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Identification of novel and recurrent mutations in the calcium binding type III repeats of cartilage oligomeric matrix protein in patients with pseudoachondroplasia

L.H. Cao[1], L.B. Wang[2], S.S. Wang[1], H.W. Ma[2], C.Y. Ji[1] and Y. Luo[1]

[1]Research Center for Medical Genomics, China Medical University, Shenyang, China
[2]Department of Developing Pediatrics, Shengjing Hospital, China Medical University, Shenyang, China

Correspondence author: Y. Luo
E-mail: luoyang@mail.cmu.edu.cn

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ABSTRACT. Pseudoachondroplasia is an autosomal dominant osteochondrodysplasia characterized by disproportionate short stature, joint laxity, and early onset osteoarthrosis. Pseudoachondroplasia is caused by mutations in the gene encoding cartilage oligomeric matrix protein (*COMP*). We looked for mutations in the *COMP* gene in three sporadic Chinese pseudoachondroplasia patients and identified two novel mutations, c.1189G>T (p.D397Y) and c.1220G>A (p.C407Y), and one recurrent mutation, c.1318G>C (p.G440R), in the calcium binding type III repeats of *COMP*. This study confirms the relationship between mutations of the *COMP* gene and clinical findings of pseudoachondroplasia; it also provides evidence for the importance of the calcium binding domains to the functioning of *COMP*.

Key words: PSACH; *COMP*; Gene mutation; Skeletal dysplasia

INTRODUCTION

Pseudoachondroplasia (PSACH, MIM #177170) is an autosomal dominant osteochondrodysplasia characterized by disproportionate short stature, early onset osteoarthrosis, deformity of the lower limbs, brachydactyly, loose joints, and ligamentous laxity. The height of the affected individual is normal at birth, and is usually identified at 2 years of age on the basis of decreased linear growth, a waddling gait and lax joints. Characteristic radiographic features include platyspondyly with anterior beaking of the vertebral bodies and generalized dysplasias of epiphyses and metaphyses of the long and short tubular bones. PSACH is classified into two subtypes, the severe Maroteaux-Lamy type and the mild Kozlowski type (Kozlowski, 1976). Multiple epiphyseal dysplasia (MED) is a group of dominantly inherited skeletal dysplasias involving epiphyses of the long and short tubular bones and their epiphyseal manifestations are very similar to PSACH. MED appears in two forms, the severe Fairbank type (Fairbank, 1946) and the mild Ribbing type (Ballo et al., 1997). MED patients do not show the significant metaphyseal and vertebral dysplasias that are characteristic of PSACH, and the statures are normal or mildly short. PSACH and MED have a broad phenotypic overlap and then they together comprise a "bone dysplasia family".

Almost all PSACH and about 80% of MED cases are caused by mutations in the cartilage oligomeric matrix protein (*COMP*, MIM 600310) gene (Briggs et al., 1998; Ikegawa et al., 1998; Deere et al., 1998, 1999; Maddox et al., 2000; Hashimoto et al., 2003; Nakashima et al., 2005; Kennedy et al., 2005a), which is on 19p13.1 and encodes a 550-kDa homopentameric glycoprotein, which predominantly localizes in the extracellular matrix of cartilage, tendon and ligament (Hedbom et al., 1992). *COMP* is the fifth member of the thrombospondin family (TSP5) comprising a coiled-coil domain that participates in pentamer assembly, four type II EGF-like repeats (T2), eight calcium binding type III repeats (T3) and a large carboxyl terminal globular domain (CTD) (Oldberg et al., 1992; Newton et al., 1994; Malashkevich et al., 1996). Type III repeats are thought to play a role in binding calcium ions and have 13 calcium binding loops that conform to the consensus sequence of an EF-hand calcium binding loop such as those found in calmodulin (Chen et al., 2000). Most mutations in the *COMP* gene have been identified within these repeats, and the majority are found in the C-terminal portion of this domain (Unger and Hecht, 2001; Briggs and Chapman, 2002).

