis of phenotypes it is often useful to begin by searching for evidence of linkage to candidate genes or loci. These candidate loci may be ascertained in several ways. The first is to analyse loci that are known to be involved in translocations found in patients who have the disorder or a related phenotype. Another approach is to analyse genes that may be involved in other phenotypes that share some similarity to the disorder under study. A third approach is to use data from animal models or other experimental data that may point to a candidate gene.

The use of the candidate locus linkage analysis in familial PHS has shown that the disorder is not linked to four loci suggested by previous clinical analyses. These excluded loci include the 7qter and 3pter loci suggested by the unbalanced translocation described by Kuller *et al*.¹³ It is important to note that this person did not have polydactyly but did have microphthalmia and would not be considered to have typical PHS on clinical grounds. The

Figure 6 This plot of the ratio of 7-dehydrocholesterol to cholesterol for four groups of subjects shows that the patients with PHS are not distinguishable from normal controls and have lower ratios than SLOS patients or parents.

presence of a hypothalamic hamartoma in this person and the absence of linkage supports the notion that hypothalamic hamartoma is a non-specific malformation.⁶ This notion is also supported by the description of patients with McKusick-Kaufmann syndrome and hypothalamic hamartoma²³ and isolated hypothalamic hamartoma.²⁴

The absence of linkage of the PHS phenotype to the 7q32.1 SLOS candidate locus and the absence of any biochemical abnormalities of the final steps of cholesterol metabolism indicate that PHS is not allelic to this disorder. It is important to note that this biochemical analysis neither excludes all forms of cholesterol metabolism defects in PHS, nor the existence of more than one gene that can cause SLOS-like phenotypes. These results are in agreement with the unpublished results showing normal cholesterol levels on two PHS patients of Verloes *et al.*⁵

The presence of proximal symphalangism in the father of the PHS founder in family 1 suggested that the two disorders could be pathogenetically related. These negative linkage results suggest that this family has two genetically distinct and rare dominant malformation syndromes. We conclude that the family has both disorders on the basis of chance alone.

The negative results of the linkage analyses could be the result of two factors. The first is the existence of a single locus in an area of the genome not analysed by this study. This is the most obvious explanation and is our working hypothesis for future work. The second is that PHS could be aetiologically heterogeneous and that a subset of the families in this analysis may have genetic alterations in one of the four candidate regions. This is consistent with the notion of Verloes *et al.*⁵ and Neri *et al.*⁴ that syndromal hypothalamic hamartoma is aeti-

ologically heterogeneous. Families 3 and 4 are too small to allow individual linkage analysis. Separate linkage analyses with families 1 and 2 show negative linkage scores at all four loci (data not shown). Therefore, given the limits of power owing to family size, we conclude that the first explanation is more likely. A definitive answer awaits a determination of the locus for hereditary PHS and resolution of the issue of genetic heterogeneity for this disorder.

The authors thank the families for their willingness to participate in this research project. In addition, we thank Judith Hall, Robert Nussbaum, and Anthony Wynshaw-Boris for their advice and critical reviews of the manuscript. The cholesterol metabolism analysis was supported by NIH grants DK44933 and HD24061.

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