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Report

A Minimalist Approach to Gene Mapping: Locating the Gene for Acheiropodia, by Homozygosity Analysis

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Acheiropodia is an autosomal recessive disease that results in hemimelia (lack of formation of the distal extremities). We performed a complete genome screen of seven members of an extended pedigree that included three siblings with acheiropodia. Homozygosity mapping was used to identify regions most likely to harbor the gene for acheiropodia in this pedigree. In these two key regions (14p and 7q), further genotyping of one additional affected member of this pedigree plus seven additional unaffected siblings provided evidence, through linkage analysis, that the 7q36 region contains the acheiropodia gene. In this region, a maximum two-point LOD score of 3.81 (4.2 with multipoint analysis) was achieved, and a homozygous haplotype spanning a region of 11.7 cM was seen in all affecteds in this pedigree. Finally, genotypic analysis of two additional cases of acheiropodia with no known relation to the other samples revealed homozygous sharing of a portion of the same haplotype on 7q36, which reduces the chromosomal location of the acheiropodia gene to an 8.6-cM region. Localization of this gene, at the screening level, by use of data from only three affected subjects, provides an example of how certain genes may be mapped by use of a minimal number of affected cases.

Acheiropodia (MIM 200500) is a rare genetic disease characterized by malformation of the distal limbs of both upper and lower extremities. The disease was first described in the Guarapuava province of Brazil, in 1929 (Peacock 1929), and has been seen almost exclusively in Brazil, where it presents as a congenital quadruple amputation of the limbs and nonformation of the hands and feet. Acheiropodia has been described as having autosomal recessive inheritance, complete penetrance, and no associated anomalies or involvement of other organs (Freire-Maia 1975). Although persons with the illness have decreased fitness, fertility, and viability in comparison with unaffected siblings, there is no significant difference, in biological fitness, in unaffected siblings compared with the general population (Freire-Maia et al. 1975*b*). Twenty-two sibships with acheiropodia were identified in 1975, all within Brazil, and the frequency of acheiropodia in the Brazilian population at that time was estimated as $\sim 3 \times 10^{-7}$ (Freire-Maia et al. 1975*a*). The mutation responsible for this phenotype is presumed to have originated in Brazil ~20 generations ago (Morton and Barbosa 1981).

Homozygosity mapping has been described (Lander and Botstein 1987) and has been successfully used (Houwen et al. 1994; Sheffield et al. 1995; Jamieson et al. 1999) to map rare autosomal recessive diseases with a minimal number of affected cases. We performed homozygosity mapping in a limited sample (including only three affected individuals and three known carriers) from an extended family from Brazil and identified two regions that are most likely to contain the acheiropodia gene. With the addition of further samples, we were then able to perform linkage and haplotype analyses to identify which of these regions harbors the acheiropodia gene. The identification of two other unrelated individuals with acheiropodia who are from this population,

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in conjunction with further genotyping, have now narrowed the region for this gene locus to the distal portion of chromosome 7 (7q36).

The families included in this study derive from the São Paulo (pedigree 1) and Santa Catarina (pedigrees 2 and 3) states of Brazil, near the area estimated, by Morton and Barbosa (1981), as being the most likely center of origin of the disease mutation. An extended pedigree with a total of five cases of acheiropodia (four living) was ascertained in the state of São Paulo (fig. 1). This pedigree has been described elsewhere (Toledo and Saldanha 1969), although the pedigree structure is revised and updated in this report. The affected individuals have absent forearms, hands, and feet, which is typical of this disease. Two of the affected individuals possess one digit implanted into each humeral stump bilaterally. Since the original description of this pedigree, the affected individuals have matured without manifesting any additional signs of illness, with the oldest affected individual now being age 46 years. As in the vast majority of cases of acheiropodia, this pedigree demonstrates consanguinity between both sets of parents who had children affected with the disease. From this family, we ultimately were able to collect DNA samples from the four surviving persons with acheiropodia, eight unaffected siblings, and the three surviving parents of the acheiropodia cases. Two other small pedigrees from the state of Santa Catarina were identified, each of which contributed one additional affected individual. The affected individual from pedigree 2, a 51-year-old woman, has three normal children. There is no known consanguinity in this pedigree. The affected individual from pedigree 3 has parents who are second cousins. The affected individuals in these last two pedigrees show the typical features of ach-

