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Original Article

Clinical and molecular analysis in a series of Mexican patients with clinical diagnosis of Fibrodysplasia Ossificans Progressiva (FOP)

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Abstract: Fibrodysplasia Ossificans Progressiva is a very rare autosomal dominant genetic disease. It is characterized by heterotopic ossification triggered by mild physical trauma leading to progressive disability. A classical and a non-classical form of the disorder have been described. Mutations in the *ACVR1* gene have been found in both groups of patients. This study reports the clinical and molecular data of a series of Mexican mestizo patients, including familial cases, with symptoms related to this disorder. The aim of this study is to provide useful clinical and molecular information to achieve an opportune diagnosis and contribute with the awareness of this severely incapacitating disease. All patients who attended from 2009 to 2013 with clinical findings suggesting the diagnosis were invited to participate. Clinical and radiological assessment revealed findings related to FOP. Molecular analysis of the *ACVR1* gene identified the c.617C>A mutation in one allele confirming the diagnosis. Two patients presented symptoms not previously reported in classical FOP. Diagnosis was delayed or mistaken in most cases and therefore, patients were exposed to surgeries or other damaging procedures. A detailed physical examination and radiographs are indispensables for detection of FOP. Diagnosis can be confirmed through molecular analysis of the *ACVR1* gene. Clinicians must perform an exhaustive assessment in all suspicious individuals to avoid misdiagnosis. An opportune diagnosis and proper orientation are essential for preventing invasive procedures and for improving patient's quality of life.

Keywords: Fibrodysplasia, progressive ossification, heterotopic bone, *ACVR1*

Introduction

Fibrodysplasia Ossificans Progressiva (FOP) (OMIM 135100) is an autosomal dominant rare genetic disease and it is one of the most incapacitating disorders. The frequency calculated worldwide is 1 per 2 millions of live births. Although most cases are sporadic, as a result of *de novo* mutations, some families with two or more affected generations have been reported [1-3]. Affected individuals usually present hallux valgus at birth. During the first or second decade of life, painful swellings (flare-ups) appear, they can be sporadic or associated to minor physical trauma [4]. The swellings become bone through an endochondral ossification process (heterotopic ossification). Heterotopic ossification (HO) affects skeletal muscle, tendons, fascia and ligaments, compromising joint movement and producing a progressive no reversible disability. Interestingly, extra-ocular, diaphragm, tongue and cardiac muscles are spared [5-7]. Other clinical and radiological findings include cervical vertebrae fusions, tall nar-

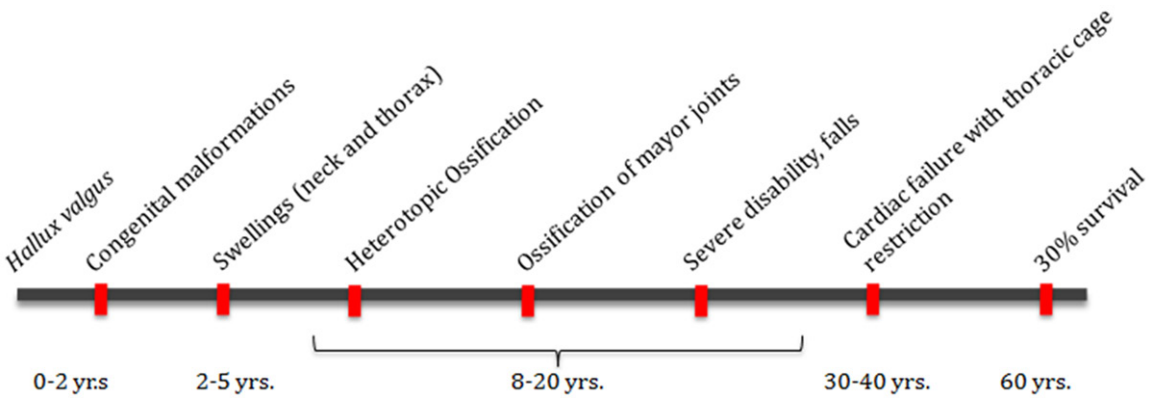

Figure 1. Natural history of FOP. At birth, the only manifestation present is hallux valgus. During the first and second decades of life swellings, at proximal body regions appear, leading to HO. As a consequence of HO joints' movement is compromised, and falls are frequent causing trauma which triggers new HO episodes. Patients usually die around the 4th and 5th decade of life. Just 30% of patients reach the age of 60 years.

row vertebral bodies, short and broad femoral necks, tibial osteochondromas, and conductive hearing loss [8, 9].

According to the clinical presentation, FOP is classified as classical and non-classical forms. The hallmarks of the classical form are the progressive HO and hallux valgus [10]. Non-classical forms are subdivided as FOP plus (when the classical features are accompanied by atypical manifestations, i.e. cataracts, intellectual disability, severe growth retardation, among others) and FOP variants (when variation in the classical features is observed, i.e. normal first toes) [11].

The low frequency of this disease complicates a timely diagnosis. Some authors have reported that up to 87% of patients have a delayed diagnosis, unfortunately by that time some of them have undergone invasive procedures triggering HO [1, 12]. Misdiagnoses include sarcomas, juvenile aggressive fibromatosis, lymphedema, Klippel-Feil syndrome, posttraumatic ossificans myositis and progressive osseous heteroplasia (POH). Perhaps the most important differential diagnosis is POH, which is an autosomal dominant disease that presents a membranous ossification without great toes malformation [1, 5, 8, 13, 14].

Bone bridges across the joints can lead patients to remain in fixed postures, diminishing their ability to walk and perform daily activities. Movement limitation has a great impact on patients' quality of life. Equally important is the impact on their family environment because relatives or couples have to assist them or contract someone to make these activities. Thoracic cage restriction, pulmonary infections, malnutrition and falls are the principal causes of death. Most patients die around 45 years old, only 30% of affected individuals survive until 60 years old [5, 13, 15]. **Figure 1** shows a schematic representation of the natural history of the classical form or the disease.

