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*Original Article*

# Molecular and clinical analysis in a series of patients with Pyknodysostosis reveals some uncommon phenotypic findings

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**Abstract:** Pyknodysostosis is a rare autosomal recessive skeletal dysplasia characterized by short stature, deformity of the skull, osteosclerosis, hypoplasia of the clavicle, and bone fragility. Radiographs show increased bone density, osteosclerosis, and acroosteolysis of the terminal phalanges. The pycnodysostosis gene is located on chromosome 1q21 and encodes an enzyme called Cathepsin K. Cathepsin K is a cysteine protease lysosomal protein associated with the degradation of bone and cartilage. In the current study, the authors described the clinical, radiological and molecular features of a group of six Mexican patients, including two familial and two sporadic cases, with Pyknodysostosis. One of the patients presented hypoacusia, an unusual finding in this disease.

**Keywords:** Pyknodysostosis, sclerosing, bone, dysplasia, Cathepsin K

## Introduction

Pyknodysostosis is a rare autosomal recessive skeletal dysplasia with complete penetrance [1]. To date, less than 200 patients have been reported in the literature. The estimated frequency is 1 to 1.7 per million. Both genders are affected equally and consanguinity has been reported in about approximately 30% of the cases [2]. The clinical manifestations were described independently in 1962 by Maroteaux and Lamy [3] and by Andren *et al* [2]. The French painter Henri de Tolouse Lautrec was retrospectively diagnosed with this disorder, however this has been subject of recent debate [4, 5]. The principal clinical features of the disease are short stature, acroosteolysis of all distal phalanges, and bone fragility with frequent fractures [6, 7]. Other features include delayed suture closure with skull deformities, prominent nose, delayed teeth eruption, partial anodontia, dental infections and short ramous of the mandible. Occasionally, exophthalmos and blue sclera are present. The trunk is not shortened although the metatarsals occasionally are abbreviated [6, 8, 9]. Short stature is an essential feature of the disease and it could be attributed not only to the sclerosing bone dysplasia affecting long bones and vertebral column, but also to associated anomalies such as congenital cardiopathy or malnutrition [10]. In addition, Soliman *et al* reported deficient growth hormone secretion in five of six patients with Pyknodysostosis in response to stimulation and low IGF-1 concentration [10]. The physiologic replacement with this hormone increased IGF-1 concentration and improved linear growth in patients with Pyknodysostosis [6, 10]. Differential diagnosis includes osteopetrosis, acroosteolysis, cleidocranial dysplasia, and mandibuloacral dysplasia [11].

Linkage analysis in inbred Arab and Mexican kindred previously showed that the Pyknodysostosis locus was located on 1q21, a position from which the gene responsible of the disease was subsequently cloned [12, 13]. The Pyknodysostosis gene, called Cathepsin K (CTSK), was originally cloned in osteoclasts from rabbits and subsequently in several

**Table 1.** Mutations described in the *CTSK* gene in patients with Pyknodysostosis. Taken from Xue *et al*. Orphanet J Rare Dis. 2011, 6: 20 and completed with data from Matsushita *et al*. Mol Syndromol 2011, 2: 254-258 and Zheng *et al*. Gene 2013, 521: 176-179