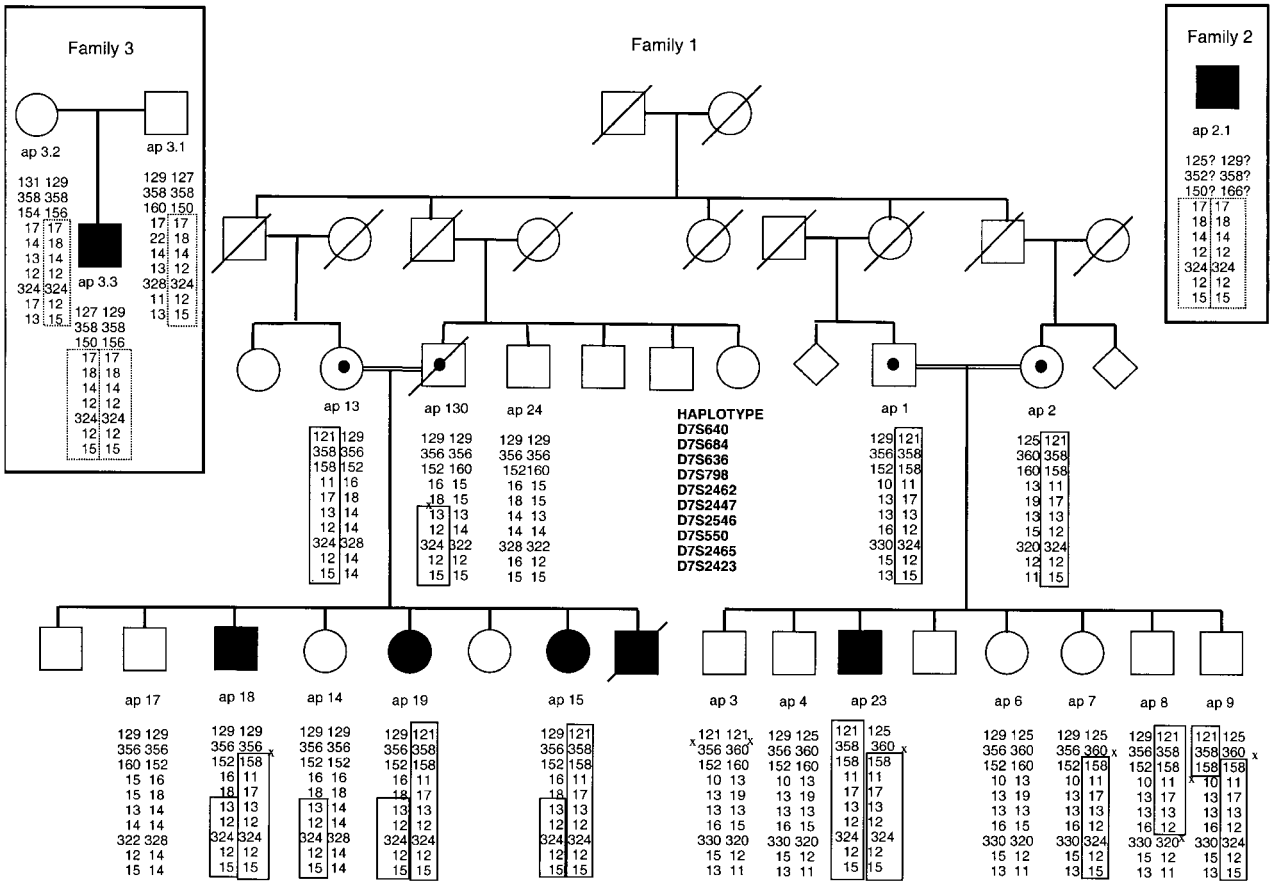

Figure 1 Pedigrees 1–3. Presumed shared ancestral haplotypes surrounding the acheiropodia gene locus are outlined. Pedigree-specific haplotypes surrounding the acheiropodia gene are outlined by either unbroken lines (pedigree 1; haplotype A) or dotted lines (pedigrees 2 and 3; haplotype B). Alleles with unknown phase are denoted by a question mark (?) following the genotype. Genotypes and haplotypes for individual ap130 are reconstructed from those of the other pedigree members. In pedigree 1, three additional affected individuals (three deceased) and five unaffected individuals (one deceased) siblings of ap23 are not shown. One affected individual (from the sibship that includes ap14, ap15, and ap17–ap19) who was still born is not shown.

eiropodia, with no additional anomalies or clinical pathology. We were also able to obtain DNA samples from both parents in pedigree 3.

