

Classic and Atypical Fibrodysplasia Ossificans Progressiva (FOP) Phenotypes Are Caused by Mutations in the Bone Morphogenetic Protein (BMP) Type I Receptor ACVR1

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Communicated by Peter Byers

Received 4 April 2008; accepted revised manuscript 27 June 2008.

Published online 9 December 2008 in Wiley InterScience (www.interscience.wiley.com). DOI 10.1002/humu.20868

ABSTRACT: Fibrodysplasia ossificans progressiva (FOP) is an autosomal dominant human disorder of bone formation that causes developmental skeletal defects and extensive debilitating bone formation within soft connective tissues (heterotopic ossification) during childhood. All patients with classic clinical features of FOP (great toe malformations and progressive heterotopic ossification) have previously been found to carry the same heterozygous mutation (c.617G>A; p.R206H) in

the glycine and serine residue (GS) activation domain of activin A type I receptor/activin-like kinase 2 (ACVR1/ALK2), a bone morphogenetic protein (BMP) type I receptor. Among patients with FOP-like heterotopic ossification and/or toe malformations, we identified patients with clinical features unusual for FOP. These atypical FOP patients form two classes: FOP-plus (classic defining features of FOP plus one or more atypical features) and FOP variants (major variations in one or both of the two classic defining features of FOP). All patients examined have heterozygous ACVR1 missense mutations in conserved amino acids. While the recurrent c.617G>A; p.R206H mutation was found in all cases of classic FOP and most cases of FOP-plus, novel ACVR1 mutations occur in the FOP variants and two cases of FOP-plus. Protein structure homology modeling predicts that each of the amino acid substitutions activates the ACVR1 protein to enhance receptor signaling. We observed genotype-phenotype correlation

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Contract grant sponsors: Center for Research in FOP and Related Disorders; International FOP Association; Ian Cali Endowment; Weldon Family Endowment; Isaac and Rose Nassau Professorship of Orthopaedic Molecular Medicine; Rita Allen Foundation; National Institutes of Health; Grant number: R01-AR41916.

between some *ACVR1* mutations and the age of onset of heterotopic ossification or on embryonic skeletal development.

Hum Mutat 30, 379–390, 2009.

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KEY WORDS: *ACVR1*; *ALK2*; BMP type I receptor; fibrodysplasia ossificans progressiva; FOP; bone morphogenetic protein; clinical variants; heterotopic ossification

Introduction

The bone morphogenetic proteins (BMPs) are a family of highly conserved extracellular signaling proteins that regulate cell differentiation fates and that have critical functions in a wide variety of cells and tissues during embryonic development and postnatal life [Chen et al., 2004; Gaggero and Canalis, 2006; Massague et al., 2005; Shi and Massague, 2003; Urist, 1965; Wagner, 2007; Wozney et al., 1988]. BMPs signal by binding to and activating transmembrane complexes of type I and type II BMP receptors. Both type I and II BMP receptors are serine/threonine kinases with similar functional domains. A single transmembrane domain links the extracellular N-terminal ligand-binding domain to the cytoplasmic C-terminal kinase domain. A unique feature of type I receptors is a cytoplasmic juxtamembrane region rich in glycine and serine residues (GS domain). Following ligand binding, serines and threonines in this region are phosphorylated by the BMP type II receptor, activating the BMP type I receptor to transmit BMP signals through SMAD and mitogen-activated protein kinase (MAPK) signaling pathways to regulate transcription of responsive target genes. BMP signaling is mediated through three known type I receptors: *BMPRIA* (*ALK3*), *BMPRII* (*ALK6*), and *ACVR1* (*ALK2*).

Mutations in *ACVR1* (MIM# 102576) were recently identified as the genetic cause of the rare human disease fibrodysplasia ossificans progressiva (FOP; MIM# 135100) [Shore et al., 2006]. FOP is a severely disabling disease that causes endochondral bone formation at extraskeletal (heterotopic) sites such as skeletal muscle, tendon, ligament, fascia, and aponeuroses [Cohen et al., 1993; Kaplan et al., 2005; Pignolo et al., 2005; Rocke et al., 1994; Roush, 1996]. Heterotopic ossification begins in childhood and can be induced by trauma or occur without warning. Bone formation is episodic, progressive, and extensive, leading to flare-ups that form in a well-defined spatial pattern to cause extraarticular ankylosis of the joints of the axial and appendicular skeleton, immobilizing the patient in a “second skeleton” of heterotopic bone. In addition to this postnatal heterotopic bone formation, alterations during embryonic skeletal development also occur [Mahboubi et al., 2001; Schaffer et al., 2005].

ACVR1 DNA sequence analysis of FOP patients who have classic disease features (progressive heterotopic ossification and great toe malformations) revealed that the same recurrent single nucleotide change in *ACVR1* occurs in each FOP patient. This mutation, c.617G>A, results in the substitution of arginine by histidine at codon 206 (p.R206H) within the GS domain of the receptor. Protein structural homology modeling predicted that this amino acid substitution results in a conformational change of the receptor that alters its sensitivity and activity [Groppe et al., 2007; Shore et al., 2006].

The goals of this study were to conduct detailed clinical evaluations of a large cohort of FOP patients to establish clinical

homogeneity or subclasses associated with FOP-like heterotopic ossification, and to determine whether the identified recurrent heterozygous *ACVR1* mutation associated with classic FOP is present in all patients with FOP-like heterotopic ossification. We identified, in addition to patients with classic FOP, a small number of patients with unusual forms of FOP. Although these patients form FOP-like heterotopic ossification, they have additional features that are not commonly associated with FOP (FOP-plus) or have major variations in the classic defining features of FOP (FOP variants). We determined that all patients with any form of FOP carry heterozygous *ACVR1* mutations. However, in addition to the recurrent *ACVR1* c.617G>A mutation found in all cases of classic FOP and most cases of FOP-plus, previously undescribed mutations in the *ACVR1* gene are associated with the wider range of FOP variable expressivity that is clinically observed in FOP variants.

Materials and Methods

Subjects and Clinical Evaluation

We evaluated 112 FOP (classic and atypical) cases from five continents (104 sporadic and eight families) who were referred to one or more of us because of progressive heterotopic ossification and/or great toe malformations. Of the 104 with sporadic FOP (32 classically affected patients from our initial study [Shore et al., 2006] plus 72 additional patients), 84 individuals had classic FOP (classic defining features of FOP). We additionally identified 20 patients with additional atypical features and/or variation of the classic FOP phenotype: eight had FOP-plus (classic defining features of FOP plus one or more atypical features), and 12 had FOP variants (major variations in one or both of the classic defining features of FOP). One family had variant FOP in three members. We previously reported seven families with inheritance of classic FOP. Routine medical history, physical examination, and clinically-relevant photographic and radiographic studies were obtained on each patient. (See Table 1 for detailed clinical features and natural history of all three FOP forms.) FOP heterotopic ossification is considered “early onset” before 2 years of age and “late onset” after 10 years. The classic course of progression and severity of heterotopic ossification has been previously defined in detail [Cohen et al., 1993; Rocke et al., 1994]. Under approval by the Investigational Review Board of the University of Pennsylvania, peripheral blood samples were obtained following informed consent from patients and unaffected individuals. Cell lines were established by Epstein-Barr Virus (EBV) transformation of peripheral blood mononuclear cells. To generate haploid chromosome 2 cell lines for Patient 11, cells were fused with the E2 mouse cell line and hybrid cell lines were obtained (GMP Companies, Fort Lauderdale, FL).