| Location in DNA sequence | Genomic DNA sequence variants | Coding DNA sequence | Effect on amino acid | Location in protein sequence | First description variants |
|---|---|---|---|---|---|
| Missense | | | | | |
| Exon 2 | g.1551T > C | c.20T > C | p.Leu7Pro | Pre | Donnarumma, *et al*., 2007 |
| Exon 2 | g.1557T > C | c.26T > C | p.Leu9Pro | Pre | Nishi, *et al*., 1999 |
| Exon 3 | g.2128C > T | c.136C > T | p.Arg46Trp | Pro | Schilling, *et al*., 2007 |
| Exon 3 | g.2227G > A | c.235G > A | p.Gly79Arg | Pro | Fratzl-Zelman, *et al*., 2004 |
| Exon 3 | g.2228G > A | c.236G > A | p.Gly79Glu | Pro | Hou, *et al*., 1999 |
| Exon 4 | g.2547G > A | c.365G > A | p.Arg122Gln | Mature | Zheng *et al*, 2013 |
| Exon 4 | g.2547G > C | c.365G > C | p.Arg122Pro | Mature | Matsushita *et al,* 2012 |
| Exon 5 | g.4120C > T | c.422C > T | p.Ala141 Val | Mature | Chavassieux, *et al*., 2008 |
| Exon 5 | g.4134G > C | c.436G > C | p.Gly146Arg | Mature | Gelb, *et al*., 1996 |
| Exon 5 | g.4192A > G | c.494A > G | p.Gln165Arg | Mature | Donnarumma, *et a*l., 2007 |
| Exon 5 | g.4258A > C | c.560A > C | p.Gln187Pro | Mature | Li, *et al*., 2009 |
| Exon 5 | g.4278G > A | c.580G > A | p.Gly194Ser | Mature | Donnarumma, *et al*., 2007 |
| Exon 6 | g.8644A > G | c.635A > G | p.Tyr212Cys | Mature | Hou, *et al*., 1999 |
| Exon 6 | g.8737 G > A | c.728G > A | p.Gly243Glu | Mature | Khan *et al*., 2010 |
| Exon 6 | g.8755T > C | c.746T > C | p.Ile249Thr | Mature | Donnarumma, *et al*., 2007 |
| Exon 6 | g.8758A > G | c.749A > G | p.Asp250Gly | Mature | Donnarumma, *et al*., 2007 |
| Exon 7 | g.9109C > T | c.830C > T | pAla277Val | Mature | Gelb, *et al*., 1998 |
| Exon 7 | g.9109C > A | c.830C > A | p.Ala277Glu | Mature | Hou, *et al*., 1999 |
| Exon 7 | g.9171T > C | c.892T > C | p.Trp298Arg | Mature | Nishi, *et a*l., 1999 |
| Exon 8 | g.9186G > A | c.908G > A | p.Gly303Glu | Mature | Toral-Lopez *et al*., 2010 |
| Exon 8 | g.11474T > C | c.926T > C | p.Leu309Pro | Mature | Haagerup, *et al*., 2000 |
| Exon 8 | g.11479G > C | c.931G > C | p.Ala311Pro | Mature | Nishi, *et al*., 1999 |
| Exon 8 | g.11482C > G | c.934C > G | p.Arg312Gly | Mature | Hou, *et al*., 1999 |
| Exon 8 | g.11501G > A | c.953G > A | p.Cys318Tyr | Mature | Bertola *et al*., 2010 |
| Exon 8 | g.11503G > T | c.955G > T | p.Gly319Cys | Mature | Donnarumma, *et al*., 2007 |
| Nonsense | | | | | |
| Exon 3 | g.2146A > T | c.154A > T | p.Lys52X | Pro | Hou, *et al*., 1999 |
| Exon 5 | g.4266C > T | c.568C > T | p.Gln190X | Mature | Hou, *et al*., 1999 |
| Exon 6 | g.8730C > T | c.721C > T | p.Arg241X | Mature | Gelb, *et al*., 1996 |
| Frameshifts (duplication) | | | | | |
| Exon 2 | g.1591-1592 | dupGA c.60_61dupGA | p.Ile21ArgfsX29 | Pro | Donnarumma, *et al*., 2007 |
| Exon 4 | g.2359 | dupA c.282dupA | p.Val95SerfsX9 | Pro | Donnarumma, *et al*., 2007 |
| Frameshifts (deletion) | | | | | |
| Exon 3 | g.2230delG | c.238delG | p.Asp80ThrfsX2 | Pro | Fratzl-Zelman, *et al*., 2004 |
| Exon 5 | g.4124delT | c.426delT | pPhe142LeufsX19 | Mature | Fujita, *et al*., 2000 |
| Splicing | | | | | |
| Intron2 | g.2112G > A | c.121-1G > A | p.del41Val-81Me | Pro | Haagerup, *et al*., 2000 |
| Exon 7 | g.9169G > A | c.890G > A; 785_890de | p.Gly262AlafsX70 | Mature | Donnarumma, *et al*.2007 |
| Stop codon | | | | | |
| Exon 8 | g.11538A > G | c.990A > G | p.X330TrpextX19 | Mature | Gelb. *et.al*., 1996 |

