Mapping of the Autosomal Recessive (AR) Craniometaphyseal Dysplasia Locus to Chromosome Region 6q21-22 and Confirmation of Genetic Heterogeneity for Mild AR Spondylocostal Dysplasia

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We report on a four-generation inbred family including 10 individuals affected with a form of craniotubular dysplasia (CTD). All affected patients were born to consanguineous healthy parents; this finding, together with the equal sex ratio among affected individuals and the occurrence of only normal individuals among their offspring, indicates that the disease in this family is an autosomal recessive (AR) trait. Taking into account the segregation pattern of the disease in the family and the radiological characteristics of two young CTD patients, the most likely diagnosis for the defect is AR cranio-metaphyseal dysplasia (CMD). CMD is a CTD, with both autosomal dominant (AD) and recessive forms. The description of the present genealogy confirms the AR pattern of inheritance of some cases of CMD and contributes to a better delineation of the clinical spectrum of AR CMD, suggesting a more pronounced diaphyseal involvement in the AR compared with the AD CMD. Through genomewide scanning, we mapped the AR CMD to a 7 cM interval, between D6S302 and D6S1639, at 6q21-22 region. We have also excluded the positional candidate COL10A1 gene as being the responsible for this disorder. Curiously, a form of AR spondylocostal dysplasia (SD) also segregates in the family, including one affected individual with both conditions. The gene DLL3, mapped to 19q13 region, was recently found to be responsible for one form of AR SD; however, we did not find evidence of linkage between this 19q region and the SD segregating in our family, thus implying in genetic heterogeneity for AR SD. Am. J. Med. Genet. 95:482–491, 2000. © 2000 Wiley-Liss, Inc.

KEY WORDS: craniometaphyseal dysplasia; craniodiaphyseal dysplasia; hyperostosis; sclerosis; spondylocostal dysostosis; linkage analysis; genetic heterogeneity

INTRODUCTION

Craniometaphyseal (CMD) and craniodiaphyseal dysplasias (CDD) are rare genetic heterogeneous skeletal disorders with abnormal modeling of the skull and tubular bones, belonging to the group of craniotubular dysplasias (CTD) [Gorlin et al., 1969; Gorlin, 1994]. Patients with CMD and CDD share similar craniofacial changes, such as hypertelorism and hyperostosis of the skull. Their distinction has been based mainly on the radiological analysis of long bones, because metaphyseal widening, especially of the lower limbs, is typically present in CMD, whereas prominent hyperostosis of the diaphyses is characteristic of CDD [Penchaszadeh...
et al., 1980; Gorlin et al., 1969; Gorlin, 1994; Tischert and Braun, 1998]. The clinical manifestations and the rate of progression of these two diseases are very variable, with a great deal of overlap. Most CMD cases thus far described present an autosomal dominant (AD) pattern of inheritance [OMIM 123000; Beighton et al., 1979; Carnevale et al., 1983; Gorlin, 1994; Beighton, 1995; Tischert and Braun, 1998], with only a few families suggesting an alternative autosomal recessive (AR) mode of transmission [OMIM 218400; Ross and Altman, 1967]. In contrast, all CDD cases, usually associated with severe facial anomalies and a very poor prognosis, are isolated, and its pattern of inheritance has yet to be confirmed [OMIM 122860; Brueton and Winter, 1990; Gorlin, 1994]. The clinical similarity among these craniofacial dysplasias suggests that genes associated with these phenotypes encode proteins that use common molecular pathways. The identification of these genes not only will be of utmost importance to clarify these questions but also will provide tools for a better classification of the craniofacial dysplasias.

The locus for the AD form was mapped to 5p on the basis of linkage analysis in a single family of German ancestry, but this gene still remains to be identified [Nürnberg et al., 1997]. In contrast, the genes for the AR CMD or the CDD forms are still not mapped and/or cloned.

In the present article, we report on a large inbred Brazilian family with an AR mode of inheritance and clinical findings consistent with a mild form of CMD. We mapped the AR CMD locus to chromosome region 6q21-22 through a genomewide search in this family. In addition, we excluded the COL10A1 gene, the most promising positional candidate, as being responsible for the disease.

