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Novel and recurrent COMP (cartilage oligomeric matrix protein) mutations in pseudoachondroplasia and multiple epiphyseal dysplasia

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要約　偽性軟骨無形成症 (PSACH) と多発性骨端異形成症 (MED) は内軟骨性骨化障害と早期の変形性関節症という共通の骨格異常がある。両者はこれまで明らかに異なる疾患単位と考えられてきたが、近年ともに cartilage oligomeric matrix protein (COMP) をコードする遺伝子の突然変異が原因であることがわかってきた。COMP突然変異を検討し表現型遺伝型の関係を調査するためにPSACHおよびMEDの15例のゲノム遺伝子を解析し８つのカルモデュリン様の繰り返しの中に10の突然変異を同定した。７つはexon 9,10,11,13,14の新たなミスセンス変異で、残る３つは。exon13の５個のGAC繰り返しの一つの欠失であった。exon13の７番目のカルモデュリン様の繰り返しのGAC繰り返しは、突然変異のhot-spotであり、その変異は重度のPSACHであり、その他では軽症型PSACHあるいはMEDであった。このような表現型遺伝型の関係を知ることは分子生物学的診断、PSACHとMEDの分類に有用でCOMP遺伝子産物の構造と機能の関係解明の端緒となる。

Introduction

Pseudoachondroplasia (PSACH) is a relatively common skeletal dysplasia characterized by short-1imbed short stature with normal facies and intelligence (Maroteaux and Lamy 1959; Hall and Dorst 1969). Its clinical features include joint laxity, limitation of the movement of joints, severe bony deformities, and early onset of osteoarthroses; radiographlc features include platyspondyly with anterior beaking of the vertebral bodies and generalized dysplasias of epiphyses and metaphyses of the long and short tubular bones (Wynne-Davies et al. 1986). This syndrome exhibits considerable clinical and genetic heterogeneity; Hall and Dorst (1969) distinguished four varieties of PSACH on the basis of severity of disease and mode of inheritance, i.e., the Maroteaux-Lamy (severe) and the Kozlowski (mild) types, each being subject to either autosomal dominant or recessive inheritance. However, recent clinical and molecular studies have demonstrated gonadal/somatic mosaicism in PSACH families that were originally considered to represent autosomal recessive inheritance, suggesting that auto-somal recessive inheritance is unlikely (Hall et al. 1 987; Hecht et al. 1995). Multiple epiphyseal dysplasia (MED) defines a group of dominantly inherited skeletal dysplasias involving epiphyses of the long and short tubular bones. MED also exhibits considerable clinical heterogeneity. This disorder appears in two forms, i.e., severe (Fairbank type; Fairbank 1947) and mild (Ribbing type; Ribbing 1 955). Although MED patients do not show the significant metaphyseal and vertebral dysplasias characteristic of PSACH, their epiphyseal mani-festations are very similar. Because of the broad phenotypic overlap between the two conditions, they are now considered to represent a continuous spectrum of disorders, being categorized as a family of skeletal dysplasias (Spranger 1988).

Genetic linkage of mild (Briggs et al. 1993) and severe (Hecht et al. 1993) forms of PSACH, respectively, has been demonstrated to a locus in the pericentromeric region of chromosome 19. A subsequent candidate-gene approach revealed that mutations of the gene encoding cartilage oligomeric matrix protein (COMP) were responsible for PSACH (Briggs et al. 1995; Hecht et al. 1995). Furthermore, COMP mutations were also identified in patients with Fairbank (Briggs et al. 1995) and Ribbing (Ballo et al. 1 997) types of MED. These findings indicated that PSACH and MED are allelic, and that mutations of the COMP gene can produce a wide spectrum of manifestations from severe PSACH to mild MED. However, the full extent of COMP mutations, and possible phenotype-genotype relationships, are unclear because so few mutations have been identified; we are aware of only 15 previously documented COMP mutations, ten of them in PSACH patients (Briggs et al. 1995, 1998; Hecht et al. 1995; Susic et al. 1997) and five in MED patients (Briggs et al. 1995, 1998; Ballo et al. 1997; Susic et al. 1997) .