In the present report, we describe two novel mutations [c.1189G>T (p.D397Y) and c.1220G>A (p.C407Y)] and one recurrent mutation [c.1318G>C (p.G440R)] in the calcium binding type III repeats of *COMP* in Chinese patients with PSACH. The identification of the disease-causing mutation was consistent with the clinical diagnosis of osteochondrodysplasia, and also provided information for genetic detection of other family members and contributed to future prenatal diagnosis of involved individuals.

MATERIAL AND METHODS

Subjects and X-ray examinations

Three sporadic cases of PSACH were diagnosed in the Department of Developing Pediatrics of the Shengjing hospital according to their clinical and radiographic manifestations. Laboratory tests including serum concentration of calcium, phosphorus, alkaline phosphatase,

and PTH were done by routine methods. All patients took X-ray examinations of wrist, lumbar vertebrae or pelvis. Peripheral venous blood samples, data from laboratory tests, and radiographs from the patients and/or their parents were obtained after their informed consent and approval of the China Medical University Institutional Review Board.

Mutation detection

Genomic DNA was extracted from white blood cells by the standard sodium dodecyl sulfate-proteinase K-phenol/chloroform method. The 19 coding exons and their flanking intronic sequences of the *COMP* gene were amplified by polymerase chain reaction (PCR), purified, and subjected to DNA sequencing by using an automated ABI PRISM3700 sequencer. Putative mutations were confirmed by duplicate PCR amplification and sequencing of the affected exons from genomic DNA of the patients and/or their parents. The mutations were also confirmed by restriction fragment length polymorphism analysis. In patient 1, the c.1189G>T mutation directly created an *Afa*I restriction site in the mutant allele. An *Nde*I restriction site was introduced into the 1220A mutant allele of patient 2 by using the mismatch primer COMPFTWF. To confirm the mutation identified in patient 3, an *Msp*I restriction site was introduced into the 1318C mutant allele by using the mismatch primer COMPWXLR. The genomic DNA of patients and 60 unrelated normal controls was used as templates, and the amplicons were digested with the corresponding restriction enzyme and separated by electrophoresis on neutral 12% polyacrylamide gel and displayed by staining with silver. The primer sequence and restriction enzyme used for mutation confirmation are given in Table 1.

Table 1. Primers and restriction enzymes used for mutation confirmation.

Case	Sequence change	Restriction enzyme	Primers used for mutation confirmation
1	c.1189G>T (p.D397Y)	*Afa*I	COMPE11-12F2-gaagtcattctggcctggtcc COMPFJLR-ctgatccgggttgctcttctg
2	c.1220G>A (p.C407Y)	*Nde*I	COMPFTWF-tggcgatggtataggggatgcat COMPFTWR-ctggtcttgatcgctgtcac
3	c.1318G>C (p.G440R)	*Msp*I	COMP13-15F2-gactttgtgggagatgcttgtg COMPWXLR-tgtcccgagagtcctgatgcc

RESULTS

Clinical findings

The clinical and radiographic features of each patient were reviewed by at least two clinical geneticists and/or radiologists. All patients had disproportionate short stature and dysplasia of epiphysis or metaphysis and there were no significant changes in laboratory tests.

Patient 1 is a four-year-old boy with short stature (84 cm high). A wadding gait was the earliest symptom recognized at the onset of walking. Clinical examinations showed pigeon chest, macrocephaly, left knee joint laxity, and limited range of movement. Radiographs showed anterior beaking of the vertebral bodies on the lateral view (Figure 1A), dysplasia of epiphysis and metaphysis of the left ulna.

Patient 2 is a six-year-old girl with short stature (102 cm high), normal face and short neck. Radiographs showed significant epiphyseal and metaphyseal changes in the joints of