Mutation Analysis

We screened genomic DNA from buccal swabs, blood, or lymphoblastoid cell lines for mutations in *ACVR1* by PCR-amplification of the nine exons containing protein-coding sequences (*ACVR1* Transcript Report Ensembl v35, accession number ENST00000263640; GenBank RefSeq NM_001105.4 and NP_001096.1) using exon-flanking primers [Shore et al., 2006]. DNA sequence analysis of genomic DNA used an ABI 3730XL sequencer (University of Pennsylvania School of Medicine DNA Sequencing Facility). The complete *ACVR1* coding region was sequenced for all atypical and variant FOP patients. Sequence data

Table 1. Clinical Features of Classic FOP, FOP-Plus, and Variant FOP Patients*

Patient # ACVR1 mutation Codon change	Atypical features of FOP-plus or FOP variant patients																			
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
Nucleotide change (cDNA position)	R206H	R206H	R206H	R206H	R206H	R206H	Q207E	G328R	G328R	G328R	G328W	G328W	G328E	G328E	G356D	G356D	G356D	G356D	R375P	P197_F198
Age of HO onset (years)	c617 G>A	c617 G>A	c617 G>A	c617 G>A	c617 G>A	c617 G>A	c619 C>G	c982 G>A	c982 G>A	c982 G>C	c982 G>T	c982 G>T	c983 G>A	c983 G>A	c1067 G>A	c1067 G>A	c1067 G>A	c1067 G>A	c.1124 G>C	c.590-592 delCTT
Gender	M, F	F	F	M	M	F	M	M	3F	M	F	F	F	F	F	M	M	F	F	F
High resolution karyotype	1-10	3	14	0.3	2	9	1.5	26	13; 22; none	21	2	8	2	2	2	0.3	?	15	14	11.5
Classic/defining FOP features:	Normal	Normal	Normal	Normal	Normal	Normal	Normal	13; 22; none	13; 22; none	21	2	8	2	2	2	0.3	?	15	14	11.5
Characteristic malformations of great toe (hallux valgus, malformed first metatarsal, and/or monophalangism)	X	X	X	X	X	X	X	X	X ^b	X	X	X	X	X	X	X (right) ^a	X	X	X	X
Progressive HO in characteristic anatomic patterns	X	X	X	X	X	X	X	X	X ^b	X	X	X	X	X	X	X	X	X	X	X
Common variable FOP features	X	X	X	X	X	X	X	X	X ^d	X	X	X	X	X	X	X	X	X	X	X
Conductive hearing impairment	X	X	X	X	X	X	X	X	X ^d	X	X	X	X	X	X	X	X	X	X	X
Cervical spine malformations ^e	X	X	X	X	X	X	X	X	X ^e	X	X	X	X	X	X	X	X	X	X	X
Proximal medial tibial osteochondromas	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
Short broad femoral necks	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
Thumb malformations (short first metacarpal, ± monophalangism)	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
Atypical FOP Clinical Features:																				
Severe variable reduction deficits of digits	0%																			
Absent finger/toe nails in digits with severe reduction deficits	0%																			
Normal or minimal changes in great toes	0%																			
Intraarticular synovial osteochondromatosis of hips and DID of hips	0%																			
Sparse, thin scalp hair (more prominent in second decade)	0%			X								X	X	X						
Mild cognitive impairment	0%			X								X	X	X						
Severe growth retardation	0%			X								X	X	X						
Cataracts	0%																			
Retinal detachment	0%																			
Childhood glaucoma	0%																			
Craniopharyngoma	0%																			
Persistence of primary teeth in adulthood	0%																			
Anatomic abnormalities of cerebellum	0%																			
Diffuse cerebral dysfunction with seizures	0%																			
Polyostotic fibrous dysplasia	0%																			
Primary amenorrhea	0%																			
Aplastic anemia	0%																			
Hypospadias	0%																			
Cerebral cavernous malformations	0%																			

*The DNA mutation numbering is based on cDNA sequence, with +1 corresponding to the A of the ATG translation initiation codon in the reference sequence (GenBank NM105.4) according to nomenclature guidelines (www.hgvs.org/mutnomen). The initiation codon is codon 1.

^aThis patient has asymmetric malformations of his great toes: right, classic; left, more severe deficit.

^bSee Table 2.

^cIntraarticular ankylosis of facet joints and early degenerative changes of cervical spine.

^dThe mother has mild orthotopic changes in c-spine; the daughters do not.

^eDistal femoral osteochondroma only.

^fRight great toe is normal; minor changes in first left metatarsal.

^gTwo members of this family, the mother and one daughter, have normal toes; one daughter has minor changes of both first metatarsals.

^hCharacteristic short monophalangeic great toe, but additionally the patient is missing middle phalanges of fourth and fifth toes bilaterally.

HO, heterotopic ossification; ?; no detected HO at the time of this report (patient is 2.3 years of age; typical age of onset is 2 to 10 years).

were analyzed using 4Peaks software v.1.6 (available at the online-only company: www.mekentosj.com/4peaks). (Electropherograms were reviewed but are not shown.) For each identified mutation, we verified the absence of a mutation in at least 98 individuals (196 alleles). Mutations are identified by nucleotide numbering that reflects the cDNA sequence, with +1 corresponding to the A of the ATG translation initiation codon in the reference sequence, according to journal guidelines (www.hgvs.org/mutnomen). The protein initiation codon is codon 1. Differences in restriction endonuclease recognition sites were identified (MacVector v.7.2 software, Cary, NC); genomic DNA (0.1 µg) was PCR-amplified and purified PCR products were digested with the appropriate restriction enzymes.

Protein Structure Homology Modeling

TGFBR1 receptor type I (TβRI; PDB# 1B6C) crystal structure coordinates were downloaded from the Protein Data Bank (www.rcsb.org). Structure-based homology models of mutant ACVR1 cytoplasmic domains were calculated through the automated SwissModel routines (Biozentrum, Basel; <http://swissmodel.expasy.org/SWISS-MODEL.html>) with the FKBP12-bound crystal structure of TβRI as a three-dimensional (3D) template. Molecular models were analyzed and figures prepared with PyMOL (DeLano Scientific, Palo Alto, California; <http://pymol.sourceforge.net>).