human tissues [13]. It is highly expressed in osteoclasts, osteoarthritic hipbones, and osteoclastoma. Targeted mutation of the Cathepsin K gene in mice resulted in many of the phenotypic features of Pyknodysostosis, including increased bone density and bone deformities [4, 5, 13]. Subsequently, point mutations in the *CTSK* gene were detected in patients with Pyknodysostosis [5]. In the review by Xu *et al*, published in 2011, 33 mutations in the *CTSK* gene had been reported in patients with Pyknodysostosis [14], afterwards only two more mutations have been reported [15, 16] (**Table 1**). CTSK is a lysosomal cysteine protease that is synthesized as a prepropeptid of 37 kDa. The mature enzyme is a monomeric protein of approximately 29 kDa. CTSK efficiently cleaves peptide bonds in different proteins

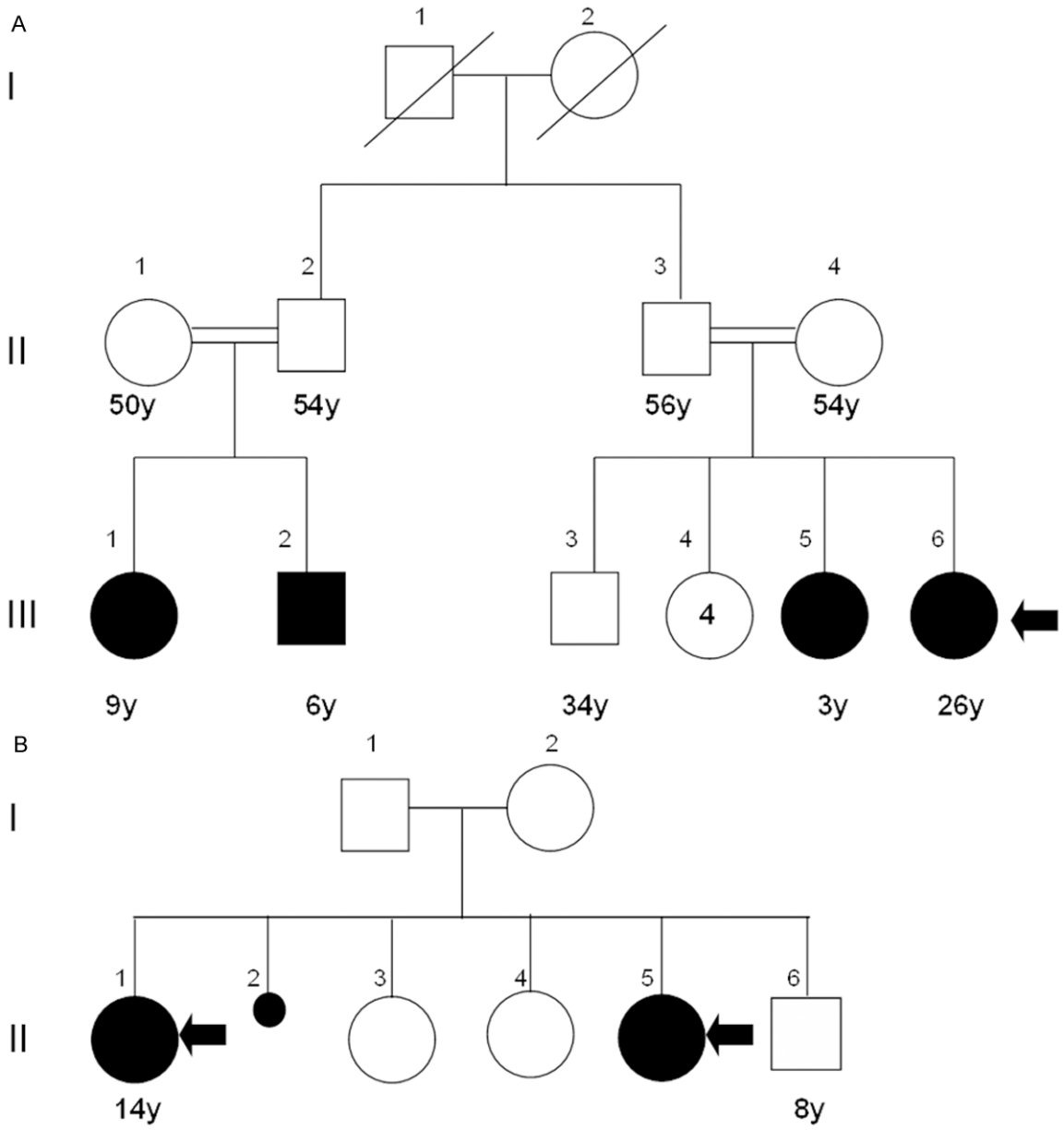


**Figure 1.** Pedigrees of the two families with pyknodysostosis. A: Consanguinity is present in family A. B: In family B there is no documented evidence of consanguinity. Squares represent males; circles represent females; filled symbols are individuals with Pyknodysostosis; the arrow indicates the propositus (patient) in each family. Roman numerals on the left indicate each generation. Numbers above the symbols identify each individual. Numbers below indicate the age in years.

including elastin and type I collagen, it is secreted to the sub-osteoclastic space where participates in bone matrix degradation [17]. In the current study the authors described the clinical, radiological and molecular features of six previously unreported Mexican patients (including two familial and two sporadic cases) with Pyknodysostosis.

*Patient data*

All patients were referred to the authors' institutions due to dysmorphic features. All were Mexican mestizos and their ages ranged from 8 to 53 years. The patients were informed about the details and aims of the study and they agreed to participate by signing an informed

