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Novel mutations in *PRG4* gene in two Indian families with camptodactyly-arthropathy-coxa vara-pericarditis (CACP) syndrome

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Background & objectives: Camptodactyly – arthropathy-coxa vara-pericarditis (CACP) syndrome is an autosomal recessive disorder caused by mutations in the *PRG4* (proteoglycan 4) gene. Hallmarks of the syndrome include congenital or early-onset camptodactyly and arthropathy with synovial hyperplasia, progressive coxa vara deformity and non-inflammatory pericardial effusions. Till date only around 25 pathogenic mutations have been reported in this gene and none have been reported from India. We report here the mutations in the *PRG4* gene in three patients of CACP from two unrelated families from India.

Methods: Molecular genetic studies were done for the three patients with the CACP syndrome, from two unrelated Indian families, through sequence analysis of all coding exons and the exon-intron boundaries of the *PRG4* gene.

Results: Two novel frame-shift deletion mutations leading to premature protein termination were found. One patient was identified to be homozygous for a 2 base pair deletion in exon 6 (c.2645_2646delGA) and the two affected siblings from the other family were found to be homozygous for a 4 base pair deletion in exon 6 (c.2883_2886delAAGA).

Conclusions: This is perhaps the first report of *PRG4* mutations from India. Further mutation studies in Indian CACP cases will help to determine the mutation spectrum of the *PRG4* gene in the Indian population and also help to further elucidate the molecular pathology and the genotype-phenotype correlation of this rare disease.

Key words CACP syndrome - Indian cases - novel mutations - *PRG4* gene

Camptodactyly-arthropathy-coxa vara-pericarditis (CACP) syndrome (OMIM # 208250) is a rare autosomal recessive condition characterized by the association of congenital or early onset camptodactyly and non-inflammatory arthropathy with synovial hyperplasia. Progressive coxa vara deformity and/or non-inflammatory pericardial or pleural effusions are

also observed in many patients with this condition^{1,2}. Using homozygosity mapping, the CACP locus was assigned to a 1.9 cM interval on the human chromosome 1q25-31³. Later, using the positional cloning approach, the causative gene for CACP was identified to be the proteoglycan 4 gene (*PRG4*; OMIM# 604283; Refseq: NM- 005807)⁴.

Till date only 15 disease causing mutations in the *PRG4* gene have been listed in the Human Genome Mutation Database (www.hgmd.cf.ac.uk), in CACP patients of Caucasian, Egyptian, Saudi Arabian, Korean, UAE and Pakistani origin. Each of these reported disease-causing mutations has been predicted to prematurely truncate the protein product⁴⁻⁷. In addition, there are two recent reports, one of five novel mutations and the first case of uniparental disomy in a European cohort of CACP cases and another of an additional five novel mutations in Saudi Arabian patients with CACP^{8,9}. Mutational spectrum of the *PRG4* gene in Indian cases of CACP has not been reported. Here we report the clinical and molecular genetic findings of three patients with CACP from two unrelated Indian families. In addition, the study also reports two novel, hitherto unreported mutations in the *PRG4* gene, which further expands the known spectrum of disease-causing mutations in the gene.

Material & Methods

Three affected individuals from two unrelated families were clinically diagnosed to have the CACP syndrome and were included in the study. Of the

three patients, two were siblings aged 10 and 5 yr, respectively (both male) (patients 1a and 1b) and the third was a 16 yr old girl (patient 2) from an unrelated family. The two affected brothers belonged to a Hindu family from the Katihar district of Bihar. They were the second and the fifth offspring respectively of non-consanguineous parents; their other three siblings were normal. They were clinically evaluated and diagnosed to have the CACP syndrome at the Medical Genetics department of the Sanjay Gandhi Postgraduate Institute of Medical Sciences, Lucknow, India in January 2009. Both siblings presented with a history of persistent painless swelling involving the knee, ankle, elbow and wrist joints and contractures involving the small joints of the hands and feet since around two years of age. Neither of them had any history of rashes or recurrent fever. Both had a normal developmental history. The only salient findings on physical examination were bilateral symmetric non-tender elbow, wrist, knee and ankle joint effusions with synovial thickening and flexion contractures of bilateral wrist, interphalangeal, metacarpophalangeal and metatarsophalangeal joints in both (Fig. 1A & B). Skeletal survey revealed increased joint spaces suggestive of effusion in the knee, elbow