Results

Individuals With Classic FOP

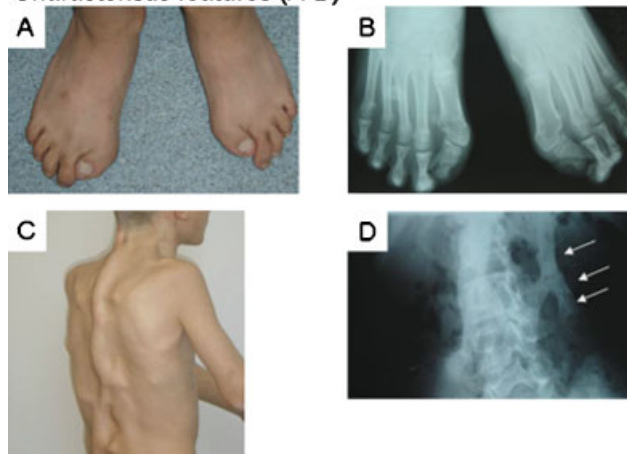
Patients with classic FOP have two defining clinical features (Fig. 1A–D): characteristic congenital malformations of the great toes and progressive heterotopic ossification in characteristic anatomic patterns [Shore et al., 2006]. In addition, common but variable features are seen in most individuals with FOP (Table 1) including proximal medial tibial osteochondromas (>90% prevalence), cervical spine malformations (>80% prevalence), short, broad femoral necks (>70% prevalence), conductive hearing impairment (>50% prevalence), and malformations of the thumb—specifically short first metacarpals and/or monophalangism of the thumbs (>50% prevalence; Fig. 1E–H) [Kaplan et al., 2005]. To date, we examined 84 sporadic cases and seven families with classic FOP, and all affected individuals were heterozygous for the canonical (c.617G>A; p.R206H) mutation in *ACVR1*.

Individuals With Classic FOP Plus Atypical Clinical Features (“FOP-plus”)

Eight individuals in our study had FOP-plus (Patients 1–7 and 15; Table 1): the classic clinical features of FOP plus one or more atypical features. Six of these individuals (Patients 1–6) had the canonical c.617G>A (p.R206H) mutation in *ACVR1*. One individual (Patient 7) had a unique missense mutation (p.Q207E) in the GS domain of *ACVR1* and one patient (Patient 15) had a missense mutation (p.G356D) in the protein kinase domain of *ACVR1*. Atypical features will be highlighted in the following brief summaries of patients with FOP-plus.

Patient 1 (c.617G>A; p.R206H) had intercurrent aplastic anemia that developed at 10 years of age and was treated successfully with an human leukocyte antigen (HLA)-matched bone marrow transplantation from an unaffected sibling [Kaplan

Characteristic features (A–D)



Variable features (E–H)

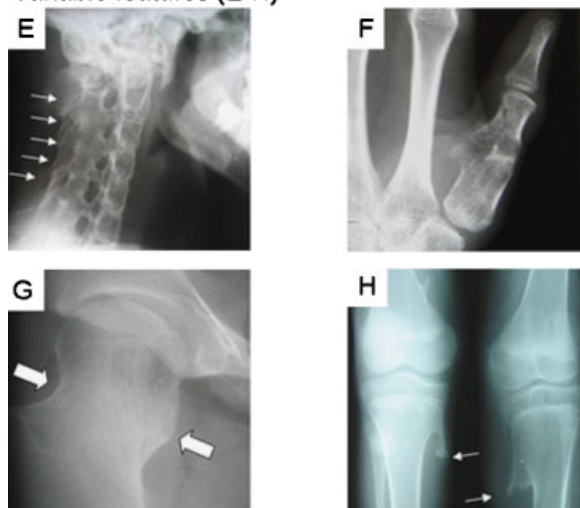


Figure 1. Characteristic and variable features of classic FOP. Composite of characteristic (A–D) and common variable (E–H) features of classic FOP. A photograph (A) and radiograph (B) of the feet in a classically-affected 15 year-old boy shows short, malformed monophalangeic great toes. A photograph of his back (C) and a radiograph of his lumbar spine (D) reveal ribbons, sheets, and plates of heterotopic bone (D, arrows). Lower panel collage (E–H) from several affected individuals depicts common variable features of classic FOP, including orthotopic fusion of subaxial facet joints of the cervical spine (E, arrows) prior to the onset of heterotopic ossification, short monophalangeic malformed thumb (F), short broad femoral neck (G, arrows), and proximal medial tibial osteochondromas (H, arrows). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

et al., 2007b]. The bone marrow transplantation cured the aplastic anemia but did not affect the progression of the FOP. At 35 years of age, the patient had 100% donor lineage in all cell lines of hematopoietic origin. DNA obtained from a buccal swab confirmed the classic *ACVR1* mutation while DNA from peripheral blood of both patient and his unaffected sibling donor did not, confirming bone marrow engraftment in the patient.

Patient 2 (c.617G>A; p.R206H) had intercurrent polyostotic fibrous dysplasia [Frame et al., 1972], a condition typically caused by somatic cell activating mutations of the *GNAS* gene (MIM# 139320). DNA was not available from affected somatic tissues and *GNAS* sequence analysis was not possible. Genomic DNA from peripheral blood was negative, as expected, for *GNAS* mutations

(data not shown) but *ACVR1* mutation screening revealed the c.617G>A (p.R206H) mutation.

Patient 3 (c.617G>A; p.R206H) was diagnosed at 10 years of age with a craniopharyngioma, which was successfully resected, although panhypopituitarism required daily replacement of adrenal steroids, thyroid hormone, and vasopressin (desmopressin acetate [DDAVP]).

Patient 4 (c.617G>A; p.R206H) was diagnosed with severe childhood glaucoma at the age of 2 years, had a bifid uvula, thin hair, and a history of mild developmental delay.

Patient 5 (c.617G>A; p.R206H) suffered from severe retinopathy of prematurity in addition to bilateral cataracts, and glaucoma of the left eye. At 4 months of age, he was diagnosed with an inguinal hernia, which was surgically repaired without the formation of heterotopic bone. He has severe motor and cognitive developmental delays with myoclonic seizures and diffuse cerebral dysfunction. He had a successful HLA-matched liver transplantation due to liver failure, attributed to cytomegalovirus infection and takes antiepileptic medications as well as sirolimus and prednisolone as chronic immunosuppression. Progressive heterotopic ossification was first noted at 9 years of age following the liver transplantation, and flare-ups of heterotopic ossification continued in characteristic anatomic patterns despite the immunosuppression. A high resolution karyotype was normal.

Patient 6 (c.617G>A; p.R206H) had growth retardation with height and weight persistently less than the fifth percentile for age. At 18 months of age, resections of large soft tissue swellings on her back exacerbated heterotopic bone formation. She rapidly developed severe scoliosis and thoracic lordosis with death at 8 years of age from heart failure that complicated thoracic insufficiency syndrome [Kaplan and Glaser, 2005].