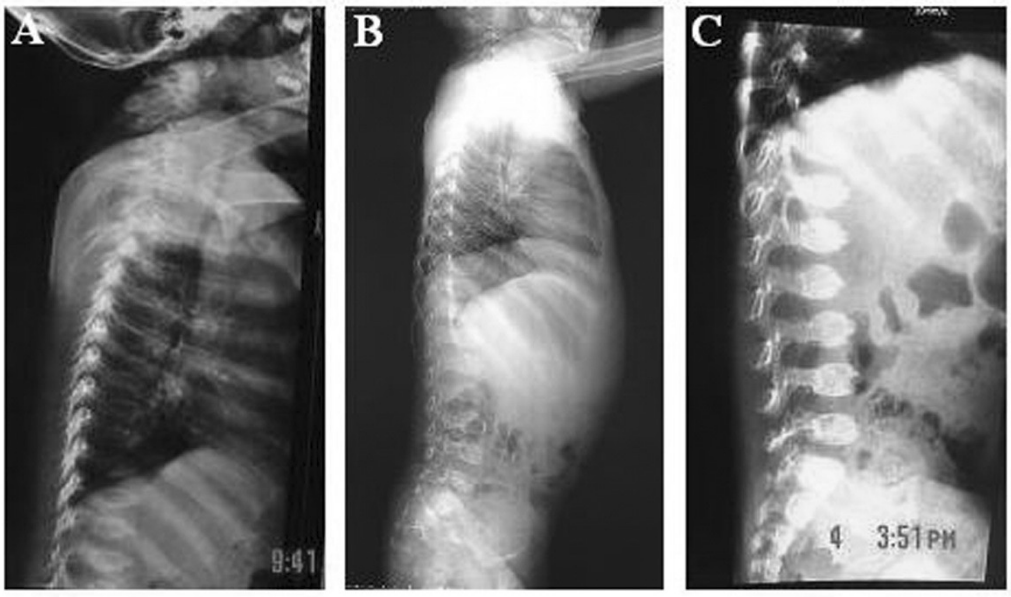

Figure 1. Lateral-view radiographs of the Chinese PSACH study patients. **A.** Radiographs showed anterior beaking of the vertebral bodies in patient 1. **B.** Radiographs indicated platyspondyly of spines and anterior beaking of the vertebrae in patient 2. **C.** Radiographs demonstrated warhead-like vertebral bodies and mild scoliosis in patient 3.

the long and short tubular bones including femur, tibia, fibula, ulna, radius, metacarpals, and phalanges. Short metacarpals and phalanges, small irregular carpal bones, platyspondyly of spines, and anterior beaking of the vertebrae (Figure 1B).

Patient 3 is a four-year-old girl with a disproportionate short stature (79.5 cm high) due to growth retardation. Clinical examinations showed genu varum and eversion of the costal margin. Radiographs showed a warhead-like vertebral bodies and mild scoliosis (Figure 1C), dysplasia of epiphysis and metaphysis of the left ulna and radius, moreover, metacarpals and phalanges were short and thick.

Identification of *COMP* mutations

Mutation analysis was performed on 3 sporadic PSACH cases by direct sequencing of the PCR-amplified DNA fragments spanning 19 coding exons and flanking intronic sequences of the *COMP* gene. Two missense mutations were identified in exon 11 and another one in exon 13. Sequencing of PCR amplicons of patient 1 revealed the heterozygous missense mutation c.1189G>T (p.D397Y) in exon 11, substituting tyrosine (Y) for the highly conserved aspartate (D) at position 397 of the *COMP*. Patient 2 had a c.1220G>A (p.C407Y) mutation in exon 11 that resulted in the substitution of a tyrosine (Y) for a cysteine (C) residue at amino acid residue 407 of the *COMP*. Patient 3 had a c.1318G>C (p.G440R) substitution in exon 13, which changed a glycine (G) 440 to an arginine (R) in the *COMP* (Figure 2). DNA samples were available from parents of patient 2, but no mutations were detected in the *COMP* sequence.