For the initial phase of the project, DNA from only seven individuals from the first pedigree was available for genotyping. We genotyped these individuals (ap1, ap2, ap13–ap15, ap18, and ap19) with 377 polymorphic markers (2,639 individual genotypes) from the Applied Biosystems (ABI) linkage mapping sets, versions 1 and 2. The average distance between markers (according to the Généthon map) was 9.58 cM. Only 14 markers (13 gaps) were separated from one another by >15 cM, and only one gap was >20 cM (a 24.1-cM gap on chromosome 8). PCR-based genotyping was performed by means of the ABI 377 automated DNA sequencer. PCR reactions were performed in a 9700 PE Biosystems thermocycler using Ampli*Taq* Gold DNA polymerase (Perkin Elmer) and fluorescent-labeled primers. Gels were read and scored by GENOTYPER 2.0 (Applied Biosystems). Each genotype was scored independently by two scorers (M.A.E., M.C.D., E.B., and J.H.), and discrepancies between scorers, for any given genotype, were discussed and corrected. All genotypes were checked for Mendelian inheritance. Non-Mendelian genotypes were again subjected to PCR, rerun, and corrected in the database.

We performed homozygosity mapping by analyzing the genome-screen data from these first seven individuals. We highlighted individual markers that met the following criteria: an identical allele shared in a homozygous manner among the three affected individuals in branch 1 (ap15, ap18, and ap19), at least one copy of the same allele in both of the parents of branch 2 (ap1 and ap2), no more than one copy of the same allele in the mother of branch 1 (ap13), and no more than one copy of the same allele in the unaffected sibling from branch 1 (ap14) (table 1). These criteria were chosen to (1) identify a region that was shared identically by all affected individuals in branch 1 and that also was carried by the two parents of the affected individual in branch 2 and (2) diminish the chance of finding a region where one allele was overrepresented in all pedigree members identical by state (IBS). This methodology assumed that one of the great-grandparents was the founder and sole introducer of the acheiropodia gene in this pedigree. Naturally, the criteria for our screen would identify only regions in which, during the three generations between the founder great-grandparent and the currently affected individuals, no recombinations had occurred between the marker of interest and the acheiropodia gene. Since these markers cover the genome at an average of just under 10 cM, this was a reasonable assumption for a first pass screen. If this first pass had been unsuccessful, we would have followed up with a less stringent screening criteria, which would have allowed for detection of markers for which the linked allele had, in at least one instance, been lost to recombination.

Table 1

LOD Scores from MLINK Analyses of Chromosomes 7 and 14

Marker	Distance[a] (cM)	Z_{max} (θ)[b]
Chromosome 7:		
D7S684	147.0	.05 (.20)
D7S636	158.2	.66 (.20)
D7S798	174.0	1.57 (.10)
D7S2462	175.0	2.00 (.10)
D7S2447	178.1	2.12 (.00)
D7S2546	178.2	3.81 (.00)
D7S550	183.5	3.81 (.00)
D7S2465	184.8	3.27 (.00)
D7S2423	186.7	2.38 (.00)
Chromosome 14:		
D14S261	6.0	.01 (.40)
D14S283	13.1	1.02 (.10)
D14S990	13.8	.42 (.20)
D14S972	20.7	.84 (.10)
D14S264	21.9	.30 (.20)
D14S64	21.9	1.23 (.10)
D14S80	27.2	.22 (.20)
D14S275	28.5	.70 (.10)

[a] Between this marker and the p telomere. Marker order and intermarker distances are based on the Généthon online database. Placement of the uppermost marker on each chromosome (used as anchor points for this table) was estimated on the basis of the Center for Medical Genetics, Marshfield Medical Research Foundation.

[b] LOD scores were calculated on the basis of allele frequencies in a control sample of 35 unrelated individuals from the same population, with θ being varied from 0 cM to .5 cM, in 0.1-cM increments.

Of the 377 markers genotyped in the homozygosity-mapping stage of this project, 24 were homozygous for the same allele in all three affected siblings of branch 1. Forty-nine markers showed a minimum of five identical alleles, of a total of six "affected" chromosomes in the three affected siblings. When the optimal criteria for haplotype mapping were applied to our sample (as described in the paragraph above), only two markers met all the requirements: D7S550 and D14S283. Although our strategy for screening for the acheiropodia gene was to find a region by homozygosity mapping, we also performed a LOD-score analysis for all markers tested in the initial screen. For this analysis, we used the GENEHUNTER program and assumed a 100% penetrance rate, a 0% phenocopy rate, autosomal recessive inheritance, rare prevalence (.0001) of the disease allele, and equally common alleles at each site. LOD scores completed at this stage ranged from 1.0 to 2.0, for both of these markers (LOD scores for other markers ranged from <-1 to 2.0).