The molecular basis of FOP was unraveled in 2006 when Shore *et al.*, found a heterozygous c.617G>A mutation in the gene encoding for the Activin A Receptor Type I, (*ACVR1*), in a series of patients with the classical form of the disease. This missense mutation results in the substitution of an Arginine for a Histidine at position 206 (p.R206H) in the *ACVR1* protein [16]. Almost all the patients with classical FOP have the c.617G>A mutation. Other mutations reported in the non-classical forms are c.619C>G (p.Q207E), c.982G>A (p.G328R), c.982G>C (p.G328R), c.982G>T (p.G328W), c.983G >A (p.G328E), c.1067G>A (p.G356D) c.1124G>C (p.R375P), c.774G>C (p.R258S). The p.G328W and the p.G328E mutations have been associated to a severe phenotype, while the p.G328R mutation to a mild phenotype with slight first toe malformation [11, 17].

The *ACVR1* gene is located at locus 2q23-24 and encodes a type I receptor of the bone morphogenetic proteins (BMP). Type I receptors are activated through phosphorylation of their Ser/Thr residues in the intracellular GS rich domain mediated by the kinase activity of the type II receptors [18]. The active receptor phosphorylates downstream effectors of the BMP signaling pathway, which induce expression of genes

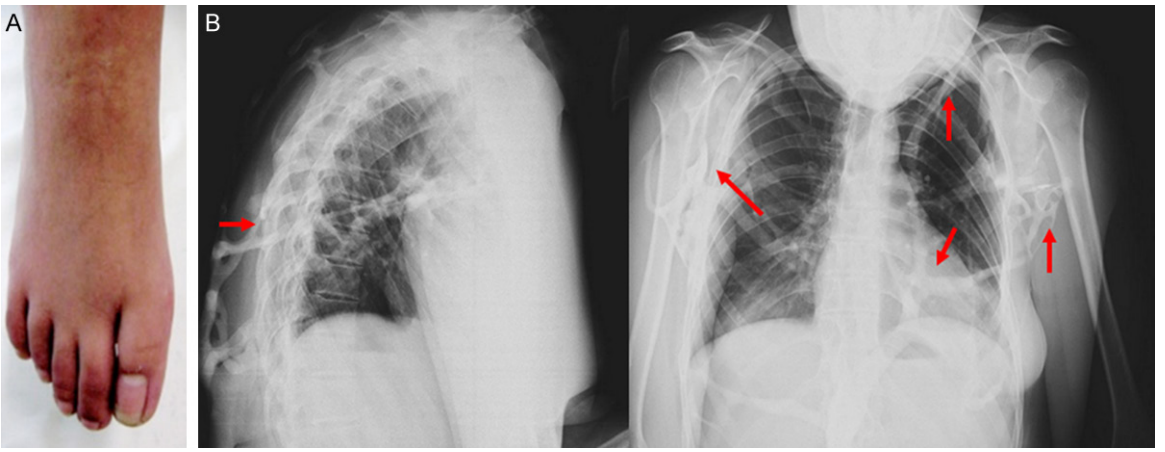

Figure 2. Photographs and image studies from patient 1. A. Congenital malformation of great toe was surgically corrected. B. Lateral and anteroposterior chest radiograph showing HO at the thorax and proximal humerus.

that promote a pro-osteogenic environment [19]. It has been demonstrated that the p.R206H mutation confers a gain of function to the *ACVR1* receptor, even in the absence of ligand, leading to an increase in the signaling of the BMP factors. There is evidence showing that also other mutations found in FOP patients can induce a similar effect [20].

The diagnosis of FOP is based on the recognition of the classical clinical manifestations. Radiographs should be performed in all patients with congenital malformation of the great toes with or without heterotopic ossification. Clinical diagnosis can be corroborated by sequencing of the *ACVR1* gene. Molecular analysis is becoming widely used and is very useful to establish a genotype-phenotype relationship.

Currently there is no definitive treatment for FOP. Among the medications recommended by the International Clinical Consortium on FOP, as supportive measures are: corticosteroids, nonsteroid anti-inflammatory drugs (NSAIDs) and COX-2 selective inhibitors, mast cell stabilizers and biphosphonates such as Etidronate [21]. In addition, therapists have a primordial role, they can help patients to preserve their independence. Furthermore, it is necessary to adapt patients' home to avoid accidents. Patients' attention represents a considerable economic expense, unfortunately not all families can afford it [22].

This article presents the clinical and molecular data of a group of patients from Mexican mestizo origin with diagnosis of FOP. We attempt to contribute with the knowledge of this devastating disorder and divulge the importance of an opportune diagnosis and appropriate management.

Methods

Patients

Patients with clinical and radiological data suggesting FOP were recruited from six hospitals (see below) in Mexico from 2009 to 2013. All participants were informed about the details and aims of the study and they agreed to participate by signing an informed consent form. Clinical files, including radiographs and CT scan images (when available) were collected and reviewed. A total of 15 patients met the classical manifestations of FOP: nine patients from the National Institute of Rehabilitation (NIR) in México City, two patients from the Civil Hospital in Guadalajara, Jalisco, two patients from the University Hospital in Monterrey, Nuevo Leon, one patient from the Children's Hospital in Tlaxcala, Tlaxcala and one patient from the Regional Hospital in Leon, Guanajuato. The study was approved by the Ethics Committee of the above mentioned institutions.