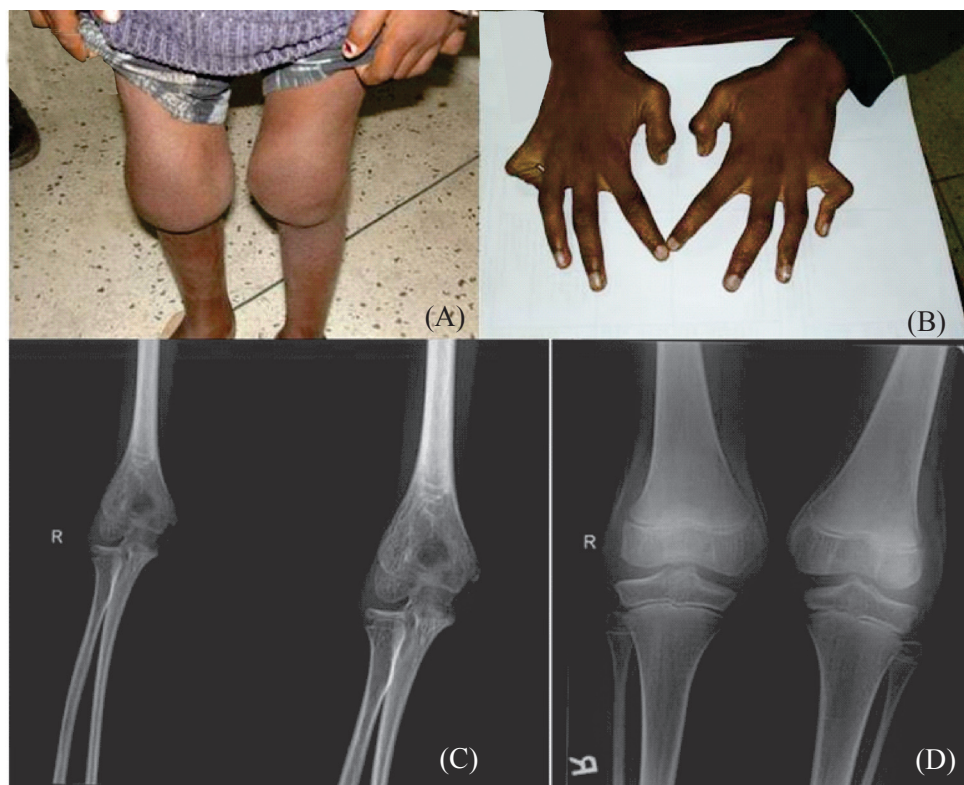


Fig. 1 (A). Photograph of the knee joints of patient 1b showing bilateral knee joint swelling. (B) Hands of patient 1a showing contractures of the metacarpophalangeal and interphalangeal joints. (C) Elbow radiograph of patient 1a showing widening of the joint space and severe periarticular osteopenia. (D) Knee radiograph of patient 1a showing widening of the joint space and periarticular osteopenia.

and wrist joints with periarticular severe osteopenia especially in the elbow joints along with soft tissue swelling (Fig. 1C & D). The female patient was 16 yr old and was the first offspring of non-consanguineous parents, belonging to a Hindu family from the Nalgonda district of Andhra Pradesh. She was clinically evaluated and diagnosed to have the CACP syndrome at the Medical Genetics department of the Nizam's Institute of Medical Sciences, Hyderabad, Andhra Pradesh, India, in December 2011. She presented with a history of persistent painless swelling involving the knee, ankle and elbow joints and contractures involving the small joints of the hands since early childhood. Her developmental milestones were normal and she did not have any other systemic symptoms. There was no history of any other similarly affected individuals in her family. The salient findings on physical examination were bilateral symmetric non-tender elbow, knee and ankle joint effusions with synovial thickening and contractures of bilateral wrist, interphalangeal, metacarpophalangeal and metatarsophalangeal joints. Skeletal survey in this patient revealed increased joint spaces suggestive of effusion in the knee and elbow joints with periarticular osteopenia.

Though all three patients were initially misdiagnosed to have juvenile rheumatoid arthritis at other centers, they were clinically suspected to have CACP syndrome on the basis of the painless joint

effusions, arthropathy and camptodactyly. None of the three patients had pericarditis. However, the absence of pericarditis does not rule out this syndrome as in some cases pericarditis has been reported to be absent or to set in later in the course of the disease².