Our strategy was to follow up any initial regions that satisfied the above criteria, by typing additional markers in the region of interest, performing additional LOD-score analyses, and searching for haplotypes that were inherited identically by descent (IBD) in the affected subjects in the pedigree. In this second stage, we were also able to genotype seven additional family members (ap3, ap4, ap6–ap9, ap17, and ap23) who had not previously been available. We genotyped these additional individuals only in the two regions (7q and 14p) that, after the initial screen, had been identified as most likely to harbor the acheiropodia gene. Since several additional samples were now available, we performed a "final" LOD-score analysis for markers in the two key regions, using the MLINK program and the same assumptions with regard to the disease parameters. Allele frequencies were estimated on the basis of genotyping of 36 control samples from unrelated individuals drawn from the same region from which this pedigree (pedigree 1) had been drawn. Since the allele frequencies of the markers in the general population can affect the range of the LOD score, and since the heterogeneous nature of the Brazilian population makes the estimation of frequencies especially difficult, we also performed the linkage analysis for one of the key markers, under three additional scenarios: the high-risk allele is rare in the population ($P = .003$), the high-risk allele is common in the population ($P = .30$), and the high-risk allele is extremely common in the population ($P = .70$).

Once genotyping was completed for all the markers at 7q and 14p in all available samples (seven individuals from the initial screen plus the eight newly acquired samples), LOD-score analyses resulted in a maximum LOD score (Z_{max}) >2.1, in all five markers in the distal region of chromosome 7q (table 1). In contrast, Z_{max} values for the chromosome 14 markers were <1.3 (table 1). The Z_{max} values under this model were at D7S550 (3.81), D7S2546 (3.81), and D7S2465 (3.27). A multipoint analysis using the MLINK program revealed a Z_{max} of 4.2 for markers D7S2546 and D7S550. Under the alternate models with regard to the frequency of the linked allele on chromosome 7, D7S2546 showed Z_{max} values ranging from 3.44 (recombination fraction [θ] 0; high-risk-allele population frequency .30) to 4.29 ($\theta = 0$; high-risk- allele population frequency .003). The Z_{max} for D7S2546 does not fall to <3.0 unless the population frequency of the high-risk allele is raised to the extremely high level of .70 ($Z_{max} = 2.95$ at $\theta = 0$).

Visual inspection of the haplotypes in the 7q36 region (fig. 1) revealed an identical haplotype (13-12-324-12-15) shared, in homozygous form, by all four patients with acheiropodia, at markers D7S2447-D72546-D7S550-D7S2465-D7S2423. The fact that the parents of the patients with acheiropodia are not homozygous for these alleles suggests that this shared homozygous haplotype is inherited in an IBD manner by all the affected individuals. Similar inspection of the chromosomes of unaffected siblings in both branches of this pedigree shows that no portion of the 13-12-324-12-15 haplotype is seen in homozygous form.

Genotypes were completed for the five markers in the 7q36 region of the two additional patients with acheiropodia (pedigrees 2 and 3 in figure 1), who have no known relationship to the main pedigree. Both of these patients showed homozygous haplotypes (17-18-14-12-324-12-15) throughout the region (extending from D7S798 through D7S2423), and the two parents in pedigree 3, both carriers of the disease gene, each had only one copy of this haplotype (fig. 1). The lower four markers in the haplotype (D7S2546-D7S550-D7S2465-D7S2423) displayed alleles (12-324-12-15) identical to those seen in all patients with acheiropodia who were from the main pedigree. Of these last four markers, only the associated allele at D7S2423 is seen at a high frequency in the background population (the frequency of the associated allele at each of these markers, as seen in our sample of 36 control subjects, was 12%, 13%, 18% and 41%, respectively).

