A Novel Missense Mutation in Cathepsin K (*CTSK*) Gene in a Consanguineous Pakistani Family With Pycnodysostosis

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Background: Deficiency of cathepsin K (*CTSK*), a lysosomal cysteine protease, has been shown earlier as a cause of an autosomal recessive osteosclerotic skeletal dysplasia pycnodysostosis. The objective of the present study was to identify the potential sequence variants in *CTSK* gene in a large consanguineous Pakistani family with pycnodysostosis.
Methods: Genotyping of 4 affected and 6 unaffected members of the family was performed using polymorphic microsatellite markers linked to *CTSK* gene on chromosome 1q21. To screen for pathogenic mutation, exons and splice junctions of *CTSK* gene were polymerase chain reaction amplified from genomic DNA and sequenced directly in an automated DNA sequencer.
Results: Microsatellite analysis showed linkage of the family to *CTSK* gene on chromosome 1q21. Sequence analysis revealed a novel missense mutation c.728G>A (p.G243E) in exon 6 of the *CTSK* gene.
Conclusions: A novel missense mutation was identified in *CTSK* gene in a Pakistani family with 5 individuals affected with autosomal recessive pycnodysostosis

Key Words: autosomal recessive pycnodysostosis, cathepsin K, missense mutation

(*J Investig Med* 2010;58: 720–724)

Bone development is a complex process in which a balance between bone formation and resorption is delicately maintained. The major effector cells of bone formation and resorption are the osteoblasts and osteoclasts. Perturbation of the balance between bone formation and resorption can result in a variety of genetic bone diseases including osteoporosis and osteopetrosis.[1] Pycnodysostosis is a relatively rare autosomal recessive osteopetrotic disease characterized by short stature, bone fragility, osteosclerosis, acroosteolysis of the distal phalanges, and skull deformities such as delayed cranial suture closure and loss of mandibular angle.[2]

The genetic defect causing pycnodysostosis was mapped to chromosome 1q21 by genetic linkage analysis, and the responsible gene was found to be cathepsin K (*CTSK*).[3–5] Since then, several mutations disrupting the structure and function of CTSK protein have been identified.[6–11] Targeted mutation of the *CTSK* gene in mice results in many of the phenotypic features of pycnodysostosis including increased bone density and bone deformity.[12,13]

Cathepsin K, a member of the papain family of cysteine proteinases, highly expressed in osteoclasts,[14] has been shown to degrade bone collagen as well as other bone matrix proteins[15,16] and as such was proposed to play a major role in osteoclastic bone resorption. Like most proteinases, cathepsin K is synthesized and secreted from the cell in an inactive proenzyme that is converted into its mature active form by proteolytic cleavage.[17]

In the present study, we have identified a novel missense mutation c.728G>A (p.G243E) in exon 6 of *CTSK* gene in a consanguineous Pakistani family with 5 individuals demonstrating autosomal recessive pycnodysostosis.

MATERIALS AND METHODS

Patients

A consanguineous family, originating from Sindh province of Pakistan, demonstrating autosomal recessive pycnodysostosis was ascertained for the present study. The family has 5 individuals affected with pycnodysostosis (IV-2, IV-4, IV-7, IV-9, and IV-10) (Fig. 1).

Blood samples were obtained with informed consent from 4 affected (IV-2, IV-4, IV-7, and IV-9) and 6 unaffected (III-1, III-2, IV-3, IV-5, IV-6, and IV-8) individuals of the family. Genomic DNA was extracted from whole blood using the standard phenol-chloroform procedure. Approval of the study was obtained from the institutional review board of Quaid-i-Azam University, Islamabad, Pakistan.

Genotyping

Genotyping was performed using microsatellite markers (D1S789, D1S534, D1S2344, D1S2345, D1S305, D1S1595, D1S1653, D1S398, D1S2771) flanking the candidate *CTSK* gene on chromosome 1q21. The polymerase chain reaction was performed as 40 ng of genomic DNA, 20 pmol of each primer, 200 μM of each deoxyribonucleoside triphosphate, 1 U of Taq DNA polymerase, 2.5 μL 10× reaction buffer, and 1.5 μL $MgCl_2$ (MBI Fermentas, York, UK) in a final volume of 25 μL. The thermal cycling conditions used included 95°C for 1 minute, followed by 40 cycles at 95°C for 1 minute, 57°C for 1 minute, 72°C for 1 minute, and final extension at 72°C for 10 minute. Polymerase chain reaction–amplified products were resolved on 8% nondenaturing polyacrylamide gel, stained with ethidium bromide, and genotypes were assigned by visual inspection.