**Table 2.** Summary of the clinical and radiological features found in this series of patients

| Clinical Features | Patient 1 | Patient 2 | Patient 3A | Patient 3B | Patient 4A | Patient 4B |
|---|---|---|---|---|---|---|
| Gender | Male | Female | Female | Female | Female | Female |
| Age (years) | 53 | 40 | 26 | 9 | 14 | 8 |
| Presentation | Sporadic | Sporadic | Familial | Familial | Familial | Familial |
| Parental consanguinity confirmed | No | No | Yes | Yes | No | No |
| Height | 1.53 m | 1. 50 m | 1.30 m | 95 cm | 1.27 m | 97 cm |
| Frequent fractures | Yes | Yes | Yes | No | No | No |
| Increased bone density | + | + | + | + | + | + |
| Frontal bossing | + | + | + | + | + | + |
| Delayed suture closed | + | + | + | + | + | + |
| Hypoplasia of the mandible | + | + | + | + | + | + |
| Proptosis | + | + | + | + | + | + |
| Blue sclera | + | + | + | + | + | + |
| Prominent nose | + | + | + | + | + | + |
| Delayed teeth eruption | + | + | + | + | + | + |
| Malapposed teeth | + | + | + | + | + | + |
| Enamel hypoplasia | + | + | + | + | + | + |
| Clavicular dysplasia | + | + | - | - | - | - |
| Short fingers (hands and feet) | + | + | + | + | + | + |
| Acroosteolysis (distal phalanges) | + | + | + | + | + | + |
| Dystrophic nails | + | + | + | + | + | + |
| Conductive hypoacusia | - | - | - | - | + | - |

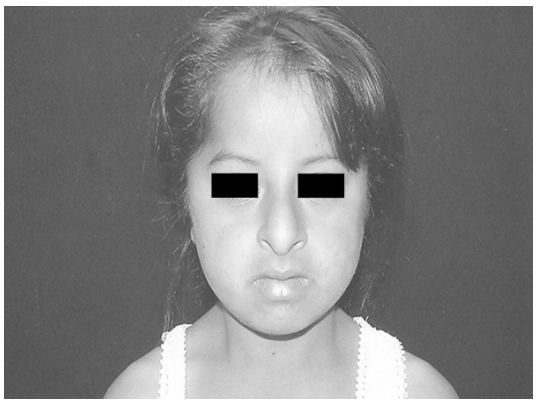

**Figure 2.** Photograph of patient 4A. Facial features of patient show ocular proptosis, beaked nose, thick bottom lip and hypoplasia of the mandible.

consent letter. **Figure 1** shows the pedigrees of the familial cases. Radiological evaluation of cranium, lumbosacral spine, long bones, hands and feet was performed on all patients. Bone mineral density (BMD) was analyzed in the hip (femoral neck, trochanter, intertrochanteric area, and Ward's triangle) and in the lumbar spine in two siblings and their mother (Family B) by dual energy X-ray absorptiometry (QDR-2000, Hologic, Massachusetts, U.S.A).