Molecular analysis

Genomic DNA was isolated from peripheral blood leukocytes with the Puregene Blood Kit (Qiagen). PCR amplification of exon 6 of the *ACVR1* gene was performed using the primers previously described [16]. Sequence primers are FOP-F 5'-CCAGTCCTTCTTCCTTCTTCC-3', FOP-R 5'-AGCAGATTTTCCAAGTTCCATC-3'. Each

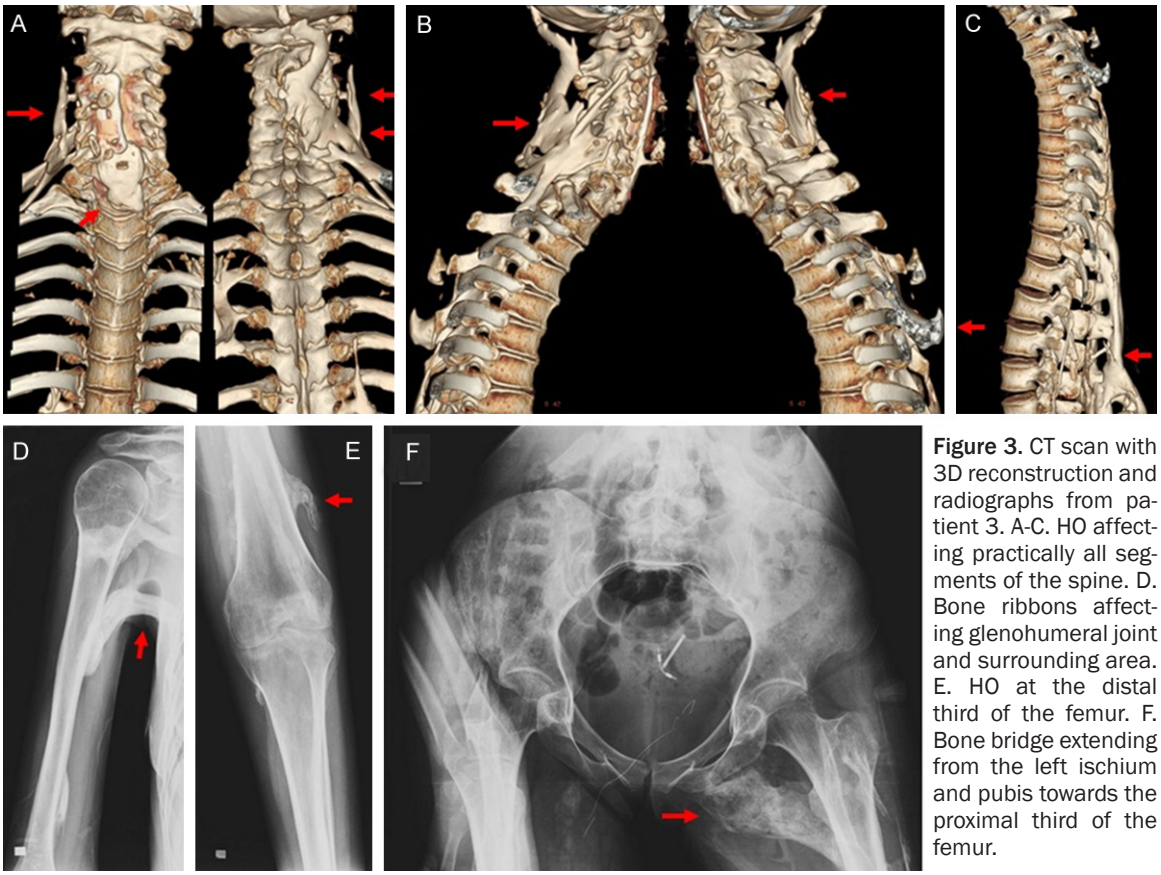

Figure 3. CT scan with 3D reconstruction and radiographs from patient 3. A-C. HO affecting practically all segments of the spine. D. Bone ribbons affecting glenohumeral joint and surrounding area. E. HO at the distal third of the femur. F. Bone bridge extending from the left ischium and pubis towards the proximal third of the femur.

20 µL PCR reaction contained 100 ng of genomic DNA, 100 nM of forward and reverse primers, 0.2 mM of each dNTP, 2 mM of $MgCl_2$, 1× PCR buffer and 2 U Taq polymerase. The cycling conditions were an initial denaturing step at 95°C for 5 min, followed by 40 cycles of denaturing at 95°C for 25 s, annealing at 67°C for 25 s, and extension at 72°C for 60 s, and a final extension step at 72°C for 10 m. The PCR products were size separated in a 1.5% agarose gel by electrophoresis. The band corresponding to the amplicon was excised and purified using the Qiaex II kit (Qiagen). Direct sequencing was performed using the BigDye 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 m. All samples were analyzed in an ABI Prism 310 Genetic Analyzer (Applied Biosystems). Sequence variants were confirmed using newly amplified fragments. Sequence data were compared to the consensus sequence of the *ACVR1* gene (GenBank accession NG_008004.1).

Results

Patients' description

Patient 1: A 14 year old female patient was referred to the clinic with the diagnosis of osteochondromatosis multiple. She was born with hallux valgus and she underwent surgery for correction within the first month of life. By the age of eight, another surgery was performed in the left knee because of the presence of ossification. She also had a tonsillectomy which caused ossification at the temporomandibular joint. At the time of examination she presented multiple swelling areas and ossifications affecting the thorax and right ankle, with consequent movement limitation. Radiographs showed fusions of cervical vertebrae, HO with branches involving thorax and subscapular area. She also presented broad and short femoral necks and osteochondromas in both tibias (**Figure 2**).

Patient 2: A 15 year old male patient was referred to the clinic without diagnosis. He was born with congenital malformation of great toes, thumbs and cryptorchidism. He presented