DNA extraction & sequencing: The approval of the Institute Ethics Committee of the Centre for DNA Fingerprinting and Diagnostics was obtained for the study. As all three patients were minors, informed written consent was procured from their parents for the study. Five ml of peripheral blood (anticoagulated with potassium - EDTA) was collected from each of the three patients following the clinical diagnosis. In addition, 5 ml peripheral blood samples (anticoagulated with potassium - EDTA) were also collected from the parents of the two affected siblings (Patients 1a and 1b). Genomic DNA was extracted from the whole blood samples using the standard salting out method. PCR primers were designed to amplify the coding sequences and intron-exon boundaries of the candidate gene using Primer 3 input (version 0.4.0) tool (http://www.primer3plus.com/web_0.4.0/input.htm; accessed on February 12, 2012) (Table). Polymerase chain reaction (PCR) was performed using the Eppendorf Mastercycler gradient (Eppendorf Scientific, Hauppauge, NY; www.eppendorf.com). For amplification of *PRG4* from genomic DNA, the PCR mixture (12.5 µl) contained 100 mM Tris HCl (pH 8.8), 500 mM KCl, 0.8% (v/v)

Table. List of primers used for PCR amplification of the coding sequences and intron-exon boundaries of the *PRG4* gene [primers self-designed using the Primer 3 input (version 0.4.0). tool]

| Exons | Forward primer | Reverse primer |
|------------|-------------------------|------------------------|
| Exon 1 | ACATGCCATACTTTGGCTAGA | TTCCAAAGCACTTACAAACAGG |
| Exon 2 | TATCAGCTGGGCCTATTGCT | AGACTCGCAGTGTTGCTTGA |
| Exon 3 | TAAGGTGGGAAATGGCTGTC | TCCTCTATGTTCCCCCTCTG |
| Exon 4 | CTGGCTGTCAACAGCATCTA | TGGGCTTAGATAAGCAGTCACC |
| Exon 5 | ATCTGTGATAAGCCCAGGTG | TCATCTCTGGTGCCATCTTG |
| Exon 6.1 | GAACTTGCCACTGACTTCCA | GTAGTTGGGGCAGGCTTCTT |
| Exon 6.2 | CCACAAAAGCCCTGATGAAT | TTCACAAGCTAACAAGGTCAGG |
| Exon 7 | AAGGGAGGGCAATCAGAACT | TAGCACTCTCCACGGCTTT |
| Exon 8 & 9 | TCGGACTTGGGAAATACCTG | GCACATTTGTGAAGCGAAAG |
| Exon 10 | TGCCAAACTGAGCACTTGTC | GGGCTGTTTGATCAGTATCCA |
| Exon 11 | CATCAGAGGGCTAAACTGAGA | GAGCAGAATTGAATTGCAGTG |
| Exon 12 | TTCATAAAGGAGAGCTGAACCTG | TACAGGTGTGAGCCATGCAA |

Nonidet P40, 10 mM dNTPs, 25 mM MgCl₂, 100 ng of genomic DNA, 10 pmol of the forward and reverse primers, and 5 units Taq polymerase (Fermentas Life Sciences, Ontario, Canada). The cycling conditions included an initial denaturation at 94°C for 4 min followed by 30 cycles at 94°C for 30 sec, 55-65°C for 55 sec and 72°C for 55 sec with a final extension at 72°C for 5 min. The PCR products were resolved by agarose gel electrophoresis. The PCR products were purified using QIAquick PCR purification kit (Qiagen, Hilden, Germany) and sequenced on ABI prism 377 automated DNA sequencer (Applied Biosystems, USA).

Mutation detection: Sequence data were analyzed using the chromasLite V 2.01 software (http://www.technelysium.com.au/chromas_lite.html) and aligned with reference Genbank cDNA sequence NM_005807.2 using Clustal W multiple sequence alignment tools (http://www.ebi.ac.uk/tools/psa/emboss_

[needle/nucleotide.html](http://www.ebi.ac.uk/tools/psa/emboss_needle/nucleotide.html)). The disease-causing potential of sequence alterations was evaluated using the Mutation taster software tool (<http://www.mutationtaster.org>). The mutations were checked with already available *PRG4* gene mutations from the Human Gene Mutation Database (www.hgmd.cf.ac.uk).