Our studies provide several lines of evidence for the acheiropodia gene being located in the 7q36 region. In stage 1, homozygosity mapping (with our initial, limited sample of three affected individuals and four unaffected individuals) in an extended pedigree first identified D7S550 as one of two markers most likely to be linked to the acheiropodia gene, in a screen of 377 markers. In stage 2, additional markers typed in the vicinity of D7S550 in four affected individuals from this pedigree (the three original affected members plus one affected individual who entered the study at this later stage) revealed a shared, homozygous haplotype stretching over 11.7 cM, most likely inherited IBD from a single chromosome of one of the great-grandparents of these affected individuals. Unaffected siblings within the two branches did not show regions of homozygosity in the key 7q36 region. Also during stage 2, a final, formal linkage analysis of this pedigree (with a total of 4 affected individuals and 11 unaffected individuals) revealed several markers at this locus that had $Z_{max} > 3.3$, and these LOD scores were robust to a reasonable range of population-level frequencies of the high-risk allele.

The shared homozygous haplotype seen in the four affected patients in the extended pedigree delimits the locus containing the gene to an 11.7-cM region extending from below D7S2462 to the q telomere. Examination of the 7q haplotypes in the two new cases of acheiropodia, which have no known relation to the extended pedigree (stage 3), further delimits the region most likely to harbor the acheiropodia gene, to an 8.6-cM segment from below D7S2447 to the q telomere. Although, in this study, the subjects with acheiropodia are drawn

from only three unconnected families, the presence of an identical homozygous haplotype in the 7q36 region of all affected individuals strongly supports the theory that all acheiropodia cases in Brazil derive from one ancestral mutation (Freire-Maia et al. 1975*b*).

The 7q36 region to which acheiropodia maps includes at least three known candidate genes for the illness: the human version of the sonic hedgehog gene (SHH [MIM 600725]), HLXB9 (MIM 142994) (which, like sonic hedgehog, is a homeobox gene) (Deloukas et al. 1998; Heus et al. 1999), and a gene with homology to the engrailed gene (MIM 131310) (Marigo et al. 1995). SHH is a promising yet problematic candidate gene. SHH is expressed in the zone of polarizing activity (ZPA) in the limb-bud zone of developing human embryos (Odent et al. 1999), and the ZPA is well known to direct the formation of limb structures (Johnson and Tabin 1997). Induced homozygous disruptions of SHH in a mouse model (Chiang et al. 1996) have been shown to cause failure of development of the forelimbs (quadruple congenital amputation)—a result identical in nature to the phenotype seen in acheiropodia—in addition to defects similar to those in holoprosencephaly. Arguing against SHH being the acheiropodia gene are the following facts: In humans, several different mutations of the SHH gene have been identified that lead to some form of holoprosencephaly (Belloni et al. 1996), yet no associated limb abnormalities have been found in any of these families (Odent et al. 1999); previous sequencing studies of SHH in families with various inherited limb abnormalities have found no causal mutations (Vargas et al. 1998); and, in humans, loss of one SHH allele appears to be sufficient to cause holoprosencephaly (Roessler et al. 1996), yet none of the carriers in the pedigree with acheiropodia show evidence of holoprosencephaly. HLXB9 is a homeobox gene that lies in the region flanked by D7S550 and D7S2423 and whose disruption has been implicated in hereditary sacral agenesis (Ross et al. 1998). Finally, engrailed, like SHH, has been shown, in animal models, to have a function in development of the limb structures (Manouvrier-Hanu et al. 1999).

Two other genetic illnesses involving limb malformations have been mapped to the 7q36 region, but the causal gene or genes for these illnesses have not yet been identified. Complex polysyndactyly (CPS), a congential malformation with both pre- and postaxial abnormalities, maps close to D7S559 in the 7q36 region (Tsukurov et al. 1994; Lynch et al. 1995). Preaxial polydactyly (PPD) also maps to the 7q36 region (Heutlink et al. 1994). The location of the PPD gene within 7q36 has been refined to a 1.9-cM region that excludes the SHH gene (Zguricas et al. 1999). Although the candidate region for PPD contains HLXB9, no disease-causing mutations have been found within this gene thus far (Heus et al. 1999). It therefore remains possible that there are distant regulatory mechanisms or as-yet-undiscovered genes within the 7q36 region whose disruption is responsible for CPS, PPD, and/or acheiropodia. Further sequencing of this region, which seems especially critical for the formation of limb structures, should ultimately lead to clarification of the precise molecular mechanisms that underly acheiropodia and these other diseases.

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Electronic-Database Information

Accession numbers and URLs for data in this article are as follows:

Généthon, http://www.genethon.fr (for intermarker distances)
Center for Medical Genetics, Marshfield Medical Research Foundation, http://www.marshmed.org/genetics
Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/Omim (for acheiropodia [MIM 200500], engrailed gene [MIM 131310], HLXB9 [MIM 142994], and SHH [MIM 600725])

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