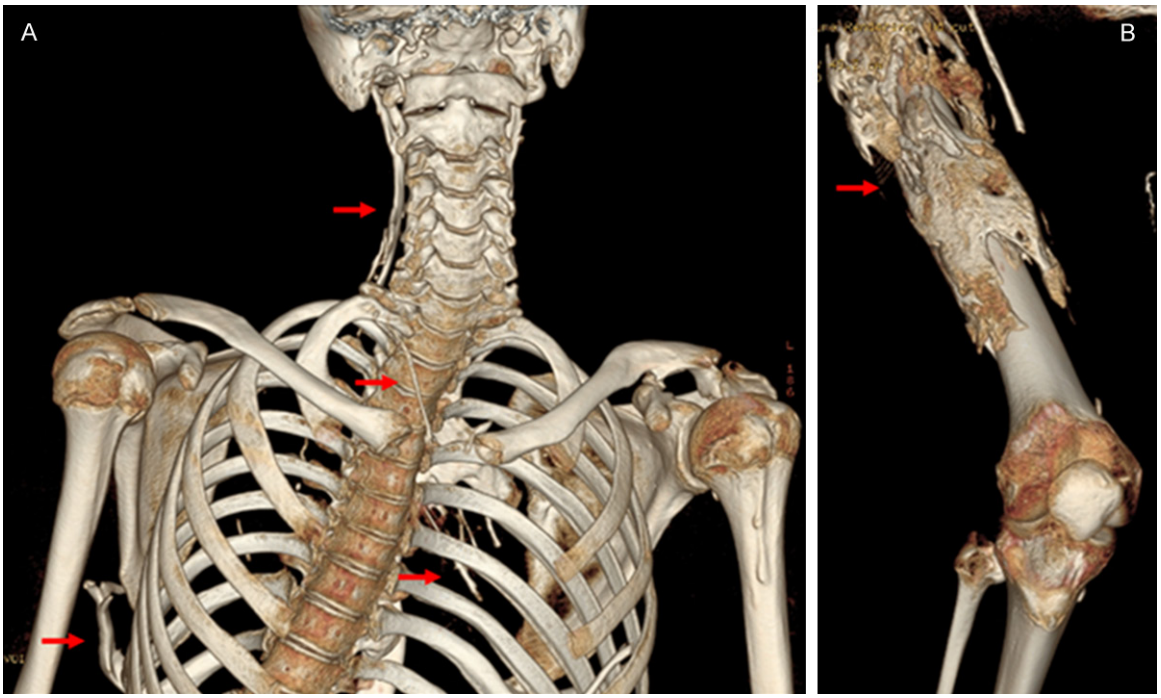

Figure 4. CT scan with 3D reconstruction from patient 5. A. CT scan with 3D reconstruction showing bone ribbons at the cervical region and thorax. B. CT scan with 3D reconstruction showing HO at the proximal region of the femur.

spontaneous HO by the age of 12 starting at the scapula-costal region and spreading along the thoracic cage. Patient does not remember any trauma before the onset of HO, but confirmed that painful and erythematous swellings were present before HO. Nowadays, movement of the thoracic cage is restricted, but ambulation and feeding are conserved. (Supplementary Figure 1).

Patients 3 and 4: This is a familial case. The mother is 26 years old female, who referred that flare-ups appeared when she was two or three years old. She presented hallux valgus at birth. When HO was noticed she was misdiagnosed with osteochondromatosis multiple and several surgical procedures were carried out. The surgeries accelerated the progression of the disease. Additionally, she suffered from Patent ductus arteriosus and epilepsy. The seizures caused frequent falls, triggering HO, she was quickly confined to wheelchair. Reduced mobility and HO leaded to the formation of skin ulcers at the lumbar region. She had a child with hallux valgus at birth. Molecular test confirmed the diagnosis in both. She stopped attending to the clinic because she was severely affected (**Figure 3**). Her family informed us of her death, but no cause was explained. Follow up of her child was not possible after she died.

Patient 5: A 20 years old male patient, who presented hallux valgus at birth. HO started at the cervical region when he was 10 years old. Afterwards HO appeared at the temporomandibular joint, upper and lower limbs. He developed scoliosis and restrictive pneumopathy (**Figure 4**).

Patients 6 and 7: This is a familial case. The mother is 22 years old, she was born with deformity of the great toes. She noticed the first episode of swellings with HO approximately when she was 16 years old. Currently she presents HO at the neck, thorax, and upper and lower limbs (**Figure 5**). She has a 15 months old son; he presented great toes malformation at birth. His development has been normal so far, he has not presented swellings or HO. Her mother requested the sequence analysis of the *ACVR1* gene for her son. It was confirmed that he carries the mutation.

Patient 8: A 19 years old female patient was referred to the clinic because she presented HO at the neck and thorax. Clinical examination