Curiously, another AR disorder, a form of spondylocoetal dysostosis (SD), also segregates in this family. SD (OMIM 277300), a heterogeneous disorder of vertebral dyssegmentation with both AR and AD inheritance, is associated with great clinical variability [Rimoin et al., 1968; Norum, 1969; Cantú et al., 1971; Beighton and Horan, 1981; Temple et al., 1988; Floor et al., 1989; Lorenz and Rupprecht, 1990; Turnpenny et al., 1991; Satar et al., 1992]. SD patients may present a severe phenotype with death in infancy or a mild form with longevity not necessarily decreased [Aymé and Preus, 1986; Temple et al., 1988; Turnpenny et al., 1991; Turnpenny et al., 1999]. Recently, it was shown that mutations in the DLL3 gene, mapped to 19q13, cause a relatively mild form of AR SD [Turnpenny et al., 1999; Bulman et al., 2000]. Linkage analysis between markers from the 19q13 region and the SD phenotype segregating in our family support heterogeneity for relatively mild forms of this group of skeletal dysplasias.

CLINICAL AND RADIOLOGICAL REPORTS

The pedigree of the family is summarized in Figure 1, showing the presence of 10 affected individuals, five being male and five female. Five of the 10 affected individuals are deceased (II-2, II-3, II-5, II-11, and II-12), and they were born to healthy parents without stated consanguinity. Their status as affected individuals was confirmed in all instances by more than one member of the family, and photographs of deceased individuals II-5 and II-11 showed the same facial characteristics of the propositus (V-1) and the other living affected individuals. The cause of their death, which took place in the age range of 46–70 yr, was clearly unrelated to the bone disease (Chagas disease and accidental). These five affected individuals had a total of 31 descendants, all normal. The other five affected individuals (IV-10, IV-26, IV-27, V-1, and V-4) were born to healthy consanguineous parents and were evaluated personally.

The current age of the CMD patients belonging to the 4th and 5th generations are as follows: IV-10 = 46 yr; IV-26 = 27 yr; IV-27 = 21 yr; V-1 = 3 yr; and V-4 = 2 yr. All patients, including the youngest one, presented flattening of the nasal bridge and hypertelorism (Fig. 2A). Prominence of the frontal bones was observed in the two sisters, IV-26 and IV-27. All affected individuals had difficulties breathing, but none had visual or hearing complaints. Except for IV-10, who has short stature, the two other adult patients have normal height (IV-26: 1.78 m; IV-27: 1.72 m). In addition, affected individuals have normal intellectual development. The neurological development of the two affected children is within the normal range.

In addition to the facial findings, IV-10 presents a short neck and severe scoliosis, alterations that were also observed in one of her cousins (V-3) without facial changes. Detailed clinical examination and radiological studies were performed in four affected individuals and are summarized as follows.

V-1 (Propositus)

V-1 (Propositus) is a male, aged 3 yr, with nasal flattening and hypertelorism observed at the age of 11 months, with worsening of the facial changes since then (Fig. 2A). Genu valgum was also present. Radiographs and three-dimensional reconstructed computerized skull tomography showed sclerosis of the cranial vault, base, and hyperostosis of the nasal root, mandible, and maxilla (Fig. 2B,C). The long tubular bones of the limbs were wide and the cortex of the diaphyses thick (Fig. 2D). Biochemical analysis showed normal calcium levels, but the urine pyrroline had a twofold increase (3,400 nM bone collagen equivalents [BCE]/mM creatinine; normal children up to 1,752 BCE/mM creatinine).

V-4

V-4 is a female, aged 2½ yr, with flattening of the nasal bridge observed at the age of 1 yr, with worsening of the facial appearance since then. Her height (97 cm, 90th centile), weight (13.0 kg, 50th centile), and head circumference (49.5 cm, 75th centile) were normal. Ra-
Fig. 1. Pedigree segregating two autosomal recessive diseases: craniometaphyseal dysplasia (CMD) and a form of spondylocostal dysostosis, as indicated by different symbols.
diographs showed hyperostosis of the skull, nasal root, mandible, and maxilla (Fig. 3A,B); diaphyseal hyperostosis in the limb bones with mild metaphyseal widening (Fig. 3C,D); and widening of the proximal and middle phalanges of both limbs (Fig. 3E,F).

V-3

V-3 is a male aged 5 yr, without the facial appearance of CTD; head circumference (49 cm) within the normal age range; height (97 cm) and weight (14.5 kg) below the 3rd centile; elongated face; synophrys; low set ears; microstomia; hypoplasia of the zygomatic region; short, barrel-shaped thorax with pectus carinatum; short neck with low posterior hairline; and protuberant abdomen. Spine radiographs showed cervical hemivertebrae, without posterior fusion (Fig. 4B–D); skull and limb radiographs were normal (Fig. 4A,E).