## Methods

### *Molecular analysis*

The study's procedures were approved by the Institutional Review Board and informed consent form was obtained from each patient. DNA was extracted from peripheral blood leukocytes in patients 4A and 4B (subjects II-1 and II-5 respectively in **Figure 1B**) using standard procedures. PCR amplification of the 7 coding exons of the *CTSK* gene was achieved using 4 pairs of primers (sequences available on request). Each 50 ml PCR reaction contained 1X PCR buffer, 100-200 ng of genomic DNA, 0.2 mM of each dNTP, 2U Taq polymerase, 1 mM of forward and reverse primers, and $MgCl_2$ between 1 and 3 mM. Amplification was carried out using a touchdown PCR protocol. Touchdown PCR included initial denaturing step at 95°C followed by 30 cycles of denaturing at 95°C for 30 s, annealing ranging from 50°C to 65°C (temperature was increased 0.5°C with each cycle) for 30 s and extension at 72°C for 60 s, final extension step at 72°C for 10 minutes. PCR products were size separated in 1.5% agarose gels and the bands corresponding to the amplicons were excised. The DNA was subsequently

purified using the QIAEX II kit (Qiagen). Direct sequencing was performed using the Big Dye Terminator Cycle Sequencing kit (Applied Biosystems) by adding ~10 ng of template DNA to each reaction. PCR program included 25 cycles of denaturation at 97°C for 30 s, annealing at 50°C for 15 s, and extension at 60°C for 4 min. All samples were analyzed in an ABI Prism 310 Genetic Analyzer (Applied Biosystems) and both DNA strands were investigated. Sequence variations were confirmed in each case using newly amplified fragments.

## Results

The results of the clinical and radiological analyses for all patients are summarized in **Table 2**. **Figure 2** shows the clinical features found in Patient 4A.

*Radiographic studies*

In all patients the observed features were concordant with radiological data previously described for patients with Pyknodysostosis [18] (**Figures 3**-**6**). Skull analysis in all patients showed dolichocephaly, frontal and occipital bossing, opened cranial sutures and wide fontanels. The bones of the calvaria were augmented in density and the mastoid air cells often were not pneumatized. Lateral radiographs of the mandible showed an obtuse mandibular angle with relative prognathism and the facial bones were underdeveloped.

All patients had shortening of hands and feet and the radiographic images revealed acroosteolysis of the distal phalanges, which is a pathognomonic feature of Pyknodysostosis. Long bones showed osteosclerosis without complete obliteration of the medullar canals. X-ray images revealed transverse bands in parallel to the epiphyseal lines, which are also a typical radiographic finding. Four of the six patients had evidence of multiple fractures in one or more long bones. In addition, generalized osteosclerosis and increased radiopacity were observed, especially in the long bones, spine, and cranial base.

The authors analyzed the BMD in Patients 4A, 4B and their mother. The mother was 33 years old and she had a height of 152 cm and weight of 45 kg. The values were corrected by the vertebral surface area scanned and expressed as the bone mineral density area (gm/cm$^2$). In this woman, the BMD in the spine was 0.870 gm/cm$^2$, this represents a 17% reduction with respect to healthy controls. Normative standards for BMD were previously established in Hispanic population (Software spine and hip V4.76A). The bone mineral density in the femoral neck was 0.797 gm/cm$^2$, also a 17% reduction compared to healthy controls. The decreased BMD in the mother was not associated with an increased fracture risk. The analysis of BMD in Patient 4A showed a 40% increase in the lumbar spine and a 15% increase in the hips. In the case of Patient 4B, the bone mineral density was elevated 20% in the lumbar spine and 40% in the hips.

An interesting finding in Patient 4A was the presence of bilateral conductive hypoacusia. In this patient, hearing loss could be attributed to chronic mastoiditis as seen on a computed tomography (CT) scan. To the authors' knowledge, this feature has been reported only once before in a patient from India [19].