Results

The entire coding region of the *PRG4* gene was assessed in our patients through bidirectional DNA sequencing, with the exception of a 1702 bp region in exon 6 which was not amenable to amplification due to its highly repetitive character. Analysis revealed two novel frame-shift mutations in our CACP patients. The affected siblings (patients 1a and 1b) were found to be homozygous for a 4 base pair deletion in exon 6 (c.2883_2886delAAGA; p.Glu961glufsX39) (Fig. 2A). Molecular genetic testing of their parents confirmed that each of them was a heterozygous carrier of this 4 base

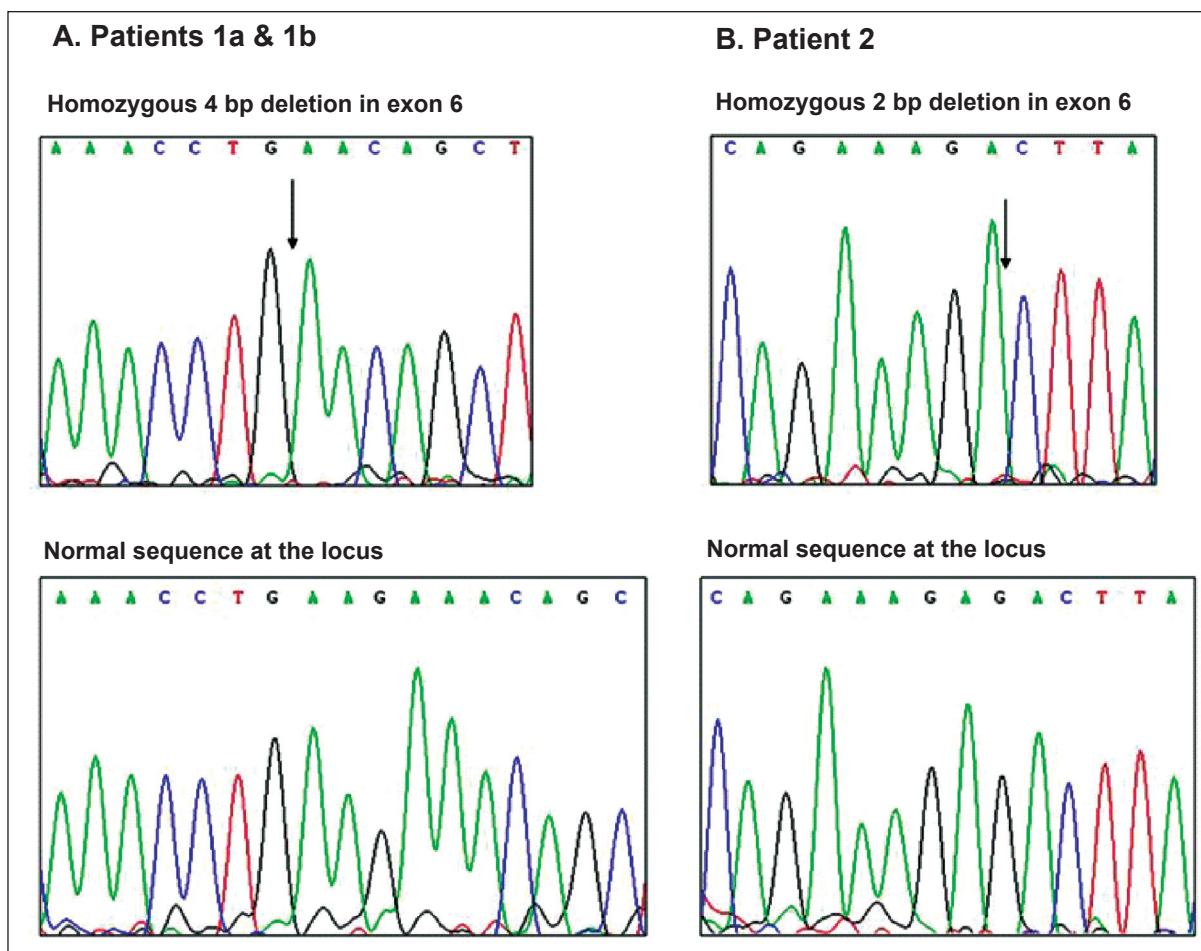


Fig. 2 (A & B). Electropherogram of wild type alleles (top) and mutant alleles (bottom) of patients 1a and 1b and patient 2. The sites of homozygous deletion are indicated by arrows.