Mutation Analysis

After establishing linkage of the family to *CTSK* gene on chromosome 1q21, the gene was sequenced in all those affected and unaffected individuals of the family from whom DNA samples were available for the study. The primer sequences used to amplify 8 coding exons and flanking splice junctions of the *CTSK* gene were the same as reported by Naeem et al.[11]

Sequencing of the *CTSK* gene was performed bidirectionally (forward and reverse strands) with Big Dye Terminator v3.1 Cycle Sequencing Kit, together with an ABI Prism 310 Genetic Analyzer

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Received February 5, 2010.
Accepted for publication February 20, 2010.
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ISSN: 1081-5589
DOI: 10.231/JIM.0b013e3181da50bd

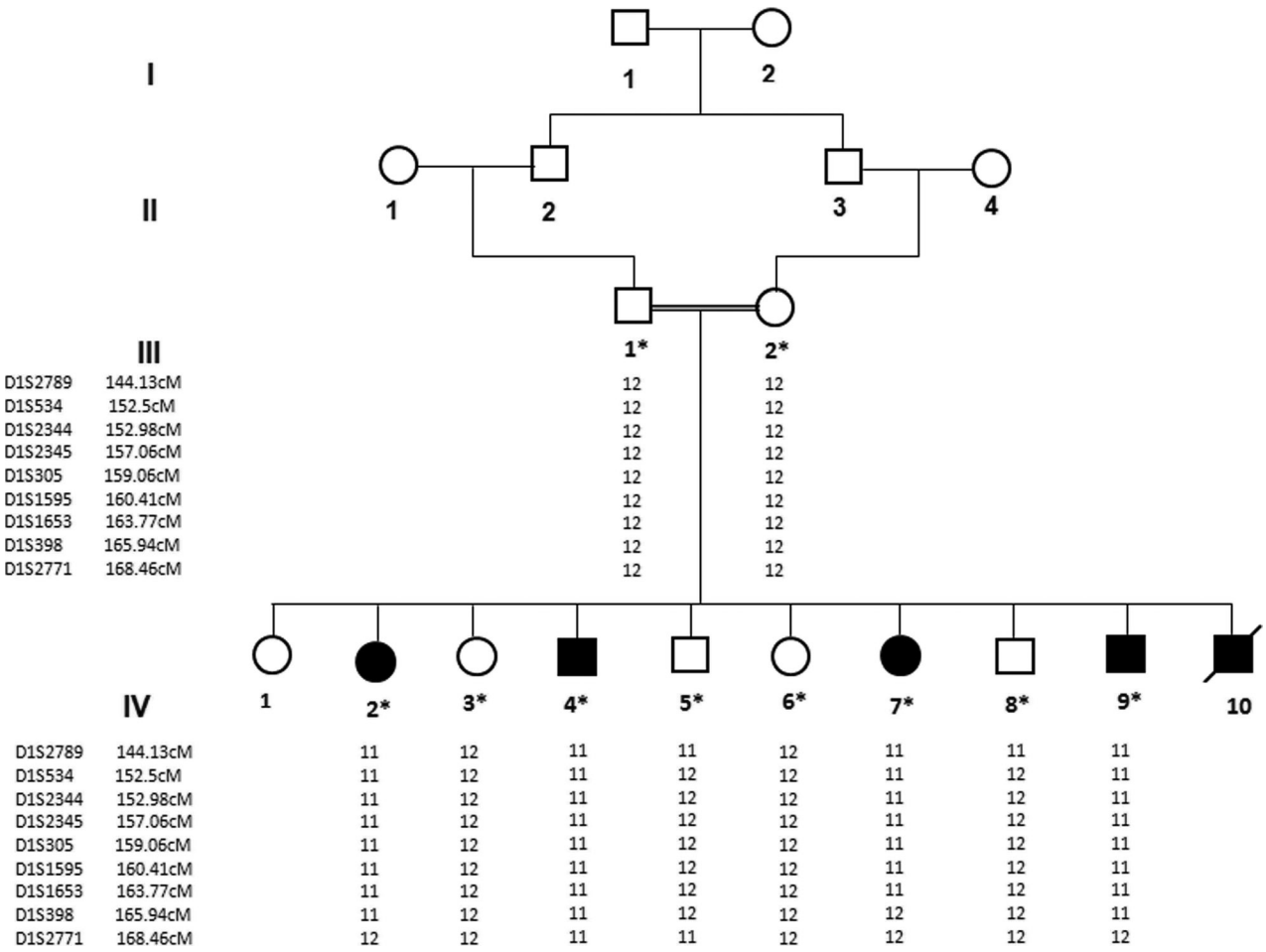

FIGURE 1. Pedigree of a Pakistani family segregating autosomal recessive pycnodysostosis. Double lines are indicative of consanguineous union. Clear symbols represent unaffected individuals, whereas filled symbols represent affected individuals. For individuals with genotyping, haplotypes of the closely linked microsatellite markers on chromosome 1q21 are shown beneath each symbol. Genetic distances in centimorgans (cM) are depicted according to the Rutgers combined linkage-physical map (build 36.2).[27]

(Applera, Foster City, CA). Sequence variants were identified via BIOEDIT sequence alignment editor version 6.0.7 (Carlsbad, CA).

RESULTS

Clinical Features

Affected individuals showed typical clinical features of the hereditary pycnodysostosis, including short stature, skull deformities, osteolysis, dental malformation, generalized increase in bone density, and stubby feet and hands with flattened nails resembling acroosteolysis (Fig. 2, A–D). All the affected individuals had a history of bone fracture. Fracturing of the