To characterize additional COMP mutations and investigate possible phenotype-genotype correlations, we screened the COMP gene in 15 patients with PSACH or MED, by direct sequencing. We report here the identification of ten mutations, seven of them novel, and pro-

vide ev-idence to indicate a correlation of genotypes with phenotypic manifestations.

Materials and methods

Patients

Patients were identified and followed at special clinics for skeletal dysplasias in the National Rehabilitation Center for Disabled Chil-dren, the Saitama Children's Medical Center, or the Dokkyo University Hospital. Diagnosis of PSACH was made on the basis of clinical and radiographic examinations. Criteria were (1) short-limbed short stature, not identifiable at birth but recognized in early childhood; (2) normal facies and intelligence; (3) joint laxity, limitation of the movement of joints, and severe bony deformities; (4) platyspondyly with anterior beaking; and (5) generalized dysplasias of epiphyses and metaphyses of the long and short tubular bones. Diagnosis of MED was also made on the basis of clinical and radiographic criteria, including generalized dysplasias of epiphyses of the long and short tubular bones and the absence of spinal dysplasia.

Fifteen patients were included in the study; ten of them were diag-nosed as having PSACH, and five as MED. None had family histories of the disease except for two of the MED patients. On the basis of the severity of the disease, the PSACH patients could be divided into the severe and mild types. Four PSACH patients were classified as the se-vere type, six the mild type. The heights of PSACH patients of the se-vere type were below -6 SD, and those of the mild type, between -4SD and -2SD. Limitation of the movement, and the deformities of the joints were more severe in the severe type. However, radiographs showed no significant qualitative difference between the two types.

DNA samples and polymerase chain reaction (PCR)

Blood samples were obtained from patients and members of their families with informed consent. Genomic DNA samples were extracted by standard procedures and amplified by the PCR. For exons lO and 13 of the COMP gene, primers were as described previously (Briggs et al. 1995; Hecht et al. 1995). Other exons were amplified by sets of primers designed according to the published COMP cDNA sequence (L32137 in GCG) and its genomic structure reported by Briggs et al. (1995). Primer sequences for exon 9 were i7i9/F (sense): 5'-TTGAGGCCGGCTTGGGTG-3' and i7i9/R (anti-sense): 5'-CCCGTAGATCTACCTTTTCATTGGG-3'. Primer sequences for exon Il were i lOil l/F (sense): 5'-CATCCTAATGAAGTCATTCTGGC-3' and ilOill/R (anti-sense): 5'-ATCCAACTTGCAGTTCACCC-3'. Primer sequences for exon 14 were e 14/F (sense): 5'-GACGTGTGCCAGGA-CGACTT-3' and el4/R (anti-sense): 5'-CCCACCTGGTrGAGCAC-CAC-3'. The PCRs were performed with the Takara exTaq system (Takara Shuzo, Otsu, Japan) according to the instructions of the manufacturer, in a total volume of 25 μl using as templates 50 - 100 ng of each genomic DNA sample. The PCR conditions were as follows: initial de-naturation (94°C, 2 min) followed by 35 cycles of denaturation (94°C, 30 s), annealing (55°-63°C according to the Tm of the primers, 30 s), extension (72°C, 30 s), and final extension (72°C, 5 min).

Nucleotide sequence analysis

The PCR products were purified by Ultrafree-MC (Millipore) and sequenced directiy by means of the AB1377 automated sequencer and the Prism Ready Reaction DyeDeoxy Terminator Cycle Sequencing Kit (ABI). For confirmation of mutations, PCR products were subcloned to T-vector (Invitrogen) and sequenced by the automated sequencer using M 13 universal primers. Nucleotide sequences were determined on both strands.

Restriction digestion of PCR products

The PCR products were digested for 6-8 h with l0-20 U of each appropriate restriction enzyme per microgram of DNA, at the optimal temperature for each enzyme, then electrophoresed on 3% or 4% NuSieve GTG agarose gels (EMC, Rockland, Me., USA).

Results

Ten COMP mutations were identified, nine for PSACH and one for MED. Three mutations comprised a 3-bp deletion, and the remaining seven were all missense mutations.