IV-10

IV-10 is a female, aged 46 with typical face of craniotubular dysplasia, short trunk and neck, and low posterior hairline. Radiographs of the skull showed hyperostosis of skull, nasal root, mandible, and maxilla (Fig. 5A,B); wide tubular bones of the upper limbs with thick diaphyseal cortex (Fig. 5C); abnormalities in vertebrae segmentation (Fig. 5D,E), similar to those in V-3.

FAMILY AND METHODS FOR MOLECULAR ANALYSIS

Family

Peripheral blood was drawn from 25 individuals (5 affected and 20 normal subjects) belonging to the family here reported (Fig. 1 and Fig. 6 for linkage analysis), after obtaining their informed written consent.

Genomewide Screen for the CMD Locus

Genomic DNA was isolated by use of standard protocols [Miller et al., 1988]. A set of 499 highly polymorphic microsatellite markers (from Isogen and/or Research Genetics), spaced 10–20 cM and spanning all human autosomes were amplified. DNA amplification was done in a 10 μL reaction containing 50 ng of genomic DNA, 10 mM Tris-HCL (pH 8.4), 1.5 mM MgCl₂, 0.01% gelatin, 200 μM each of dTTP, dGTP, and dATP, 2.5 μM dCTP, 0.35 μCi α-[³²P]dCTP, 4 pmol of each primer, and 0.15 U of Taq polymerase (HT Biotechnology). Thermal cycling (9700; Perkin-Elmer, Oakbrook, IL) consisted of a first denaturing cycle of 94°C for 4 min, followed by 30 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min. The PCR products (Gibco BRL) were denatured (at 96°C for 5 min) and were separated on a 6.5% polyacrylamide 4 M urea gel.
The gels were exposed to autoradiography film with intensifying screens for 2–24 hr at room temperature. For an initial screen, we analyzed four affected CMD patients (Fig. 6: IV-11; IV-21; IV-22; V-4) and a DNA pool containing equal amounts of DNA from three non-affected relatives (Fig. 6: IV-14; IV-19; IV-20). Once a homozygosity region was detected among the affected individuals but not in the pool, all the other members of the family were analyzed for the respective markers.

Analysis for the Autosomal Recessive Spondylocostal Dysostosis (AR SD) Mapped at 19q13 Region

The family was tested for the AR SD gene DLL3 mapped to 19q13 using seven polymorphic microsatellites markers from this candidate region: D19S75, D19S224, D19S876, D19S47, D19S422, D19S217, and APOC2. For this analysis, both patients with SD (Fig. 7: IV-11 and V-3) were considered as affected individuals, whereas all the others, including those with only CMD, were classified as normal subjects (Fig. 7).

Linkage Analysis

Two-point linkage analysis was performed by using the MLINK program of the LINKAGE package, version 5.1 [Lathrop et al., 1984]. An AR model of disease inheritance was used, and disease-allele frequency was set equal to 0.001. Equal recombination rates for both sexes were considered. Except for the markers that showed evidence for linkage to the AR CMD gene, we considered equal allele frequencies for all the others. On the basis of the analysis of 48 control chromosomes, we estimated the frequency for each allele segregating in the family for the four markers (D6S261, D6S433, D6S287, D6S1657) linked to the CMD locus.

COL10A1 Gene Analysis

The National Center for Biotechnology Information (NCBI) database was examined for identification of candidate genes in the chromosomal region linked to the CMD locus (6q21-22). We identified the COL10A1 gene as possible candidate. The three coding exons of this gene (including the intron/exon splice junctions) were screened for mutations through SSCP analysis and/or direct sequencing. The primers used are available under request. DNA amplification was performed in a 25-μL reaction containing 50 ng of genomic DNA, 10 mM Tris-HCl (pH 8.4), 1.5 mM MgCl₂, 0.01% gelatin, 250 μM each of dTTP, dGTP, dATP, and dCTP, 25 pmol of each primer, and 1 U of Taq polymerase (HT Biotechnology). Thermal cycling (9700; Perkin-Elmer) consisted of a first denaturing step of 94°C for 4 min, followed by 35 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min.
Fig. 6. Summarized pedigree of the family showing haplotypes at marker loci on chromosome 6. Dots represent sites that have occurred recombination. Haplotypes in brackets are deduced from those in offspring and shaded haplotypes represent the 7 cM genetic interval for the AR CMD locus.
Fig. 7. Summarized pedigree of the family indicating as affected only the two individuals with a form of autosomal recessive spondylocostal dysplasia (SD). Haplotypes with 19q markers are shown. Dots represent sites where have occurred recombination. Haplotypes in brackets are deduced from those in offspring.
RESULTS