bones, especially tibia, femur, and clavicle, started at an early age. Affected individuals started walking late, probably at the age of 3 years. Seizures at the age of 18 years were reported in 2 affected individuals (IV-2 and IV-4). Two other individuals (IV-7 and IV-9) are younger than 18 years and have not complaint of the occurrence of the seizures so far. Analysis of laboratory investigations revealed normal values for leukocyte and thrombocyte counts, plasma phosphate, and alkaline phosphatase. No obvious phenotypic abnormality was observed in the heterozygous parents and other unaffected individuals of the family.

Radiographic examination of 3 affected individuals (IV-4, IV-7, and IV-9) revealed the presence of open sutures with patent fontanelles. Wormian bones were also seen in the region of coronoid sutures. The maxilla and mandible were hypoplastic with zero mandibular angle. Dental malformations and presence of deciduous teeth were also observed (Fig. 3A). Bilateral clavicle and scapula were hypoplastic (Fig. 3B). Bilateral epiphyses of the head of the humeri appeared fragmented (Fig. 3C). Erlenmeyer deformity was present on the distal end of the femur (Fig. 3D). Dense thick ribs and scoliotic deformity were seen with concavity toward the left side (Fig. 3E). Spondylosis of thoracic and upper lumbar vertebrae with spool shape and increase in bone density of the pelvis was observed (Fig. 3, E and F).

Genotyping and Mutational Analysis

Genotyping data and haplotype analysis showed linkage of the family to *CTSK* gene on chromosome 1q21 (Fig. 1). Subsequently, the *CTSK* gene was sequenced in all those affected and normal individuals of the family from whom DNA samples were available for the study. Sequence analysis of the *CTSK* gene was performed using a control reference obtained from the Ensembl database (Ensembl accession ID ENST00000271651).