Identification of a recurrent COMP mutation in patients with severe PSACH

Direct sequencing of exon 13 of the COMP gene identified 3-bp deletions in three patients IPS-SI (M), PS-S2(H), PS-S3(O)]; each deletion had eliminated one of the five copies of GAC from the trinucleotide repeat region at nucleotides 1405-1419 (nucleotides are numbered from the translation start site). This mutation, confirmed by sequencing the subcloned PCR products, had been describeci previously in patients with severe PSACH (Hecht et al. i995), It resulted in loss of an aspartic acid residue (D473del) within the 7th calmodulin-like repeat of the gene product. The phenotype of all three of our patients with this mutation was also severe, their adult heights being less than 110 cm. They were all sporadic cases.

Identification of a novel COMP mutation
in a patient with severe PSACH

One patient [PS-S4(1)] was heterozygous for a single-base change at nucleotide 1418 (A 141 8~,G) . This novel missense mutation, which also occurred within the GAC repeat in the 7th calmodulin-like repeat encoded in exon 13, would cause replacement of a conserved aspartic acid residue with glycine at codon 473 (D473G). Direct sequencing of DNA samples from the patient's clinically unaffected parents failed to find this mutation, nor was it detected in normal controls or any other PSACH or MED patients. The patient was a sporadic case. His height at the age of 17 years was 108 cm (-l0 SD).

Identification of novel COMP mutations i
n mild PSACH cases

Five of the six patients with mild PSACH carried novel missense mutations elsewhere than in exon 13 of the COMP gene. These mutations occurred at sites encoding conserved amino acids in the calmodulin-like repeats of the protein. Patient PS-M1 (O) was heterozygous for G868->A (D290N) in exon 9; patient PS-M2(D) for AI046->G (D349V) in exon 10; patient PS-M3(W) for Tl 159->G (C387G) in exon 11 ; patient PS-M4(1) for G1552->A (D518N) in exon 14; and patient PS-M5(K) for G895->A (G299R) in exon 9. These mutations were confirmed by PCR-RFLP (restric-tion fragment length polymorphism) analyses : the D349V and C387G mutations created Fnu4HI and Sau961 sites respectively, and the D290N , D518N, and G299R mutations abolished MvaI, TaqI and DdeI sites, respectively. None of these sequence changes were present among 50 unrelated, unaffected individuals or in other PSACH or MED patients of our panel. All patients were sporadic cases.

Identification of a novel COMP mutation in MED

We found a novel missense mutation in exon 10, A 1082->T (D361V), in a patient [MED-1 (M)] with Fairbank-type MED The aspartic acid at codon 361 is a highly conserved amino acid in the 3rd calmodulin-like repeat. This mutation was confirmed by PCR-RFLP analysis, as the change had created a Tsp451 site. The mutation was not detected in 50 unrelated, unaffected individuals or in other PSACH or MED patients. The patient had an affected mother and an affected younger sister, who also had the mutation.

Sequence variations

In all patients examined, nucleotides 766-767 and 854-855 were GC, not CG as in the published sequence (L32137). This difference would mean substitution of an arginine residue for alanine at codon 256 (CGC->GCC) and a phenylalanine residue for arginine at codon 285 (CCG->CGC). However, neither of these sites represents a conserved amino acid. We also found two patients who were heterozygous for C279->A, but this change did not cause an amino acid substitution.

Discussion

COMP is an extracellular matrix protein specific to cartilage; it is localized mainly in the territorial matrix surrounding chondrocytes. The COMP monomer is a 110-kDa glycoprotein containing an amino-terminal domain, four contiguous epidermal growth factor-like repeats, eight contiguous calmodulin-like repeats, and a carboxy-terminal domain (Newton et al. 1994; Briggs et al. 1995). Calmodulin-like repeats are thought to bind calcium by means of aspartic acid residues lining calcium-binding pockets. The consensus sequence of the calmodulin-like repeats of COMP is N-(D)Q-D-D-DG-GDAC(D)-D-D-D...DNPC- (DiCesare et al. 1994). Because the amino acids in the repeats are highly conserved, replacement would alter the conformation and function of the COMP gene product. All COMP mutations reported in the previous and present studies have involved calmodulin-like repeats, except for two cases (Briggs et al. 1998), underscoring the