Genomewide Search for the CMD Locus

After testing 499 polymorphic microsatellite markers, we identified only one single region of homozygosity, with D6S261, among all affected individuals (Fig. 6). Twelve additional polymorphic markers from this region were tested, as follows: cen-D6S1563-5cM-D6S302-2cM-D6S261-2cM-D6S433/D6S287/D6S1657-1cM-D6S1608-2cM-D6S1639-3cM-D6S407-3cM-D6S1620-1cM-D6S262-2cM-D6S1656-4cM-D6S975–tel [Dib et al., 1996]. All the CMD affected individuals share a common homozygous haplotype with the markers D6S261-D6S433/D6S287/D6S1657-D6S1608. Lod scores higher than 3.0 were observed with two of them, D6S261 and D6S1657 (Table I), thus confirming linkage between these 6q region and the AR CMD gene. Based on the homozygous region and two recombinant individuals (Fig. 6: II-3 and IV-30 or any of her ancestors), we placed the putative AR CMD gene within a 7 cM interval on the long arm of human chromosome 6, flanked by the markers D6S302 and D6S1639.

We identified only the COL10A1 gene as a potential candidate for this disease through the examination of the 6q21-22 region. However, we excluded it as candidate because of the analysis of its coding region (including the splice sites) in our CMD patients failed to identify pathogenic mutations.

Analysis of the 19q Region as Candidate for the Autosomal Recessive SD Form Mapped at 19q13.1-13.3

Twenty-five individuals of the genealogy, including 2 affected and 23 nonaffected individuals for this skeletal dysplasia were typed for seven markers from the 19q13.1-13.3 region. Haplotypes and lod scores for these markers are shown respectively in Figure 7 and Table II. Homozygosity for one of the 19q markers (D19S217) is observed in both SD affected individuals but also in five nonaffected relatives of the family. Besides, both SD patients and four nonaffected individuals share a common haplotype for this 19q region. Assuming full penetrance for this condition, these findings suggest that the skeletal dysplasia segregating in our family is not linked to the 19q13.1-13.3 region.

DISCUSSION

Clinical Characterization of the Family

All affected individuals with the craniofacial disorder in the family reported here have had only normal offspring; this finding, the presence of ubiquitous consanguinity, and a 1:1 sex ratio among the affected individuals indicate that the defect is inherited in an autosomal recessive mode.

The marked craniofacial alterations observed in this family are typical of a craniofacial dysplasia. Among affected children, we observed not only metaphyseal widening but also diaphyseal hyperostosis in the upper limbs and significant enlargement of the metapysis in the femur (Figs. 2 and 3). In the 46-year-old patient (Fig. 5), the upper limb bones were thick and undermodeled, with relatively dense diaphyses. In all three cases, the short tubular bones of the hands showed a moderate degree of cortical hyperostosis. These observations, together with the typical autosomal recessive pattern of inheritance, indicate that the most likely diagnosis of the condition segregating in this family is AR CMD. A pronounced involvement of the diaphyses of the upper limb bones was also described in some of the AR CMD patients belonging to the few familial cases so far reported [Penchaszadeh et al., 1980], suggesting that diaphyseal involvement even in adulthood is a common feature in AR CMD. In contrast, diaphyseal sclerosis can be present in children with the AD form, but it tends to disappear with age [Gorlin et al., 1969; Gorlin, 1994]. The significant diaphyseal abnormalities in the upper limbs of the affected adult individual of our family (IV-10; Fig. 1) would not allow CDD to be ruled out as an alternative diagnosis for this family. However, inheritance in CDD is not likely to be autosomal recessive because all CDD cases so far reported are isolated [Brueton and Winter, 1990; Sinow et al., 1996] and might therefore represent new mutations of an autosomal dominant form. As it has been further illustrated in the present report, the overlap among AD CMD, AR CMD, and CDD is significant and can lead to diagnostic confusion.