*Sequence analysis*

The sequence of the 7 coding exons of the *CTSK* gene was obtained from patients 4A and 4B. **Figure 7** depicts a fragment of the sequence corresponding to exon 5 from patient 4A (right) and from a healthy control sample (left). The patient's DNA showed a homozygous G-to-C transversion at nucleotide position g. 4134 (arrow), predicting a Gly (GGT) to Arg (CGT) substitution at residue 146 (p.Gly146Arg) of the Cathepsin K protein. In all cases, the molecular analysis showed this substitution. Only this homozygous missense mutation was detected in both patients 4A and 4B.

## Discussion

Pyknodysostosis is a rare hereditary bone disease characterized by short stature, osteosclerosis, acroosteolysis of distal phalanges, increased tendency of pathologic fractures, and delayed suture closure [2, 18]. All patients in the current study had the characteristic clinical and radiological findings of Pyknodysostosis, which reinforces the previously stated notion that Pyknodysostosis presents with a uniform clinical phenotype both between and within affected families [18]. Although knowledge about the basic genetic defect in several scle-

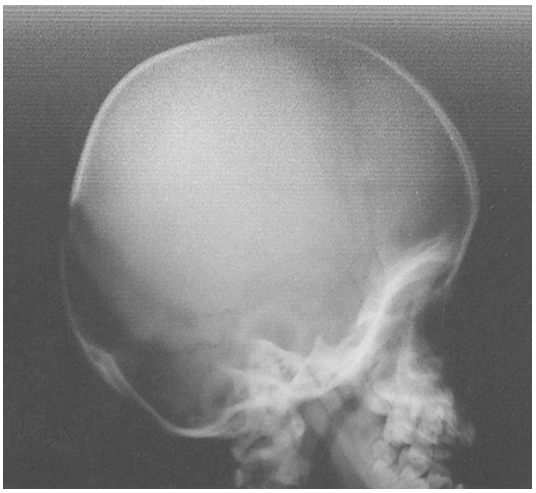

**Figure 3.** X-Ray image of the skull of Patient 3B. Image shows opened cranial sutures and obtuse angle of the mandible.

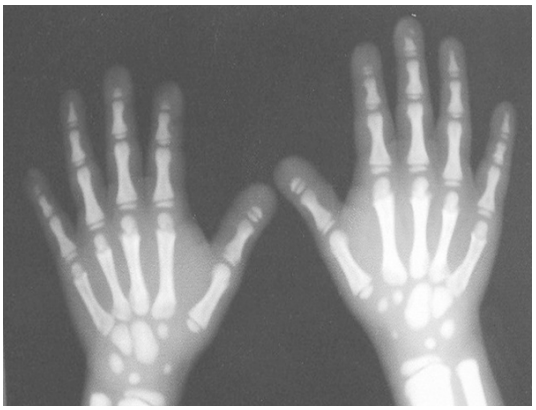

**Figure 4.** Radiograph of the hands of Patient 4B. X-ray shows brachydactyly and acroosteolysis of all distal phalanges.

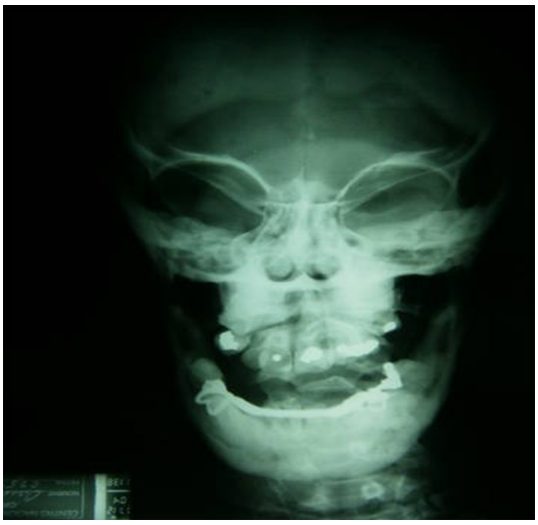

**Figure 5.** Radiograph of the skull from patient 2. Sign of the mask due to increased BMD is observed, also opened cranial sutures are noted.