functional importance of this domain. If one includes the results of the present study, a total of 22 different COMP mutations have been identified in 37 patients, 19 of them with severe PSACH, 12 with mild PSACH, and 6 with MED . All 19 patients with severe PSACH carried mutations in the 7th calmodulin-like repeat encoded in exon 13, 17 of them within the GAC repeat sequence at nucleotides 1405-1419; 15 of these reflected deletion of one trinucleotide. Hence, the GAC repeat is a mutational hotspot of the COMP gene; more than one-third of the identified mutations, and almost half of mutations in PSACH comprised this delption. Mutations in the 7th calmodulin-like repeat in exon 13 produce the severe PSACH phenotype except one at the top of the repeat. In contrast, patients with mutations in exons other than 13 showed mild PSACH or MED phenotypes. These genotype-phenotype correlations should facilitate molecular diagnosis and classification of PSACH and MED, and provide insight into the function of COMP and the physiological consequences of different mutations. Among the 22 COMP mutations documented here and elsewhere, 18 were missense mutations and 4 were inframe deletions; most of them substituted or deleted conserved aspartic acid or cysteine residues. The type of mutation is unlikely to be related to phenotype, however, because although missense mutations such as D473G could produce a severe PSACH phenotype, the most drastic change among the reported mutations, a four amino acid deletion (V513-K516del), resulted in mild PSACH (Susic et al. 1997). To date, no mutations producing truncated gene products, for example nonsense mutations or insertion/deletions causing frameshifts, have been identified so far. It remains to be determined whether these kinds of mutation would produce phenotypes within the PSACH-MED spectrum of skeletal dysplasias, or cause syndromes belonging to a completely different category. The molecular mechanism by which COMP mutations cause PSACH and MED remains unclear. Haplo-insufficiency is unlikely, in view of the wide spectrum of disease phenotypes associated with known mutations. No individuals with a karyotypic deletion of 19p have shown pheno-types similar to PSACH and MED; no case of PSACH or MED with deletion of the COMP locus has been reported. A dominant-negative mechanism has been postulated, on the ground that COMP forms a pentamer (Morgelin et al. 1992). However, incorporation of mutant monomers into the COMP pentamer has not been proven. COMP belongs to the thrombospondin family of extra-cellular calcium-binding proteins. Thrombospondins participate in calcium-dependent interactions with a number of extracellular matrix proteins, including type V collagen, laminin, and heparin (Mumby et ai. 1984; Takagi et al. 1 993). Chondrocytes from PSACH and MED patients show cytoplasmic inclusion bodies that stain with antibodies against core protein of proteoglycan (Stanescu et al. 1982). Patients carrying mutations in the type IX collagen gene (COL9A2) also exhibit a MED phenotype (Muragaki et al. 1996). These lines of evidence suggest that COMP might interact with these molecules. If so, dysfunction of COMP would likely result in structural and functional disintegration of the extracellular matrix. Identification and charac-terization of additional COMP mutations in PSACH and MED patients would improve our understanding of the molecular pathogenesis of these diseases and provide more information about the relationship between the structure and function of the COMP gene product.

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References

Ballo R, riggs MD, Con DH, Knowlton RG, Beighton PH, Ramesar RS (1997) Multiple epiphyseal dysplasia, Ribbing type: a novel point mutation in the COMP gene in a South African family. Am J Med Genet 68:396~400

Briggs MD, Rasmussen IM, Weber JL, Yuen J, Reinker K, Rimoin DL, Cohn DH (1993) Genetic linkage of mild pseudoachondro-plasia (PSACH) to markers in the pericentromeric region of chromosome 19. Genomics 18:656-660

Briggs MD, Hoffman SMG, King LM, Olsen AS, Mohrenweiser H, Leroy JG, Mortier GR, Rimoin DL, Lachman RS, Gaines ES, Cekleniak JA, Knowlton RG, Cohn DH (1995) Pseudoachondroplasia and multiple epiphyseal dysplasia due to mutations in the cartilage oligomeric matrix protein gene. Nat Cenet I O:330-335