The clinical manifestations in the patients reported here are milder than other reported cases of autosomal recessive CMD, where the skull is more severely involved with generally complete nasal obstruction and cranial nerve compressions leading to visual or hearing loss [Ross and Altman, 1967; Millard et al., 1967; Penchaszadeh et al., 1980; Gorlin, 1994]. Obviously, these differences might be only due to different allelic muta-

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tions. The spectrum of clinical variability of the AR CMD is not well defined because there is a tendency to label severe isolated cases of CMD as the AR form [Beighton, 1995; Elçioglu and Hall, 1998], which might actually represent new dominant mutations.

Patient IV-10, in addition to the typical facial and radiographic changes of CMD, also had a short neck and trunk, probably due to vertebral segmentation abnormalities. Similar spine anomalies were also observed in patient V-3, who does not have CMD. Given the consanguinity in this family, it is possible that IV-10 has CMD in addition to a second autosomal recessive skeletal disorder, possibly a form of spondylocostal dysostosis (SD), affecting V-3 in isolation.

Mapping of the AR CMD to 6q Region and Genetic Heterogeneity for AR SD

We have located a gene for AR CMD to chromosome region 6q21-22 by homozygosity mapping of a large Brazilian white family, providing further evidence that AR and AD CMD are two distinct conditions. The candidate interval was defined in 7 cM.

The field of human genetics, including the skeletal dysplasias, has boomed in the last 10–12 yr; not only it has been shown that mutations within the same gene may cause different clinical disorders but also that different mutations in the same gene may cause more than one clinical phenotype, sometimes with different pattern of inheritance [Wolf, 1997; Passos-Bueno et al., 1999; Barletta et al., 2000]. In view of these observations, we considered the COL10A1 gene as a positional candidate gene for AR CMD, even though it is well known that mutations in this gene cause Schmid metaphyseal chondrodysplasia [Warman et al., 1993; Wallis et al., 1994; Wallis et al., 1996]. The COL10A1 gene, which encodes a short-chain, nonfibrillar, homotrimer collagen synthesized exclusively and transiently by the hypertrophic chondrocytes of the growth plates [Schmid and Linsenmayer, 1985; Kirsch and von der Mark, 1990, Marriott et al., 1991], was shown here not to be the faulty gene in CMD patients. Therefore, we have yet to identify potentially interesting candidate genes in this chromosomal area in which has already been mapped many expressed-sequenced tags with unknown function.

Bone is a dynamic tissue in which the process of resorption and formation is integrated through systemic and local interactions to achieve skeletal remodeling and morphogenesis during growth and development. Although our understanding of bone development has advanced tremendously in the past 10 yr, particularly on osteoclast differentiation and therefore bone resorption, there is still much more to be learned on bone remodeling or more specifically on osteoblast differentiation [Karsenty, 1999]. These processes are possibly coordinated through an interaction of many genes, and a defect in any of them might lead to an impaired bone morphogenesis and/or bone turnover [Francomano et al., 1996; Karsenty, 1999].

The nature of the basic defect in AR CMD is still unknown. It has been discussed that the local hyperostosis and metaphyseal long bone dysplasia in CMD are due to osteoclast dysfunction or alternatively to increased bone remodeling [Millard et al., 1967; Fanconi et al., 1988; Yamamoto et al., 1993]. In three isolated cases of severe CMD, alkaline phosphatase and urinary excretion of hydroxyproline were increased [Fanconi et al., 1988; Key et al., 1988; Yamamoto et al., 1993]. Similarly, in one of our cases, the calcium levels were normal, but urine pyridinolines were increased twofold. These biochemical findings are consistent with increased bone remodeling in CMD, and a defect on osteoblast differentiation might be involved.

Haplotype analysis of the 6q21-22 region revealed that patient V-3, who has only spine deformities and a diagnosis of nonsyndromic SD, did not share the same haplotype of the CMD patients. These observations indicate that the skeletal alterations seen in patients V-3 and IV-11 (Fig. 7) are indeed due to mutations in another locus and that patient IV-11 is homozygous for two recessive disease loci, CMD and SD. This skeletal dysplasia does not seem to be caused mutations in the DLL3 gene mapped at 19q13 [Turnpenny et al., 1999; Bulman et al., 2000], because our results do not support linkage between this chromosomal area and the SD disease segregating in our family. Thus, we suggest that there are at least two loci for relatively mild forms of nonsyndromic SD.

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