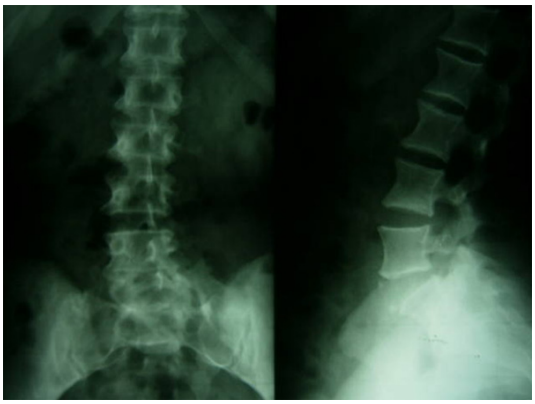

**Figure 6.** Patient's 1 spine X-Rays. Radiographs show scoliosis at the lumbar segment.

rosing bone displasias is progressing, the analysis of clinical and radiological features is still necessary to achieve a correct diagnosis. In the current series, the radiological analysis showed that, in the majority of patients, nonunion and persistence of stress fracture lines are common finding, which suggests a diminished bone healing capacity. It is interesting to note that the mother of patients 4A and 4B, an obligate heterozygous carrying a mutant allele for the *CTSK* gene, showed a reduction in BMD. This observation does not agree with the expected result (increment in BMD), since Pyknodysostosis is characterized by increased bone density. Only patients 1, 2 and 3A (aged 53, 40, and 26 years respectively) had history of fractures. In these cases the fractures occurred in long bones after the second decade of life. Fracture open reduction was necessary in most cases. Nowadays, the appropriated management of fracture healing in this disorder is still controversial. However, the majority of fractures in affected individuals are managed with open reduction.

An uncommon finding in Patients 1 and 2 was their height of 153 cm and 150 cm respectively, which does not correspond to the short stature expected for patients with this disease. However, the majority of the clinical and radiological signs of Pyknodysostosis were present in both patients. Interestingly, there is a recent

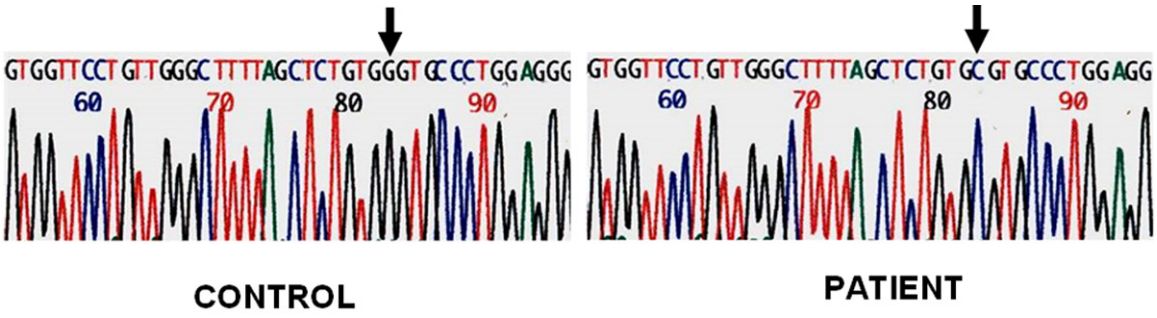


**Figure 7.** Partial Cathepsin K DNA sequence analysis. The PCR-products of exon 5 from patient 4A (right) and from a control sample (left) were sequenced. The patient's DNA showed a homozygous G-to-C transversion at nucleotide position g.4134 (arrow), predicting a Gly (GGT) to Arg (CGT) substitution in residue 146 (p.Gly146Arg) of the Cathepsin K protein. The same mutation was found in patient's sister (patient 4B data not shown).

report from a Chinese patient with confirmed molecular diagnosis who has normal height [15]. These observations should warn clinicians not to discard the diagnosis of Pyknodysostosis because of the absence of short stature when the other clinical and radiological findings are present.

Another unusual finding in this series was conductive hearing loss in patient 4A. This alteration is probably related to chronic mastoiditis as seen on a CT scan. Deafness had been reported just once in a patient from India. However, there is no sequence analysis available from that patient, neither CT scan images in order to look for signs of chronic mastoiditis or other defects associated with hypoacusia [19]. This clinical feature broadens the gamma of clinical manifestations associated to Pyknodysostosis. Therefore, physicians should be aware of this complication and indicate audiological assessment in patients with Pyknodysostosis in order to provide appropriate medical attention.