Patient 7 had a clinical appearance nearly identical to Patient 6, but he had not undergone invasive diagnostic biopsies. Nevertheless, he had failure to thrive with height and weight persistently below the fifth percentile. A unique heterozygous mutation (c.619C>G; p.Q207E) in the GS domain of *ACVR1* was detected in his genomic DNA.

Patient 15 had persistence of primary teeth into adulthood, and primary amenorrhea. Sequence analysis of genomic DNA revealed a heterozygous mutation (c.1067G>A; p.G356D) in the protein kinase domain of *ACVR1*.

Individuals With Variant FOP Features ("FOP Variants")

Twelve cases (Patients 8–14 and 16–20) are variants of classic FOP, with major variations in one or both of the classic defining features of FOP (Table 1). Variations from the classic FOP features are highlighted in the following brief summaries.

Patient 8 had short thumbs at birth, although his toes appeared normal. He had no soft tissue lesions or heterotopic ossification during childhood. At 26 years of age, he tore the right anterior cruciate ligament, underwent a surgical repair, and formed heterotopic bone at the operative site. Surgical resection of mature heterotopic bone was attempted but failed. By age 30 years, he had a stooped posture, a painless reciprocal gait, 80% residual movement of the cervical spine, 150 degrees abduction of the shoulders, and full range of motion of the jaw, elbows, wrists, and ankles. Radiographs revealed heterotopic ossification in the neck, back, and left knee. Both hips showed mild degenerative changes. Radiographs of the right great toe were normal; however, there was a small bony irregularity of the left distal first metatarsal (Fig. 2A). A CT scan of the head and neck revealed a hypoplastic cerebellum. Sequence analysis of genomic DNA revealed a

heterozygous mutation (c.982G>A; p.G328R) in the protein kinase domain of *ACVR1*.

Patient/Family 9 included three individuals, a mother (Patient 10.II.2) and two daughters (Patients 10.III.1 and 10.III.2; Table 2) who were reported previously (as Family 2) [Viridi et al., 1999] with a mild FOP phenotype characterized by either normal toes or mildly-affected toes and no or late onset of heterotopic ossification (Table 1). No substantial progression of FOP has occurred in any of the individuals since the previously published report. Sequence analysis of genomic DNA revealed a heterozygous mutation (c.982G>A; p.G328R) in the protein kinase domain of *ACVR1* in all three affected individuals, but not in the unaffected father (Patient 10.II.1) or maternal grandmother (Patient 10.I.2).

Patient 10 had normal hands but short malformed great toes at birth. Surgical correction was attempted and preoperative films were not available, however postsurgical radiographs showed monophalangism of the great toes, with surgical correction of hallux valgus, and absence of the middle phalanges of the fourth and fifth toes bilaterally (Fig. 2B). Heterotopic ossification began at 21 years of age in the characteristic anatomic patterns. At 32 years, he developed headaches and a CT scan of the brain revealed multiple cerebral cavernous malformations (CCMs). No genetic workup was undertaken for the CCMs. Sequence analysis of genomic DNA revealed a heterozygous mutation (c.982G>C; p.G328R) in the protein kinase domain of *ACVR1*.

Patient 11 was noted at birth to have severe reduction deficits of the great toes, with lack of toenails in the affected digits, and severe malformations of the thumbs (Fig. 2C). During the second decade of life, her scalp hair became thin, and its growth rate slowed dramatically. Her eyebrows were sparse. She had mild cognitive impairment with difficulty in abstract thinking but no difficulty in attention. A CT brain scan revealed anatomic abnormalities of the cerebellum without associated impairment in movement. An MRI of the brain was not performed. Sequence analysis of genomic DNA revealed a heterozygous mutation (c.982G>T; p.G328W) in the protein kinase domain of *ACVR1*.

Examination of polymorphic markers in the FOP linkage region on chromosome 2 also revealed a loss of heterozygosity (LOH) region in Patient 11. We determined (data not shown) that this LOH is a de novo 1.8-kb region that is not present in either of the patient's parents. Genomic DNA database analysis and comparison of the deleted region across species revealed that the deletion contains no identified gene coding regions and that the sequence is not conserved in mouse genomic DNA. No other patients examined (including Patients 12–14 reported here) contained this deletion.

Patient 12 showed severe reduction deficits of the great toes and thumbs at birth, with absent toenails in the affected digits [Connor and Evans, 1982]. Progressive heterotopic ossification in characteristic anatomic patterns began at age 8 years. She had mild conductive hearing impairment and short broad femoral necks. During the second decade of life, she developed sparse thin hair on her scalp and mild cognitive impairment without any difficulty in attention. Sequence analysis of genomic DNA revealed a heterozygous mutation (c.982G>T; p.G328W) in the protein kinase domain of *ACVR1*, the same mutation identified in Patient 11.

Patient 13 [Connor and Evans, 1982] had severe reduction deficits of the great toes and thumbs at birth, with absence of toenails in the affected digits (Fig. 2D). During the second decade of life, she developed sparse thin hair on her scalp and had mild cognitive impairment without any difficulty in attention.

FOP VARIANTS		FEET		HANDS	
A	G328R (Pt #8)				
B	G328R (Pt #10)				
C	G328W (Pt #11)				
D	G328E (Pt #13)				
E	G328E (Pt #14)				
F	G356D (Pt #16)				
G	G356D (Pt #17)				
H	G356D (Pt #18)				

Figure 2. Digital malformations in FOP variants. All patients with FOP variants whose images are depicted here (A–H) had heterozygous missense mutations at either codon 328 (A–E) or codon 536 (F–H) in the kinase domain of *ACVR1*, in contrast to all classically-affected individuals with FOP who had a recurrent mutation in *ACVR1* at codon 206 (R206H) (Fig. 1); protein RefSeq NP_001096.1. All three variants with the G328R mutation (A, B, plus members of Family 9, not shown) had either normal great toes or minimal malformations, while the hands were normal. All four variants with either the G328W mutation (C; plus Patient 12, not shown) or the G328E mutation (D, E) had severe truncation deformities of multiple digits (C–E) and/or syndactyly (C, D). Patients 13 and 14 (D, E; G328E mutation) had slightly different malformation patterns from each other: Patient 13 (D) had severe truncation of the great toes, whereas Patient 14 (E) had more severe reduction deficits of the posterior digits. The hand malformations were similar in each, although Patient 14 (E) was missing a postaxial digit on both hands. Both patients lacked nails in all severely affected digits. Three variants with the G356D mutation (F–H) had severe truncation malformations of the thumbs and great toes, although variable degrees of terminal symphalangism were noted and the digital truncations of Patient 16 (F) were asymmetrical in the hands and feet. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Sequence analysis of genomic DNA revealed a heterozygous mutation (c.983G>A; p.G328E) in the protein kinase domain of *ACVR1*.