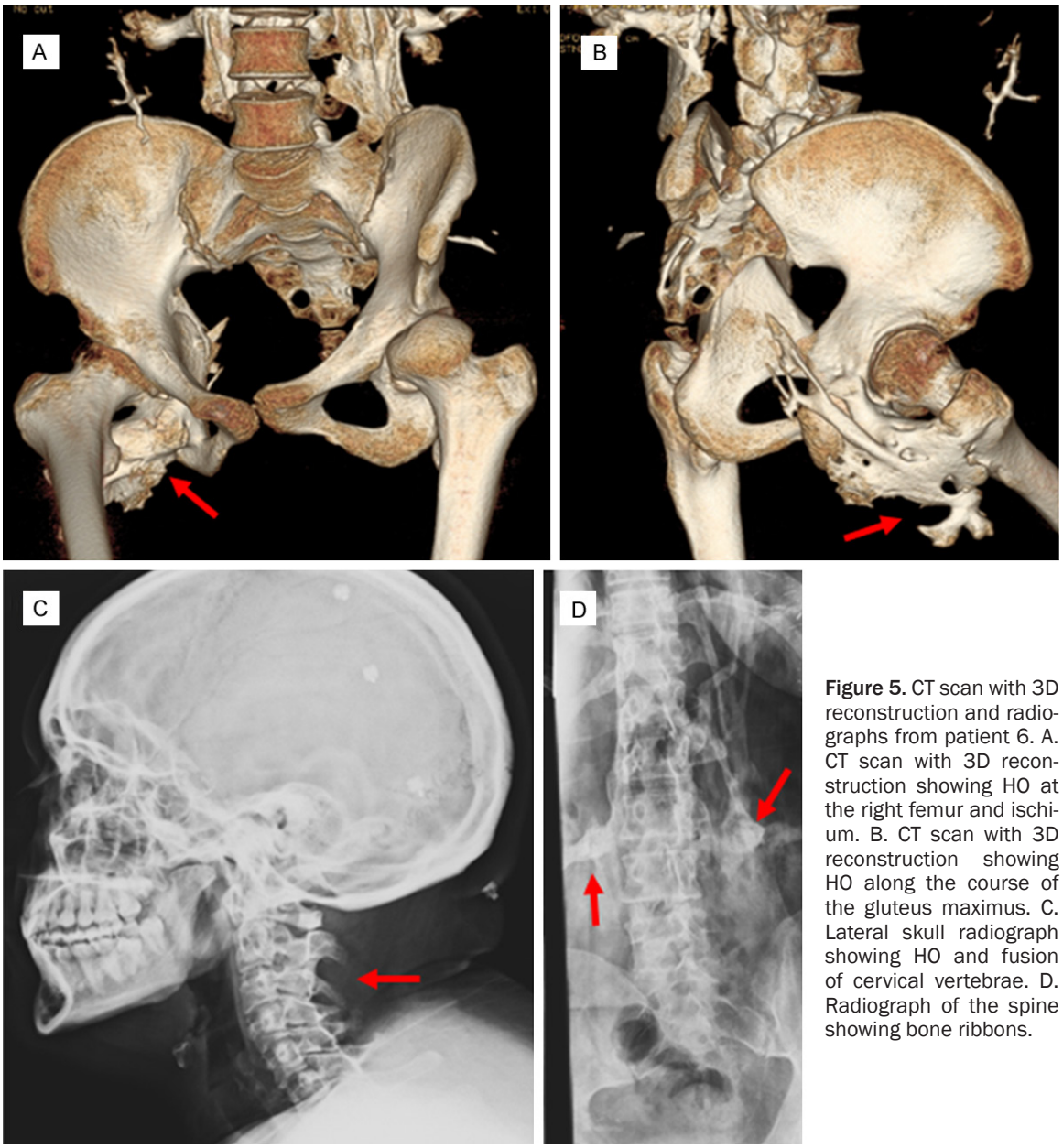

Figure 5. CT scan with 3D reconstruction and radiographs from patient 6. A. CT scan with 3D reconstruction showing HO at the right femur and ischium. B. CT scan with 3D reconstruction showing HO along the course of the gluteus maximus. C. Lateral skull radiograph showing HO and fusion of cervical vertebrae. D. Radiograph of the spine showing bone ribbons.

revealed hallux valgus, radiographs showed HO at the cervical region, left humerus, left axillary region and right hip (Supplementary Figure 2).

Patient 9: A 9 years old female patient referred to the clinic because of severe physical disability. She was born with hallux valgus, which was corrected with surgery within the first month of life. Around the age of three, flare-ups and HO appeared at the neck and dorsal region, progression occurred towards limbs causing important fixation of several joints. She also presented mixed hypoacusis, predominantly neurosensory.

Patient 10: Female patient aged three, who presented great toes deformity at birth. Her mother did not present any feature of FOP, information about her father was not available. She has two older healthy siblings without great toes malformation. When she was 2 years old her mother noticed reduced neck mobility and soon after upper and lower limbs mobility was compromised. Her mother referred that no trauma was associated with these events. Clinical examination revealed clinodactyly of the fifth fingers and short thumbs (Supplementary Figure 3).

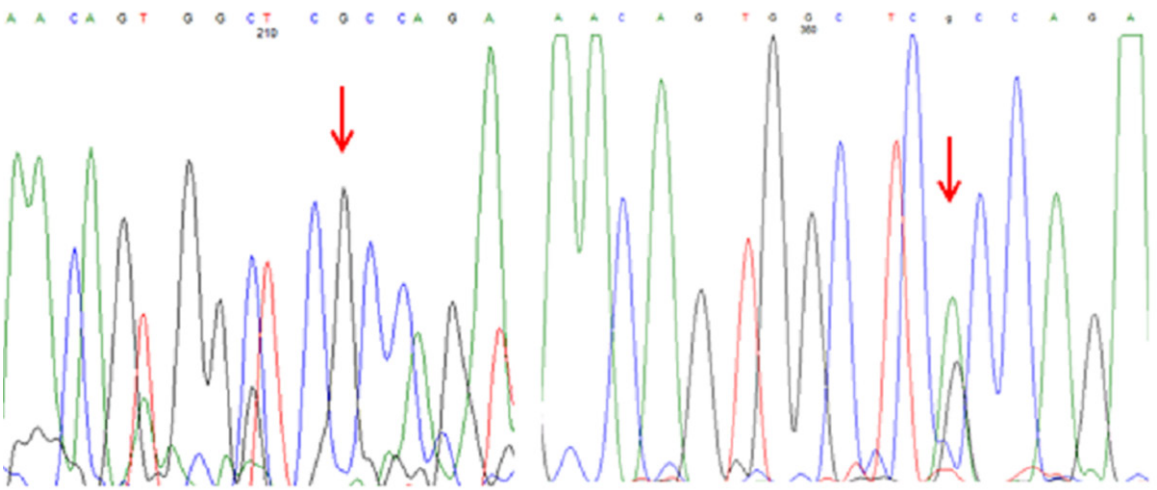

Figure 6. Partial sequence analysis of the *ACVR1* gene. The PCR-products of exon 6 from a healthy control (left) and from a FOP patient (Right) were sequenced. The control's DNA showed a homozygous G/G genotype at nucleotide position c.617 (arrow). The patient's sample showed a heterozygous G/A genotype at the same nucleotide position, predicting an Arginine to Histidine substitution in residue 206 (p.R206H) in the *ACVR1* mutant protein.

Patients 11 and 12: Is a familial case the pedigree is shown in Supplementary Figure 4. The mother is 40 years old, she was born with malformation of the great toes, she refers that her first flare-up episode appeared at the age of 20. Movement limitation started at the thoracic cage and lower extremities. Radiographs showed HO at the scapulo-humeral joint, costal border and thoracic spine (Supplementary Figure 4). Her 11 years old son presented congenital malformation of the great toes and his swellings started when he was 6 years old. To date, he has HO at the thorax and severe movement limitation of the upper limbs. She referred that her other son also presented hallux valgus at birth, however it was not possible to collect any further information about him (this is why we only account two affected individuals in this family).

Patients 13 and 14: Clinical and radiological data from these patients were not available. They were included in this study because FOP was diagnosed by a geneticist in Guadalajara, Jalisco. The responsible physician sent us DNA from both patients; however the sample from patient 14 was of poor quality for analysis.