It has been shown that Pyknodysostosis results from gene defects in the *CTSK* gene that encodes a lysosomal protease. Cysteine proteases have been implicated in bone remodeling and resorption, although *CTSK* was reported to be selectively expressed in osteoclasts and is associated with the degradation of bone matrix proteins such as type I collagen, osteopontin and osteonectin [4]. This is supported by ultra structural analysis in bones of patients with Pyknodysostosis, which suggest that osteoclasts are normal in number, but do not degrade the organic matrix adequately [20]. Most lysosomal enzyme proteins are constitutively expressed in all somatic cells and the clinical features resulting from their deficiencies reflect the accumulation of undegraded substrates in various tissues. Nevertheless, the clinical manifestations of pycnodysostosis are limited to bone tissue. Inaoka *et al*, suggested that Cathepsin K may be an important element of human stochastic bone resorption in other disorders including osteoporosis and osteoarthritis. Apparently cysteine proteases are involved in various physiologic and pathologic processes, such as osteoarthritis, osteoporosis, glomerulonephritis, Alzheimer's disease, and cancer invasion and metastasis [4].

The p.Gly146Arg mutation identified in our patients was firstly described in two Moroccan Arab siblings homozygous for this mutation and in an American Hispanic patient who was a compound heterozygous with a p.Arg241X mutation in the second CTSK allele [17]. Afterwards, the p.Gly146Arg mutation was reported in homozygous state in patients from Tunisia, Algeria and another one from Morocco [21-23]. Additionally, it has been also reported in a compound heterozygous patient from Brazil, interestingly the second allele of this patient was the same (p.Arg241X) as the one observed in the American Hispanic patient reported by Gelb in 1996 [24].

The G-to-C transversion at nucleotide position g.4134 lies on exon 5. The Gly146 is present in the mature form of the cathepsine K. This amino acid is located deep within the active site cleft of the enzyme, therefore the substitution of the non-polar and hydrophobic Glycine residue by the positively charge non-hydrophobic Arginine residue is expected to have a sig-

nificant impact in Cathepsin K function. Using the system of *P. pastoris* it was observed that the p.Gly146Arg mutation resulted in absence of the mature form of the protein as well as enzymatic function [25]. The p.Gly146Arg mutation is considered one of the hot spots of the *CTSK* gene because of the CpG content of this region [21]. It is interesting to notice that the patients reported with the p.Gly146Arg mutation are from the Mediterranean region, this observation has raised the possibility of a common origin for this allele. However, it is necessary to study other polymorphic markers in order to support this hypothesis [14]. This is the first time that this mutation is identified in a homozygous state in a non-Arab patient supporting the notion that this may be a frequent mutation [17]. Previous reports in Mexican population had found a homozygous C-to-T transition at nucleotide position g.8730 originating the p.Arg241X mutation in one large family and a G-to-A transition at nucleotide g.9186, in homozygous state, causing the p.Gly303Glu change in another family [7, 26]. The Gly303Glu mutation has been found only in this Mexican family. This means that only three mutant alleles have been described in Mexican patients with Pyknodysostosis, two of them also found in patients from the Mediterranean region and one not found in other populations yet.

Up to date, the treatment of patients with Pyknodysostosis involves the symptomatic management of fractures and other skeletal manifestations. The administration of specific enzyme inhibitors may also decrease pathologic bone resorption and growth hormone treatment has shown improvement on the patient's linear growth [6, 10]. Future investigations focused in new approaches to correct the basic defect of the disease may include bone marrow transplantation to provide fully functional osteoclasts, enzyme replacement therapy or gene therapy.

In conclusion, there are less than 200 documented patients with Pyknodysostosis and 35 disease-causing mutations in the *CTSK* gene have been described. Even when mutation hot spots have been identified, mutations are scattered along the gene making it difficult to establish an accurate genotype-phenotype correlation. The current study adds clinical and radiological data of six new patients with Pyknodysostosis to the literature and informs of the second patient with conductive hypoacusia, broadening the spectrum of clinical manifestations associated with the disease. Therefore, audiological tests must be included in the clinical assessment of all the patients with Pyknodysostosis.

## Disclosure of conflict of interest

None.

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