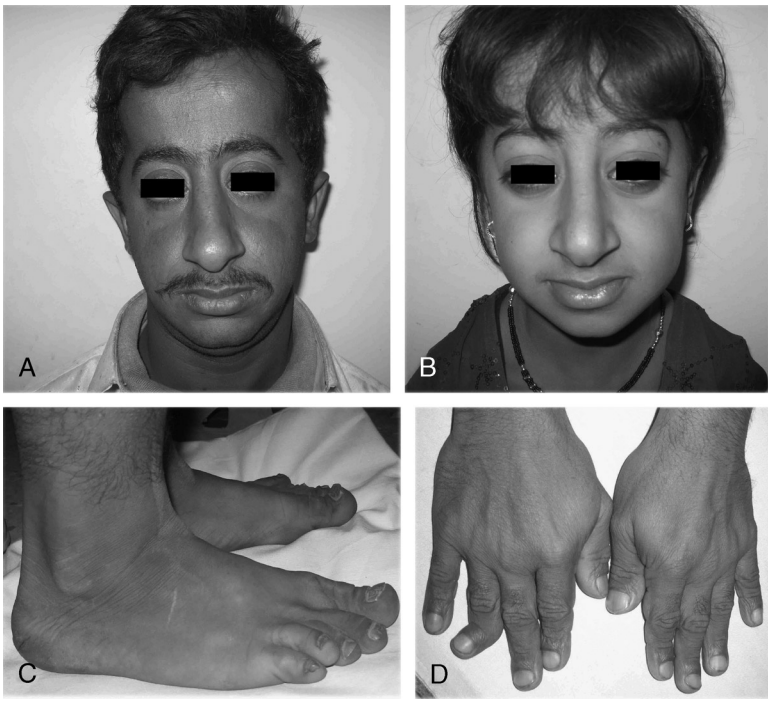

FIGURE 2. A and B, Affected individuals (IV-4 and IV-7) of the family. C and D, Stubby feet and hands with flattened nails resembling acroosteolysis.