Patient 15: A seven years old female patient who was born with congenital malformations of the great toes. She started with HO at the age of seven. Currently, the ossifications are present at the temporomandibular joint, neck and thoracic cage (Supplementary Figure 5).

Molecular analysis

Sequence analysis demonstrated that all analyzed patients are heterozygous for the c.617G>A mutation in the *ACVR1* gene confirming the diagnosis. DNA from patient 14 was of poor quality and molecular analysis could not be performed. **Figure 6** shows an example of the mutation found.

Discussion

Fibrodysplasia Ossificans Progressiva is one of the most disabling genetic diseases with no definitive treatment available. It has an autosomal dominant inheritance pattern, it means than males and females are affected equally and that affected patients have a 50% probability to inherit the disease to their offspring. Up to date there are approximately 800 diagnosed patients worldwide. A detailed phenotypic description is available for less than 200 patients. Hüning I. *et al.*, collected the data from 134 patients with confirmed molecular diagnosis, their review has been useful to establish a genotype-phenotype relationship [23].

The classical FOP phenotype has two main characteristics: congenital malformation of great toes, and HO, which is the cause of progressive disability [8]. Furthermore, some patients present additional uncommon features or variants of the disease's hallmarks, such patients have been classified as FOP plus and FOP variants respectively. Genetic analysis has allowed the

identification of mutations in the *ACVR1* gene in patients with the classical and atypical phenotypes. The c.617G>A point mutation has been found in >90% of patients with classical FOP. Studies in individuals presenting the FOP plus and FOP variant phenotypes have evidenced the presence of additional mutations [11, 16].

This study presents the first series of FOP patients from Mexican mestizo origin. According to the estimated frequency, there should be approximately 60 affected individuals in Mexico. This is the first effort to start a FOP registry in our country. All studied individuals showed the two main characteristics of classical FOP. Congenital great toes malformation was a constant feature in all patients. The appearance of HO ranged from 2 to 20 years old and it was not always triggered by physical trauma. In the review done by Hüning I. *et al*., the observed range was from 1 to 15 years old [23]. In our series, HO often started at the thorax and neck and spread towards the limbs.

Additional clinical features were observed in patient number two, who presented bilateral cryptorchidism, a feature not previously reported in the literature. Patient three suffered from epilepsy and patent ductus arteriosus. Seizures had been observed just once before (patient five from [11]). That patient presented severe neurological complications, probably secondary to cytomegalovirus infection. On the other hand, patent ductus arteriosus has not been described previously. Patient nine suffer from mixed hypo acusis, predominantly neurosensory, which has been described previously. Patient ten presented clinodactyly and short thumbs, both features previously observed in FOP patients.

One major issue observed in this series, was that an important number of patients underwent invasive procedures before the diagnosis was established. This is one important factor that aggravates patient's condition because it can trigger HO. Among the most common procedures are the correction of great toes malformation, bone tumor removal and dental interventions. The last ones can lead to HO at the temporomandibular joint, which is a life threatening situation because it compromise feeding. All invasive procedures must be avoided until diagnosis is confirmed.

All patients, from whom DNA was available for analysis, carried the point mutation c.617G>A in one allele of the *ACVR1* gene. This observation is in agreement with the genotype-phenotype correlation described previously, and reinforces the usefulness of the molecular test in the assessment of patients [11]. The underlying molecular mechanisms leading to HO are not fully understood. Functional studies have demonstrated that the c.671G>A mutation, and some others associated to atypical phenotypes, confers a gain of function to the protein. Recently, Fujimoto M. *et al*., have established a model of murine embryonic stem cells (SC) carrying this mutation. They showed that these cells can induce activation of the *ACVR1* downstream signaling in the absence of ligand, driving to bone formation. Interestingly, bone formation was considerably reduced in the presence of an inhibitor of BMP type I receptor kinases [24]. A chimeric mouse harboring the p.R206G mutation resembles the classical FOP phenotype and has been useful to study the process underneath new bone formation [25]. The investigation about the origin of the cells participating in the new bone formation suggests that they can have an endothelial origin. It has been demonstrated that the p.R206H *ACVR1* mutant protein can induce Endothelial-to-mesenchymal transition (EndMT). EndMT results in the generation of mesenchymal stem-like cells that have the potential to originate different lineages. It has been proposed that the inflammatory process, secondary to trauma or injury, could be a scenario that favors the differentiation of these mesenchymal stem-like cells into chondrocytes. The ultimate factors that trigger this differentiation are unknown though [26]. Understanding the molecular mechanism underlying the HO in FOP patients could also provide valuable insights in tissue engineering and regenerative medicine.

Previous reports have described only eight familial cases [11]. We observed three affected families, which is very unusual given the severe incapacity developed at an early age. Genetic counseling facilitated that the affected parents were able to recognize the congenital great toe malformation in their offspring. The early recognition of the disease enables to take proper care of the children and to avoid risky procedures. Prevention is the first and most valuable strategy to control the progression of the disease.

Patients and relatives need to be orientated to reduce the impact of this disorder in the family environment. The International FOP Association (IFOPA) is a nonprofit support organization that offers valuable information for children, families, caregivers and health professionals (www.ifopa.org). They also publish information related to the development of new therapies and how to participate in clinical trials.

In summary, hereby we present the clinical and molecular data of the first series of FOP patients from Mexican mestizo origin, including three families. Great toes malformation was present at birth in all patients, therefore this must be a warning sign and requires further investigation by health professionals. HO initiates between 2-20 years old and it is not necessary associates to physical trauma. Only two patients from our series displayed features not described previously in FOP patients. Invasive procedures are not uncommon and they are an important aggravating factor. Early molecular diagnosis in suspicious patients allows opportune intervention to avoid complications and unnecessary procedures that can accelerate the progression of the disease. Genetic counseling is of great importance to help the patient and the family to understand the disease, know the challenges and adopt preventive measures. Reports describing the clinical and molecular findings in FOP patients are necessary to increase the knowledge and awareness about this devastating disease among the health professionals. Even though there is no definitive treatment available, the quality of life of patients can be improved through supportive measures, approved medications and physical therapy.