Patient 14 was noted at birth to have severe reduction deficits of the great toes and thumbs as well as absent toenails of the affected digits (Fig. 2E). During the second decade of life, she was noted to have sparse, thin hair on her scalp and sparse eyebrows. She had mild cognitive impairment without any deficit in attention. DNA sequence analysis of genomic DNA revealed a heterozygous mutation (c.983G>A; p.G328E) in the protein kinase domain of *ACVR1*, the same mutation that was found in Patient 13.

Patient 16 had asymmetrical malformed great toes and thumbs with missing nails on the severely affected digits at birth. The distal interphalangeal joints in his index fingers were absent (Fig. 2F) and he had hypospadias. Sequence analysis of genomic DNA revealed a heterozygous mutation (c.1067G>A; p.G356D) in the protein kinase domain of *ACVR1*.

Patient 17 had bilateral absence of both great toes and hypoplastic thumbs with severe shortening of the first metacarpals (Fig. 2G). His eyebrows and eyelashes were sparse. He was 28 months of age at last examination and had no manifestations of soft tissue swellings or heterotopic ossification; typical age of onset

Table 2. Phenotypes of FOP Variant Patient/Family 9*

	10.II.2	10.III.1	10.III.2
Congenital malformation of great toe	None	Minor	None
Age of onset of HO	22	no HO	13
Severity of HO	Mild	–	Mild
Exacerbation of HO following trauma	+	–	+
Intraarticular synovial osteochondromatosis of the hip	–	–	+
Radiographic anomalies of the cervical spine	+	–	–
ACVR1 mutation	c.982G>A	c.982G>A	c.982G>A

*Affected mother (Patient 10.II.2) and two affected daughters (Patients 10.III.1 and 10.III.2). ACVR1 mutation numbering reflects the cDNA sequence with +1 corresponding to the A of the ATG translation initiation codon in the reference sequence (NM001105.4).

–, Absent; +, Present; HO, heterotopic ossification.

is 2 to 10 years. Since his toe and thumb malformations were suggestive of FOP, the ACVR1 gene was examined. Sequence analysis of genomic DNA revealed a heterozygous mutation (c.1067G>A; p.G356D) in the protein kinase domain of ACVR1.

Patient 18 had severe reduction deficits of the great toes and thumbs at birth, with absent or hypoplastic nails of the affected digits (Fig. 2H). Heterotopic ossification began at 15 years of age, causing mobility restriction of the neck and back; a flare-up of the right hip occurred at 16 years old. Severe alopecia, and primary amenorrhea were noted. Sequence analysis of genomic DNA revealed a heterozygous mutation (c.1067G>A; p.G356D) in the protein kinase domain of ACVR1. This is the same mutation identified in Patients 15, 16, and 17.

Patient 19 had clinically and radiographically normal toes. FOP flare-ups initiated at age 14 years and progression of disease was slow and evanescent. At 40 years of age, she had limited motion of the cervical spine and shoulders with heterotopic ossification in the neck, back, chest, and right hip, but was still ambulatory. Sequence analyses of genomic DNA revealed a heterozygous mutation (c.1124G>C; p.R375P) in the protein kinase domain of ACVR1.

Patient 20 had clinically and radiographically normal toes. At 11 years of age, painful flexion contracture of the left hip prompted imaging studies that showed a poorly defined soft tissue mass of the left iliopsoas muscle. Following a biopsy that was diagnosed as “aggressive fibromatosis,” she developed heterotopic ossification with ankylosis of the left hip. Within 6 months she had ankylosis of all major joints of the axial and appendicular skeleton. Sequence analysis of genomic DNA revealed a unique in-frame 3-bp heterozygous mutation (c.590_592delCTT; P197_F198delinsL) in the GS domain of ACVR1 that replaces amino acids proline (codon 197) and phenylalanine (codon 198) with leucine.

ACVR1 Mutations

All mutations identified in classic FOP, FOP-plus, and variant FOP patients are single nucleotide substitutions that cause missense mutations, with the exception of a three-nucleotide deletion (Patient 20) that replaces two amino acids with a single amino acid. In families with inherited FOP (such as Family 9 in this study), all affected members have a mutation and no unaffected members carry the mutation. For most cases of sporadic FOP-plus and FOP variants, both parental samples were

unavailable for analysis; however, de novo mutations were confirmed for Patients 11 and 18. Absence of each of the identified mutations was verified in at least 98 unaffected individuals. None of these ACVR1 sequence variants are reported in SNP databases (www.ncbi.nlm.nih.gov/SNP; http://www.ensembl.org/Homo_sapiens/genesnpview). Unlike the recurrent c.617G>A mutation, none of the rarer mutations in FOP-plus and variant FOP patients alter a CpG dinucleotide.

In addition to direct DNA sequence analysis, most of the identified ACVR1 mutations were verified experimentally by differential restriction endonuclease digestion (data not shown). New enzyme digestion sites are formed by the c.619C>G (NruI), and c.1067G>A (DrdI) nucleotide substitutions. Each of the single nucleotide substitutions identified in codon 328 eliminates a StyI digestion site.

ACVR1 is a protein that has been highly conserved during vertebrate evolution. Comparison of the human ACVR1 protein sequence (509 amino acids) to ACVR1 in other species shows high degrees of similarity (for example, 99.8% in chimp, 98.4% in mouse, and 97.9% in chick). Each of the mutated amino acids in the FOP variant and FOP-plus patients is conserved across species (Fig. 3). Unlike the classic FOP mutation that changes codon 206, an amino acid that may contribute to receptor specificity (ACVR1 receptors encode arginine [R] while BMPRIA and BMPRII have lysine [K] at the analogous position; see Supplementary Fig. 2 in Shore et al. [2006]), each FOP variant mutation and FOP-plus mutation is invariant among all three human type I BMP receptors: ACVR1 (ALK2), BMPRIA (ALK3), and BMPRII (ALK6).

No frameshift or nonsense mutations were identified in the ACVR1 sequence, suggesting that in each case a mutant receptor protein with altered function is produced. All of the identified mutations occur in either the GS or protein kinase domains, regions of the ACVR1 receptor that are important in conferring downstream signal transduction.

Protein Modeling

Although the family of activin-like kinases (ALKs) includes seven different receptors, only the structure of the intracellular domain of TβRI (ALK5; PDB# 1B6C) has been experimentally determined [Huse et al., 1999]. However, the extensive cytoplasmic domain homology within the ALK family allows reliable structure-based homology modeling of wild-type and mutant ACVR1 receptor kinases (Fig. 4). Our previous modeling predicted that the highly conserved p.R206H substitution, found in all classic FOP and in six patients (Patients 1–6) with FOP-plus, results in an aberrant ion pair or salt bridge that acts as a pH-sensitive switch, leading to ligand-independent activation of the receptor [Groppe et al., 2007].