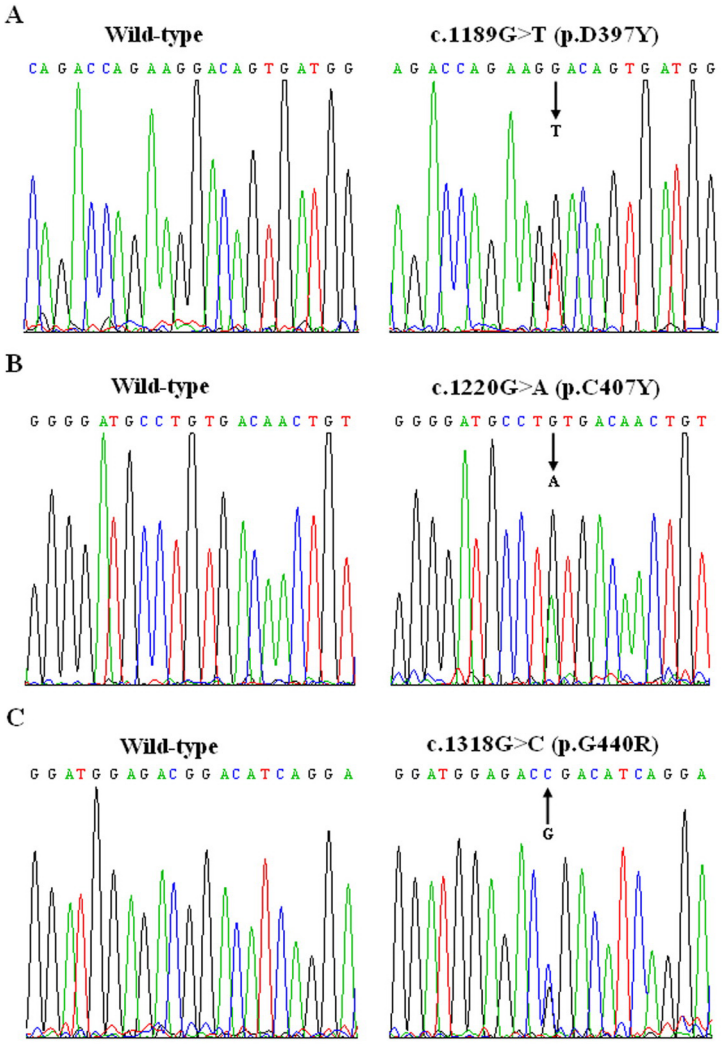

Figure 2. Three mutations of the *COMP* gene in the Chinese PSACH study patients. **A.** DNA sequencing chromatogram showing the missense mutation c.1189G>T (p.D397Y) in exon 11 of the *COMP* gene in patient 1. **B.** DNA sequence analysis demonstrating the presence of the missense mutation c.1220G>A (p.C407Y) in exon 11 of the *COMP* gene in patient 2. **C.** DNA sequencing chromatogram indicating the missense mutation c.1318G>C (p.G440R) in exon 13 of the *COMP* gene in patient 3.

The mutations c.1189G>T (p.D397Y) and c.1220G>A (p.C407Y) were novel, and c.1318G>C (p.G440R) was reported previously (Briggs et al., 1998; Loughlin, et al., 1998). All mutations lay in the calcium binding type III repeats domain of cartilage oligomeric matrix protein. By restriction analysis using *Afa*I, *Nde*I and *Msp*I, respectively, these mutations were confirmed in all affected individuals but were not detected in unaffected family members or in 60 unrelated Chinese controls (data not shown).

DISCUSSION

COMP also known as TSP5, a large extracellular glycoprotein, is abundantly expressed in proliferating and hypertrophic chondrocytes of the growth plate, articular cartilage, synovium, tendon, and ligament. Although *COMP* has recently been identified as a marker for osteoarthritis, its function is still unclear. It may play an interfacing role by mediating the interactions between cartilage fibrils and the extrafibrillar matrix (Budde et al., 2005; Hecht et al., 2005; Chen et al., 2007).