Disclosure of conflict of interest

None.

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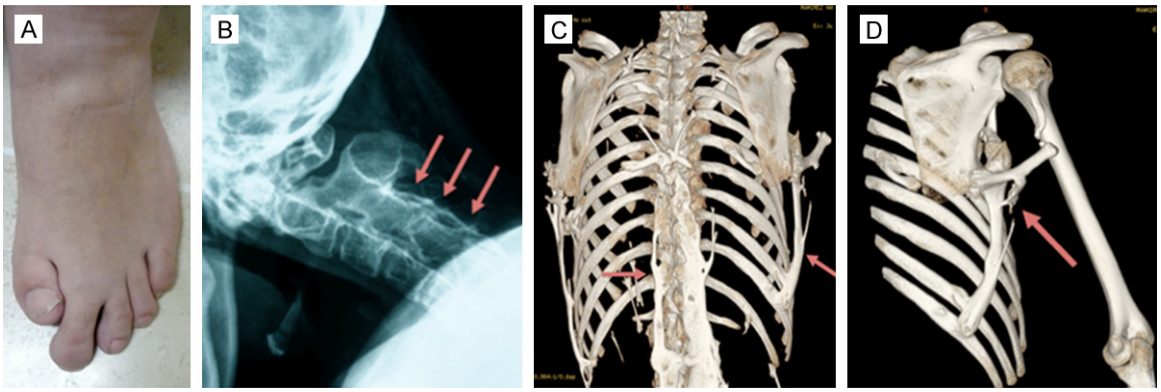
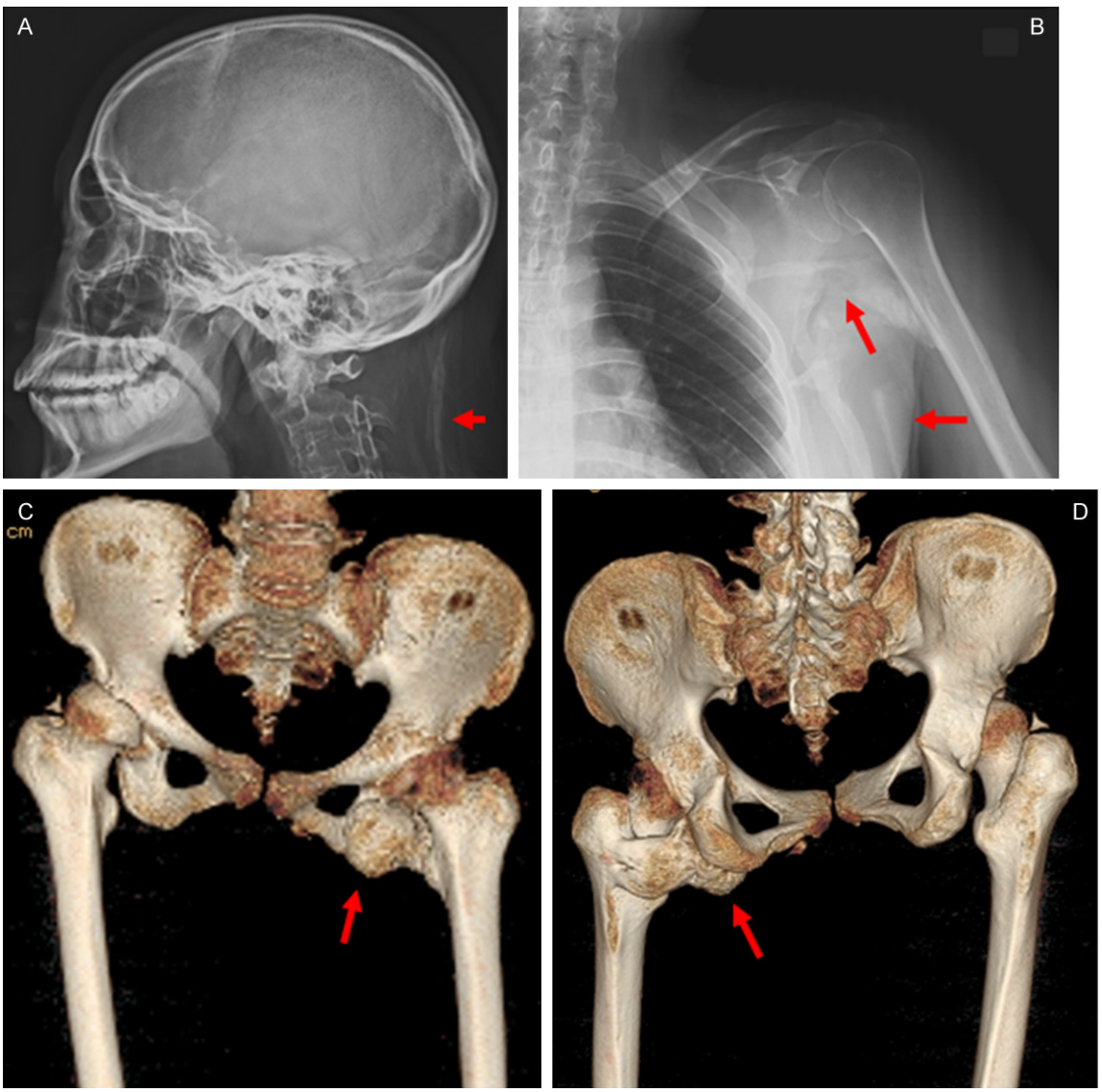
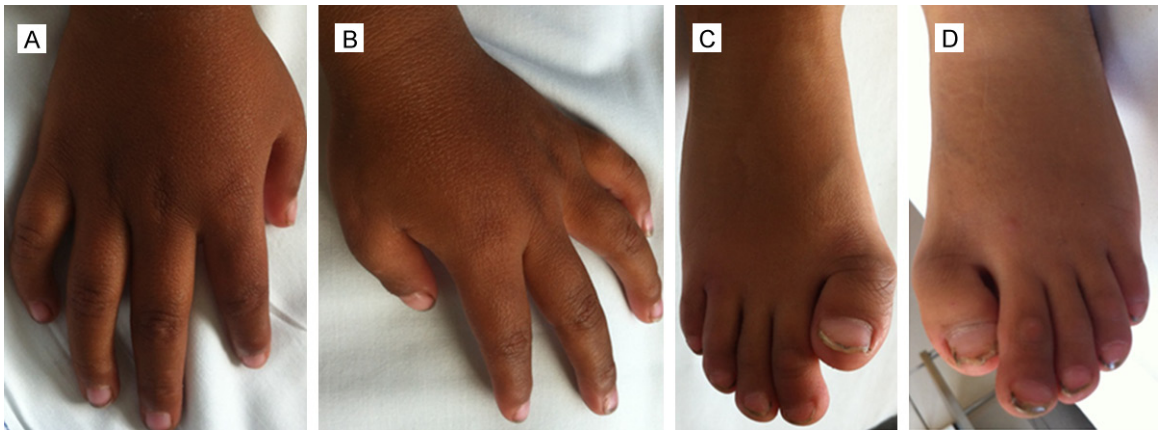