pair deletion mutation. The female patient (patient 2) was found to be homozygous for a 2 base pair deletion in exon 6 (c.2645_2646delGA; p.Asp882LysfsX5) (Fig. 2B). Parental testing to confirm their heterozygous status for this deletion mutation could not be done for patient 2, as the parental samples in this case could not be procured. The disease-causing potential of sequence alterations was evaluated using the Mutation taster software tool. The 4 base pair deletion affecting Glu 961 in patients 1a and 1b predicts premature termination after 39 residues whereas the 2 base pair deletion affecting Asp882 in patient 2 predicts premature termination five residues later. Further proof for the pathogenicity of the two detected mutations was obtained using the Mutation Taster software tool, which predicted that both mutations were 'disease-causing'.

Discussion

The *PRG4* gene is the only gene known to be associated with the camptodactyly-arthropathy-coxa vara- pericarditis syndrome. Among the products of full length *PRG4* transcripts is a secreted glycoprotein of 1404 amino acids termed lubricin, a major constituent of synovial joints¹⁰. Lubricin is a large proteoglycan that functions as the major lubricant in articulating joints, produced and secreted by synoviocytes and superficial zone chondrocytes^{11,12}. It is present in the synovial fluid and at cartilage surfaces^{13,14}. Functions attributed to lubricin within articulating joints include protection of cartilage surfaces from protein deposition and cell adhesion, inhibition of synovial cell growth, and boundary lubrication at the articular cartilage surface¹⁵. Lubricin contains multiple protein domains namely somatomedin B (SMB), hemopexin-like (PEX) domain and a large, central extensively O- linked glycosylated, mucin-like domain that likely contribute to its diverse biologic properties^{15,16}. Studies involving synovial fluid from patients with the CACP syndrome have suggested that synovial fluid lacking lubricin would be less able to dissipate the energy of impact on the joints that occurs during locomotion¹⁷.

Individuals with CACP have normal appearing joints at birth, but with advancing age develop joint pathology associated with non-inflammatory synoviocyte hyperplasia and sub-intimal fibrosis of the synovial capsule³. These patients also develop pericarditis and finger joint flexion contractures, likely due to insufficient surface lubrication between the visceral and parietal pericardium and the tendon and tendosynovium respectively. Initial studies have shown

that the genetic disruption of the *PRG4* gene in mouse recapitulates clinical features of CACP in humans¹⁵.

Since the identification of *PRG4* as the causative gene for CACP by Marcelino and co-workers in 1999⁴, 15 mutations have been reported in the Human Genome Mutation Database (www.hgmd.cf.ac.uk) in CACP patients of Caucasian, Egyptian, Saudi Arabian, Korean, UAE and Pakistani origin⁵⁻⁷. There are two recent reports of an additional 10 novel mutations in the *PRG4* gene; five each in a European cohort and a Saudi Arabian cohort of CACP cases^{8,9}.

Both of our patients were found to have mutations in exon 6 of the *PRG4* gene. Many of the previously reported mutations in the *PRG4* gene have also been found to occur within exon 6 or an adjacent intronic splice acceptor site. This could be probably accounted for by the fact that exon 6 accounts for around 67 per cent of the coding sequence of the *PRG4* gene³. It is also interesting to note that no mutations have been documented so far in exons 1-5 of this gene. This may be due to the presence of alternatively-spliced *PRG4* isoforms that exclude exons 2, 4 and 5, thus masking mutations with no phenotypic consequence¹⁸. Earlier reports have also led to the conclusion that missense mutations or in frame deletions are not pathogenic and CACP syndrome occurs due to a null phenotype (*i.e.* premature truncation)⁴⁻⁷. Our findings also support the previous reports that only frame shift and non-sense mutations in the *PRG4* gene trigger the phenotype.

It is of interest to note that though neither of the two families gave a history of parental consanguinity, homozygosity for the respective mutations was found in both families. It is known that even in the absence of actual consanguinity, inbreeding can lead to homozygosity for recessive mutations and thereby result in disease in the offspring¹⁹. This is not an uncommon phenomenon in many regions of India where inbreeding within small communities is quite prevalent. In each of the two families, both the husband and the wife hailed from the same village and belonged to the same religious and caste group.

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