Briggs MD, Mortier GR, Cole WG, King LM, Golik SS, Bonaventure J, Nuytinck L, Paepe A De, Leroy JC,

Biesecker L, Lipson M, Wilcox WR, Lachman RS, Rimoin DL, Knowlton RC, Cohn DH (1998) Diverse mutations in the gene for cartilage oligomeric matrix protein in the pseudoachondroplasia-multiple epiphyseal dysplasia disease spectrum. Am J Hum Genet 62:311-319

DiCesare PE, Morgelin M, Mann K, Paulsson M (1994) Cartilage oligomeric matrix protein and thrombospondin I. Purification from articular cartilage, electron microscopic structure, and chondrocyte binding. Eur J Biochem 223:927-937

Fairbank HAT (1947) Dysplasia epiphysealis multiplex. Br J Surg 135:225-232

E, Dorst JP (1969) Pseudoachondropiastic SED, recessive Maroteaux-Lamy type. The clinical delineation of birth defects. Birth Defects 5:242-257

Hall JG, Dorst JP, Rotta J, McKusick VA (1987) Gonadal mosaicism in pseudoachondroplasia. Am J Med Genet 28: 143-151

Hecht JT, Francomano CA, Briggs MD, Deere M, Conner B, Horton WA, Warman M, Cohn DH, Blanton SH (1993) Linkage of typi-cal pseudoachondropiasia to chromosome 19. Genomics l8 :661 - 666

Hecht JT. Nelson LD, Crowder E, Wang Y, Elder PFB, Harrison WR, Francomano CA, Prange CK, Lennon GG, Deere M, Lawler J (1995) Mutations in exon 17B of cartilage oligomeric matrix pro-tein (COMP) cause pseudoachondroplasia. Nat Genet 10:325-329

Marotcat,x P. Lamy M (1959) Les formes pseudoachondroplastiques des dysplasies sponctylo-epiphysaires. Presse Med 67:3 83-386

Morgelin M, Heinegard D, Engel J, Paulsson M (1992) Electron microscopy of native cartilage oligomeric matrix protein purified from swarm rat chondrosarcoma reveals a five-armed structure. J Biol Chem 267:6137-6141

Mumby SM, Raugi CJ, Bornsein P (1984) Interaction of thrombospondin with extracellular matrix proteins: selective binding to type V collagen. J Cell Biol 98:646-652

Muragaki Y, Mariman ECM, Beersum SEC van, Perala M, Mourik JBA van, Warman ML, Olsen BR, Hamel BCJ (1996) A mutation in the gene encoding the (x2 chain of the fibril-associated collagen IX, COL9A2, causes multiple epiphyseal dysplasia (EDM2). Nat Genet 12: I 03-l05

Newton G, Weremowicz S, Morton CC, Copeland NG, Gilbert DJ, Jenkins NA, Lawler J (1994) Characterization of human 'and mouse cartilage oligomeric matrix protein. Genomics 24:435-439

Ribbing S (1955) The hereditary multiple epiphyseal disturbance and its consequences for the aetiogenesis of local malacias: particular-ly the osteochondrosis dissecans. Acta Orthop Scand 24:286-299

Spranger J (1988) Bone dysplasia families. Pathol Immunopathol Res 7:76-80

Stanescu V, Maroteaux P, Stanescu R (1982) The biochemical defect of pseudoachondroplasia. Eur J Pediatr 138:221-225

Susic S, McGrory J, Ahier J, Cole WG (1997) Multiple epiphyscitl dysplasia and pseudoachondroplasia due to novel mutations in the calmodulin-like repeats of cartilagc oligomeric mattrix protein. Clin Genet 51 :2 19-224

Takagi J, Fujisawa T, Usui T, Aoy'am'a T, Saito Y (1993) A single chain 19-kDa fragment of bovine thrombospondin binds to type V collagen and heparin. J Biol Chem 268: 15544-15549

Wynne-Davies R, Hall CM, Young ID (1986) Pseudoachondroplasial: clinical diagnosis at different ages and comparison of autosomal dominant and recessive types. a review of 32 patients (26 kindreds). J Med Genet 23:425-434

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