In addition to the p.R206H mutation, we identified two other GS domain mutations. Protein modeling predicts that introduction of an acidic residue such as p.Q207D, an engineered constitutive activating TβRI mutation [Wieser et al., 1995], or p.Q207E (Patient 7; FOP-plus) would disrupt an ion pair formed between the neighboring basic residue (ACVR1 Arg206) and an invariant acidic residue (ACVR1 Asp269). Formation of the nonnative ion pair (in *cis*), even transiently, is predicted to sterically hinder the binding of the inhibitory FKBP12 binding protein (Fig. 4A), a protein that is required to maintain the receptor kinase in an autoinhibited state until activated by ligand-induced assembly of the heterotetrameric signaling complex [Huse et al., 2001]. The three-nucleotide deletion in FOP variant Patient 20 (c.590_592delCTT), replaces proline 197 and phenylalanine

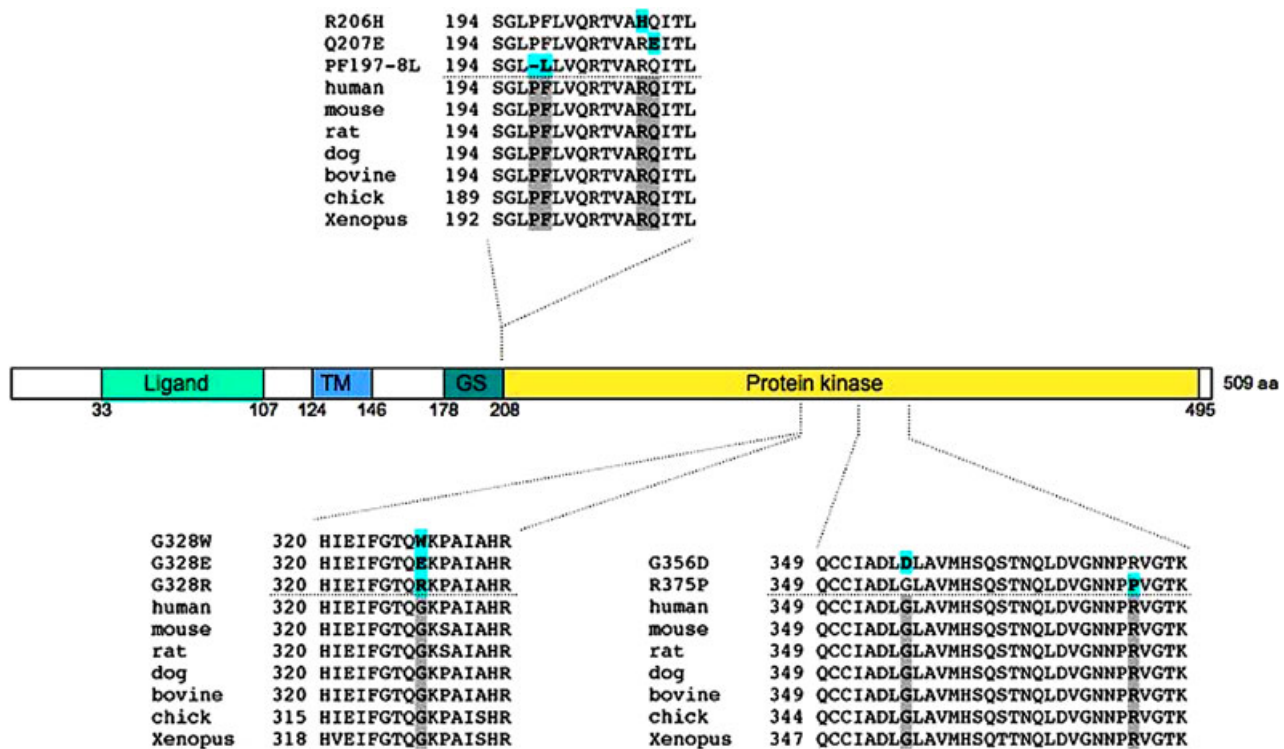


Figure 3. Position and conservation of ACVR1 amino acid changes. ACVR1 encodes a 509–amino acid protein that contains a ligand binding region, a transmembrane (TM) domain, a glycine-serine rich region (GS), and a protein kinase domain. The numbers below the protein representation indicate the amino acid codons included in each identified domain; the protein initiation codon is codon 1 (RefSeq NP_001096.1). The relative positions of all identified mutations are shown with the altered amino acids in bold with light shading. Each mutation in the *ACVR1* gene occurs in an identical amino acid at the corresponding position of ACVR1/ALK2 across species (darker shading). ClustalW was used for multiple protein sequence alignment. The schematics are drawn approximately to scale.

198 with a single leucine residue, and removes 1 of the 2 residues (Phe 198, Leu199) comprising the FKBP12 binding site (Fig. 4A); this loss of the entire FKBP12-ACVR1 binding interface would abolish all interaction with the inhibitory protein. Thus all three mutations in the GS region are predicted to share the common effect to perturb, diminish, or abolish interactions with FKBP12.

ACVR1 mutations within the protein kinase domain were also found. Glycine 328, a site of multiple mutations in FOP variants, is replaced with arginine (Patients 8–10), tryptophan (Patients 11 and 12), or glutamate (Patients 13 and 14). Glycine 328 is at the bottom of a cavity formed by a flanking surface loop, by the GS loop, and by the N-terminal end of the α C helix (4). The structural basis for the G328 mutations are not clear since none of the substitutions significantly alters the conformation of the polypeptide chain as determined by homology modeling (not shown). Introduction of bulky, charged, or hydrogen-bonding side-chains into the cavity could impair receptor function by affecting GS domain interaction with FKBP12 or SMAD proteins. Alternatively, the cleft could be a substrate-binding site for phosphorylations in the MAPK pathway, with the substituted residues causing enhanced interactions with target substrates. A third possibility is that introduction of these new side-chains results in displacement of the α C helix, a key regulatory element of all protein kinases (see below).

Two additional protein kinase domain FOP mutations, p.G356D (Patients 15–18) and p.R375P (Patient 19), occur within the receptor kinase active site (Fig. 4D). Glycine 356 substitution with aspartate has no detectable effect on the backbone conformation of the receptor (not shown). However, an ion pair

between lysine 235 and glutamate 248 (Fig. 4D), found in all protein kinases that modulate enzyme activity by α C helix conformation changes [Huse and Kuriyan, 2002], may be altered by introducing an ion pair-forming partner (aspartate 356) that causes loss of autoinhibition of the kinase. Arginine 375 forms a conserved ion pair with aspartate 354, blocking a cation-binding site required for ATP hydrolysis. Loss of this ion pair due to substitution of arginine with proline allows cation binding and promotes phosphorylation by the receptor (a similar view for T β RI interactions has been reported [Huse et al., 1999]).