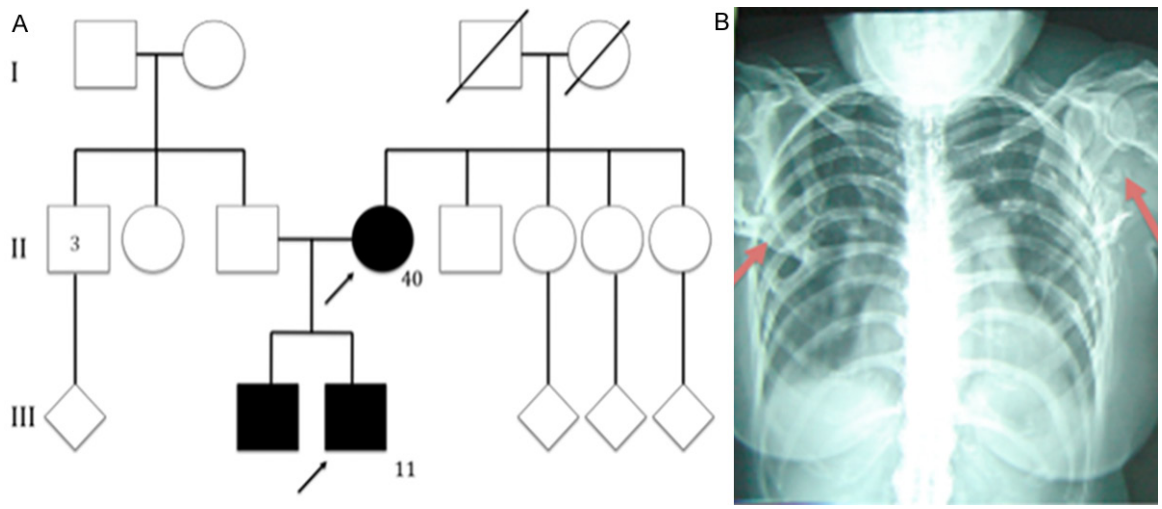
Supplementary Figure 1. Clinical, radiographic and CT scan with 3D reconstruction images from patient 2. A. Congenital malformation of the left great toe. B. Cervical spine fusion. C. Bone ribbons at the thoracic cage. D. Bone bridge from the thoracic cage to left humerus.



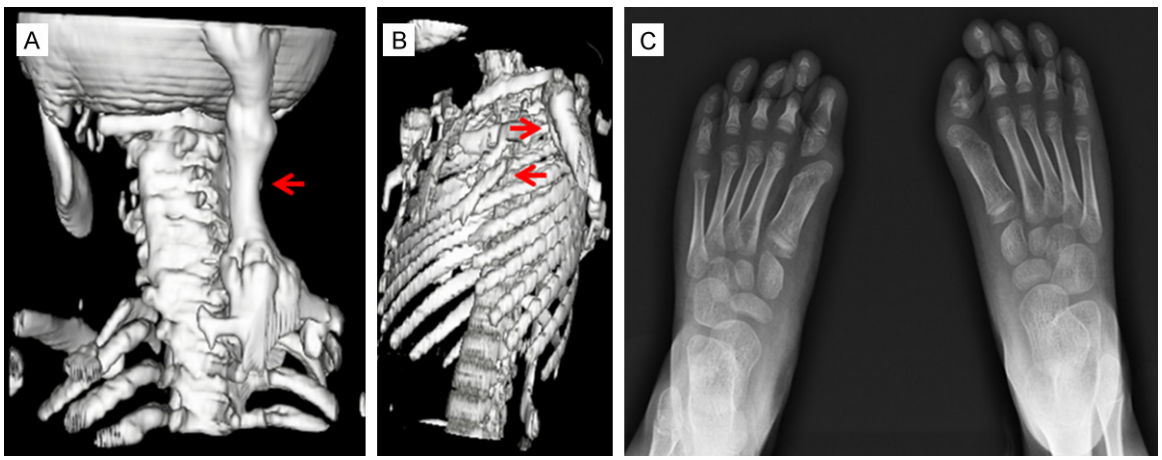
Supplementary Figure 2. Radiographs and CT scan with 3D reconstruction from patient 8. A. Lateral skull radiograph showing HO at the cervical region. B. Radiograph of left humerus showing HO ribbons. C and D. CT scan with 3D reconstruction of the pelvis showing HO spanning from the proximal femur to the ischium.



Supplementary Figure 3. Clinical pictures of patient 10 showing bilateral clinodactyly and short thumbs (A, B) and great toes malformation (C, D).



Supplementary Figure 4. A. Pedigree of cases 11 and 12, a 40 years old female and her two sons are affected. There were no data suggestive of FOP in her parents. B. Radiograph of the mother showing HO at the thoracic cage.



Supplementary Figure 5. CT scan with 3D reconstruction and radiograph from patient 15. A. HO affecting the cervical region. B. Bone rbbons at the thoracic cage. C. Congenital malformation of the great toes.