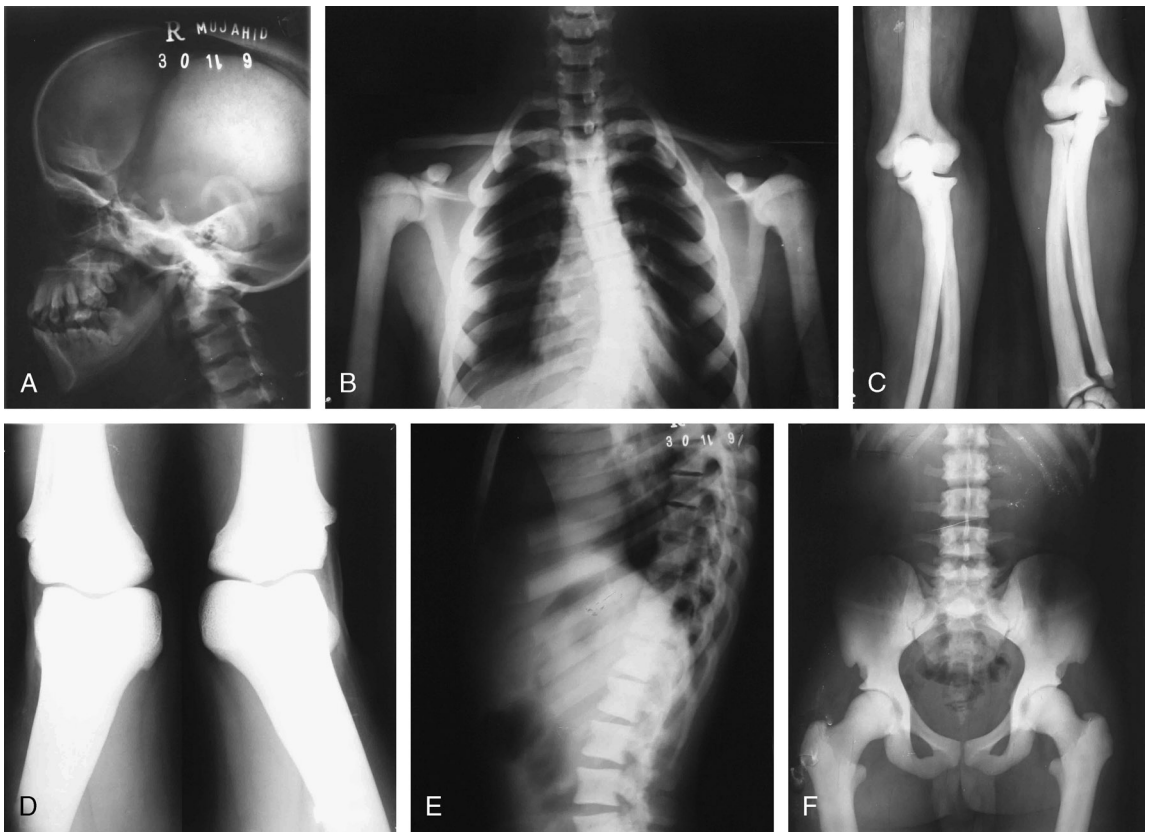

FIGURE 3. Radiographs of the patients (IV-4, IV-7, and IV-9). A, Skull deformities with frontal bossing, open sutures and fontanelles, hypoplasia of the maxilla, and dental malformation. B, Hypoplasia of clavicle and scapula. C, Epiphyses of the humeri appear fragmented. D, Erlenmeyer deformity at the distal end of the femur. E and F, Scoliotic deformity and spondylolysis of thoracic and lumbar vertebrae.

Sequence analysis of the *CTSK* gene in affected individuals of the family identified a novel missense mutation. Analysis of exon 6 of the gene revealed that the affected individual had a homozygous variant of G>A transversion at nucleotide position 728 (c.728 G>A). This resulted in substitution of glycine with glutamic acid at amino acid position 243 (p.G243E) in the CTSK protein. The pathogenic sequence variant was found in the heterozygous state in obligate carriers (Fig. 4, A–C) and segregated with the disease in the family. To ensure that the mutation does not represent a nonpathogenic polymorphism, a panel of 200 unrelated unaffected ethnically matched control individuals was screened for the mutation, and it was not identified outside the family.

Multiple sequence alignment of the CTSK sequences from different species (*Homo sapiens*, *Pan troglodytes*, *Canis lupus familiaris*, *Bos taurus*, *Mus musculus*, *Rattus norvegicus*, *Danio rerio*) revealed that amino acid glycine (G243), substituted in the novel homozygous missense mutation in the family, reported here, is an evolutionary conserved residue (Fig. 5).

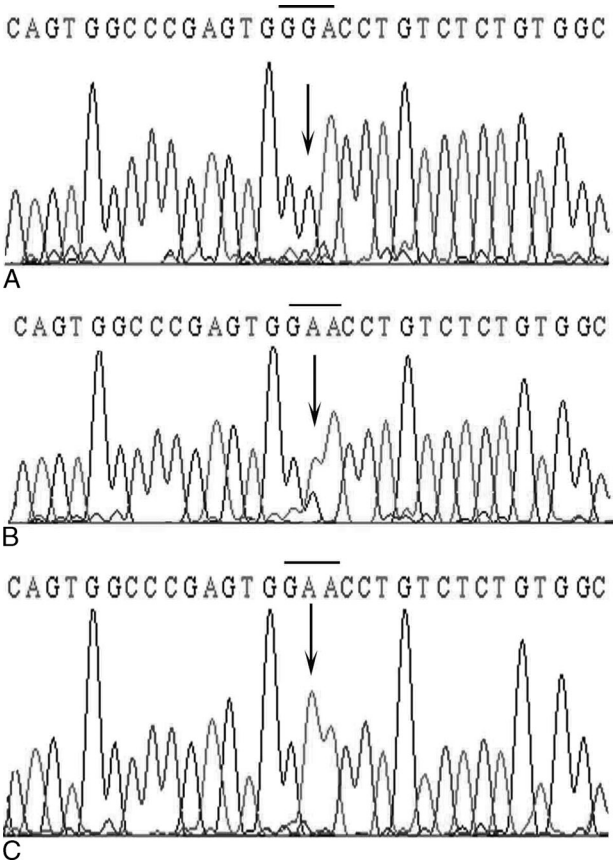

FIGURE 4. DNA sequence analysis of a novel missense mutation (c.728 G>A; p.G243E) in *CTSK* gene. The upper panel (A) represents the nucleotide sequence in the control unaffected individual, the middle panel (B) in the heterozygous carrier, and the lower panel (C) in the affected individual. Arrows represent position of the mutation.