Discussion

Small variations in genotype can give rise to large variations in phenotype that provide important insight into the mechanisms of human disease. Among patients identified with FOP-like heterotopic ossification and/or great toe malformations, we identified 20 patients who showed notable variation in the clinical presentation commonly observed in patients with FOP. All patients with “classic FOP” features have a recurrent c.617G>A (p.R206H) mutation in the *ACVR1* gene. In this study, we expanded our analysis of classic FOP patients and examined DNA from the atypical FOP patients for *ACVR1* gene mutations. This analysis has led to several significant conclusions: All patients with FOP-type heterotopic ossification have mutations in the protein-coding region of *ACVR1*, and all are missense mutations or in-frame deletions. While all patients with classic FOP have the identical single nucleotide and resulting amino acid change, some patients with atypical clinical presentation of FOP have alternate

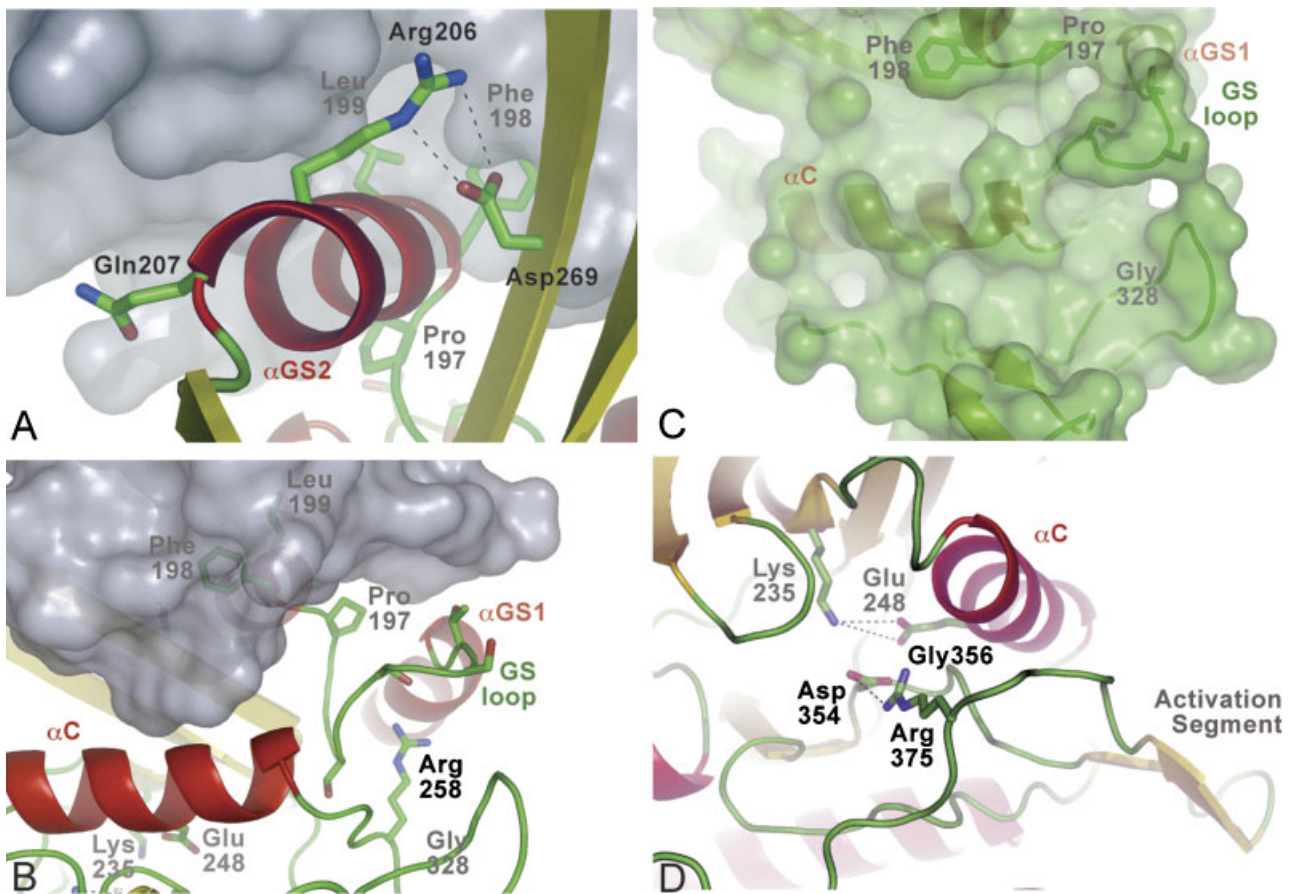


Figure 4. Sites of FOP mutations in a structure-based homology model of the ACVR1 receptor kinase domain. Panels show models of the wild-type ACVR1 protein with specific amino acids that are implicated in structural changes as a result of mutations. The protein initiation codon is codon 1 (RefSeq NP_001096.1). **A:** Mutation sites within the GS regulatory region. Arginine 206, which forms an ion pair with aspartate 269 (dashed lines), is substituted with histidine in all patients with classic FOP and in 6 out of 8 patients (Patients 1–6) with FOP-plus. The adjacent residue, glutamine 207, is substituted with glutamate in FOP-plus Patient 7. A three-nucleotide deletion replaces proline 197 and phenylalanine 198 with a leucine residue in variant FOP Patient 20. The surface of the FKBP12 binding protein at the binding protein-receptor interface is depicted in gray. **B,C:** Multiple glycine 328 missense mutations. Seven FOP variants (Patients 8–14) had substitutions of glycine 328. Codon 328, in the protein kinase domain, resides in a loop at the bottom of a surface cavity bordering the GS loop and the N-terminal end of the α C helix. The surface of the kinase domain is depicted in green. For clarity, FKBP12 is not shown. The view in (C) is similar to (B), with the FKBP12 binding site and GS loop rolled slightly forward toward the viewer. **D:** Mutation sites within the receptor kinase active site. Glycine 356 is substituted with aspartate in Patients 15–18 (one with FOP-plus and three FOP variants) and arginine 375 with proline in FOP variant Patient 19. The ion pair between arginine 375 and aspartate 354 blocks a cation binding site required for ATP hydrolysis by the enzyme. The lysine 235–glutamate 248 ion pair is conserved in all protein kinases and modulates enzyme activity by altering active site conformation. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

mutations in the *ACVR1* gene. The classic FOP mutation occurs in most patients with “FOP-plus” (classic features plus additional unusual features); their additional features may be coincidental or may be due to genetic modifiers. However, some patients with FOP-plus have novel mutations, which may explain their additional features. “FOP variant” patients (variations in the classic defining features of FOP) have novel mutations that can provide insights into the effect of altered ACVR1 function during developmental, cognitive, and homeostatic processes. Additionally, understanding the specific effect of a missense mutation on ACVR1 function could help guide the design of pharmacologic agents that will modify or prevent the cause of the disease.