To date, 109 different mutations in the *COMP* gene associated with PSACH or MED have been reported, including those described here, and most of them are missense mutations, while small deletions, splicing and small insertions are secondary, and gross deletions are rare. However, the vast majority of these mutations are found in exons 8-14, which encode the calcium binding T3 repeats, while a few mutations have been identified in exons 15-19, which encode the CTD, and no mutations have been reported in exons 1-7, which encode the coiled-coil domain and T2 repeats. This highlights the importance of the T3 domain to the structure of *COMP* (Briggs et al., 1998; Ikegawa et al., 1998; Loughlin et al., 1998; Deere et al., 1998, 1999; Chen et al., 2000; Maddox et al., 2000; Unger and Hecht, 2001; Briggs and Chapman, 2002; Hashimoto et al., 2003; Mabuchi et al., 2001, 2003; Kennedy et al., 2005a,b; Nakashima et al., 2005). A comparison between TSP 1-4 and *COMP* sequence reveals a high degree of conservation in this region. Mutations in the T3 repeats are thought to interfere with protein folding and cause retention of mutant COMP with several other cartilage extracellular matrix proteins (specifically, type IX collagen and matrilin-3) in the rough endoplasmic reticulum of chondrocytes and may result in increased cell death (Hou et al., 2000; Vranka et al., 2001; Kleerekoper et al., 2002; Hecht et al., 2004; Schmitz et al., 2006; Merritt et al., 2007; Chen et al., 2008; Kwak et al., 2009). Mice lacking *COMP* do not produce a dwarf phenotype, and no whole *COMP* gene deletion associated with PSACH has been reported. The reason for this might be that related proteins compensate for the absence of *COMP* protein or that the patient's phenotype is caused by *COMP* protein malfunction rather than the lack of *COMP* protein. But the transgenic or knock-in mice expressing the mutant D469del or Y583M *COMP* showed growth retardation, so the mutant COMP may exert a dominant negative effect mechanism and ultimately affect the morphology and proliferation of growth plate chondrocytes, eventually leading to chondrodysplasia and the short stature of affected individuals (Piróg-Garcia et al., 2007; Schmitz et al., 2008). Previously studies have suggested that circulating *COMP* is decreased in PSACH patients carrying *COMP* mutations, so that plasma *COMP* levels may reflect genetic abnormalities in *COMP*, providing a method for preliminary screening PSACH, followed by sequencing of the *COMP* gene (Mabuchi et al., 2004; Tufan et al., 2007).

According to the clinical and radiographic presentation, three sporadic Chinese cases of PSACH were ascertained by disproportionate short stature and characteristic radiographic features including platyspondyly with anterior beaking of the vertebral bodies and generalized dysplasias of epiphyses and metaphyses of the long and short tubular bones. Mutation analysis of the *COMP* gene identified 3 missense mutations, c.1189G>T (p.D397Y), c.1220G>A (p.C407Y) and c.1318G>C (p.G440R). Moreover, all 3-amino acid residue sites were highly conserved among mammals. All mammalian *COMP* proteins with sequences available in the databases, including human, rhesus, mouse, dog, opossum, and platypus, have aspartic acid at position 397, cysteine at 407 and glycine at 440, which suggests a strong functional and structural constraint.

Both mutations D397Y and C407Y lay in the seventh calcium-binding loop and the fifth calcium binding repeat. Within this region, six causative mutations have been identified, D399N, D401N, C407F, D408Y, C410Y, and N415K (Loughlin et al., 1998; Kennedy et al., 2005a; Zankl et al., 2007). Interestingly, D397Y and C407Y are associated with PSACH, but the other six mutations all caused MED, especially C407, when substituted for different amino acids, leading to diverse phenotypes. The third mutation G440R lay in the sixth calcium binding repeat and was reported by Briggs MD and Loughlin J, respectively (Briggs et al., 1998; Loughlin et al., 1998). In addition to G440R, Briggs MD also identified another mutation, G440E, at the same position associated with PSACH, and the authors speculated that G440 was positioned to form a hydrogen-bonded turn that had a major effect on the overall structure of this region of the *COMP* protein. If disrupted, it may affect the relative positioning of calcium binding pockets and cause a malfunction of the *COMP* protein (Briggs et al., 1998).

In summary, we identified two novel mutations, c.1189G>T (p.D397Y) and c.1220G>A (p.C407Y), and one recurrent mutation, c.1318G>C (p.G440R), in the calcium binding type III repeats of *COMP* in three sporadic cases of PSACH. Further study of the mutant COMP would add to our understanding about the function of *COMP*. At present, although clinical and radiological criteria for diagnosis of PSACH have been published, no biochemical test is available, and a missense mutation in the calcium binding T3 repeats is the most common cause of PSACH. Sequencing of the *COMP* gene is therefore the gold standard for confirming the clinical diagnosis and genetic counseling.

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