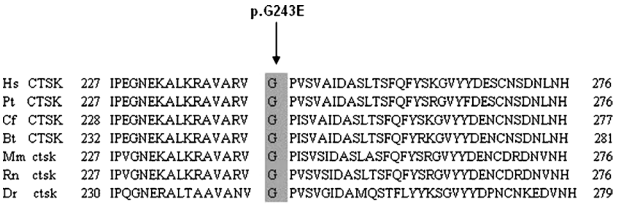

FIGURE 5. Partial amino acid sequence comparison of human CTSK with other orthologs. The shaded serine (G) residue indicates the conserved residue across different species. The missense mutation p.G243E affecting conserved serine residue in human CTSK is indicated by an arrow. Hs indicates *Homo sapiens*; Pt, *Pan troglodytes*; Cf, *Canis lupus familiaris*; Bt, *Bos taurus*; Mm, *Mus musculus*; Rn, *Rattus norvegicus*; Dr, *Danio rerio*. The accession numbers for the respective proteins are as follows: Hs, NP_714928.1; Pt, XP_517090.2; Cf, XP_853902.1; Bt, NP_777172.1; Mm, NP_067267.1; Rn, XP_223512.4; Dr, NP_001017778.1.

DISCUSSION

In the present investigation, we have presented a large consanguineous Pakistani family with 5 individuals affected with hereditary pycnodysostosis. All the affected individuals were mentally normal but became distinguishable with the disease from an early age with features such as short stature, abnormal facial appearance due to frontal bossing, stubby feet and hands, loss of the mandibular angle, and dental anomalies including persistence of deciduous teeth (Figs. 2 and 3). Most of the clinical features observed in affected individuals of our family were similar to those reported earlier. However, occurrence of seizures in affected individuals of the present family probably was not reported earlier.

The gene cathepsin K (*CTSK*), encoding a lysosomal cysteine protease and responsible for causing hereditary pycnodysostosis, was identified earlier by Gelb et al.[4] The *CTSK* gene was cloned originally from rabbit osteoclasts[18] and subsequently from several human tissues.[19–22] The predicted 329-residue CTSK polypeptide sequence is highly homologous with other proteases such as cathepsins H, S, and L.[20,21] It presents typical prepropeptide organization of cysteine proteases of the papain family, including a 15–amino-acid signal sequence (1–15), a 99–amino-acid propeptide (activation peptide) (16–114), and 215–amino-acid mature peptide (115–329) of cathepsin. It is synthesized as an inactive glycosylated preproenzyme (37 kd), which is transported to the lysosomal compartment via the mannose-6 phosphate receptor pathway for activation.[23] Activation can be catalyzed by other cathepsins, such as cathepsin D, or other cysteine protease-related enzymes or by autocatalysis.[24] Cathepsin K, predominantly expressed in osteoclasts, is a potent extracellular matrix degrading enzyme and plays a critical role in osteoclast-mediated bone resorption, which is necessary for bone remodeling. The bone resorption process is subdivided into demineralization and organic matrix degradation steps.[14,25] Different mutations reported in CTSK also lead to impaired matrix degradation due to loss of the functional CTSK protein, which is due to reduced bone resorption that results in a net increase in bone mass as observed in patients with pycnodysostosis. Osteoclasts from these patients accumulate undigested collagen fibrils.[26]

To date, 29 different mutations in the *CTSK* gene have been described, which lead to pycnodysostotic phenotype.[6–11] In the present study, we have identified a novel missense mutation (p.G243E) in *CTSK* gene in a consanguineous Pakistani family. Earlier, 20 missense mutations have been reported in *CTSK* gene. The Gly243 is highly conserved across 7 species (Fig. 5), suggesting the significance of the residue in the function of the CTSK protein. Glycine, being a simple aliphatic amino acid, has unusual conformational abilities and is commonly found in turns

of the polypeptides. The glycine is considered as helix breakers for its role in disrupting the regularity of the helical backbone conformation. On the other hand, the presence of glutamic acid tends to favor the formation of helical structures. Hence, substitution of glycine with glutamic acid probably disrupts the secondary structure by preventing the formation of U turns in the CTSK. It might also impair the conformational flexibility to the polypeptide chain normally facilitated by the glycine, which contains single hydrogen in its side chain.

ACKNOWLEDGMENTS

The authors thank the members of the family for their invaluable participation and cooperation. The work presented here was funded by the Higher Education Commission, Islamabad, Pakistan. B.K. was supported by indigenous PhD fellowships from Higher Education Commission, Islamabad, Pakistan.

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