The consequences of specific *ACVR1* mutations during postnatal life are likely different from those during embryogenesis. Studies of identical twin pairs with FOP [Hebela et al., 2005] have shown that although environmental influences have major effects on the course of heterotopic ossification, genetic factors are the major influence on developmental defects (such as embryonic

skeletal formation) in FOP. Among patients with classic FOP, FOP-plus, and FOP variants, a wide range of variability in great toe malformations is observed, and our data suggest that genotype-phenotype correlations may explain at least some of this variation. In some patients, different mutations (for example, p.R206H and p.Q207E, both in the GS domain) show similar toe malformations. We also observed that similar phenotypes are associated with mutations in different domains of the ACVR1 receptor. For example, mutations in the protein kinase domain or the GS activation domain occur in FOP variants with normal or mildly malformed great toes (Patients 8–10, 19, and 20; Table 1). By contrast, the same mutation (p.G356D) that was identified in four patients (Patients 15–18), and in an additional recent case report [Furuya et al., 2008], is associated with a wide range of phenotypic consequences, possibly due to differences in genetic backgrounds of the individuals [Shore et al., 2005].

Other developmental phenotypes may only be caused by specific *ACVR1* mutations, such as the severely shortened thumbs

and great toes, alopecia, nail dysplasia, and learning disorders [Botchkarev, 2003; Botchkarev and Sharov, 2004; Hens et al., 2007; Kobiela et al., 2007; Wang et al., 2004] that occur in four FOP variants (Patients 11–14) with glycine to tryptophan or glutamic acid codon 328 mutations. These codon 328 mutations are distinct from the glycine to arginine mutations in less severe FOP variants (Patients 8–10) who had normal/minimally affected great toes and late onset heterotopic ossification. *ACVR1*, therefore, appears to be particularly sensitive to codon 328 mutations, suggesting importance in regulating receptor function and BMP signaling during embryonic development.

Progressive postnatal heterotopic ossification is the common feature shared by all patients with classic FOP, FOP-plus, and FOP variants. Although the rate of progression and the severity of heterotopic ossification varies among individuals with classic FOP, we found correlation between the severity of heterotopic ossification and specific mutations among the *ACVR1* mutations identified in FOP variant patients. These data support that all of the identified *ACVR1* missense mutations influence the promiscuous postnatal induction of cartilage and bone cell differentiation; however, as supported by our protein modeling data, the proposed molecular mechanisms may vary.

All of the mutations in *ACVR1* associated with classic, FOP-plus and variant forms of FOP reside in or adjacent to the GS regulatory region or active site of the kinase. None were mapped to the larger C-terminal domain or lobe, which serves only as a scaffold to stabilize the receptor. Furthermore, the structural basis for loss of autoinhibition by the mutant receptors is predicted by structural modeling, with the exception of the multiple substitutions at glycine 328 that could perturb the receptor through one of several plausible mechanisms.

All of the reported mutations in FOP and its variants are predicted by protein structure homology modeling to activate the *ACVR1* protein and enhance receptor signaling. Constitutive *ACVR1* expression in embryonic chick limbs induces expansion of chondrogenic anlagen, strongly suggesting that *ACVR1* signaling alters cell fate and induces undifferentiated mesenchyme to form cartilage [Zhang et al., 2003]. In the chick, however, the heterotopic bone was seen at birth, whereas in humans with FOP, the effects were not seen at birth, and occurred only in the postnatal period. These findings suggest the intriguing possibility that the FOP mutations are mildly constitutively active and hyperresponsive to receptor stimulation. Recent data support these findings [Billings et al., 2008; Shen et al., 2007].

Enhanced expression of BMP transcriptional targets is observed in FOP cells [Fiori et al., 2006; Kaplan et al., 2006; Serrano de la Pena et al., 2005]. Overactive BMP signaling in FOP cells may lead paradoxically to orthotopic ankylosis of the joints and early degenerative joint disease as seen in FOP patients and in animal models of promiscuous BMP signaling [Ahn et al., 2003; Fiori et al., 2006; Gannon et al., 1997; Glaser et al., 2003; Kan et al., 2004; Kaplan et al., 1990, 2006, 2007c; O'Connell et al., 2007; Serrano de la Pena et al., 2005; Shafritz et al., 1996]. Aberrant *ACVR1* signaling may also be relevant to the pathogenesis of degenerative joint disease [Oshin and Stewart, 2007], as seen in early orthotopic degenerative changes of the great toe, thumb, cervical spine, and in the costovertebral joints before the appearance of FOP flare-ups and subsequent heterotopic ossification.

Animal and human studies of mutations in *BMPRIIB* (*ALK6*; another BMP type I receptor associated with brachydactyly type A2), as well as *GDF5* and *NOGGIN*, suggest that mutations in BMP type I receptors affect cartilage formation in a dominant-negative manner [Baur et al., 2000; Dawson et al., 2006; Lehmann

et al., 2003, 2006, 2007; Seemann et al., 2005; Yi et al., 2000]. We cannot yet rule out the possibility that a similar effect is present during embryonic development with the mutations in classic FOP, FOP-plus, and FOP variants that lead to malformations of the great toes. Mutant *ACVR1* may oligomerize with other type I receptors such as *BMPRIA* and *BMPRIIB*, accounting for some of the in vitro and in vivo effects seen in individuals with FOP and its variants [Gilboa et al., 2000; Nohe et al., 2002].

All of the classic and common variable features of FOP as well as many, if not all, of the atypical features evaluated in our study could plausibly be ascribed to dysregulation of the BMP signaling pathway. A recent report that mutations in *BMP4* cause eye, brain, and digit abnormalities suggests that *BMP4* signaling through *ACVR1* could lead to at least some of the atypical features found in some FOP patients [Bakrania et al., 2008]. However, it is not yet known if atypical FOP features such as aplastic anemia [Kaplan et al., 2007b], polyostotic fibrous dysplasia [Frame et al., 1972], craniopharyngioma [Davis and Camper, 2007], cerebellar abnormalities [Angle et al., 2003; Qin et al., 2006], childhood glaucoma [Plikus et al., 2008; Wordinger et al., 2002, 2007], cataracts [Andreev et al., 2006; Wordinger and Clark, 2007], persistence of primary teeth [Thesleff, 2006], primary amenorrhea [Knight and Glister, 2006], hypospadias [Morgan et al., 2003], or severe growth retardation [Lee et al., 2007] (each seen in only one or two individuals) were intercurrent findings coincidentally associated with FOP or whether they were causally related to the underlying mutations in *ACVR1* and unmasked by polymorphisms in the BMP or other signaling pathways in the affected individuals. Further studies of BMP signaling in animal models of classic and variant FOP will be critical to address these questions.

Identification of disease-causing mutations in *ACVR1* has important diagnostic and therapeutic implications. Presently, there is no definitive treatment for patients with FOP or its variants [Glaser and Kaplan, 2005] and the identification of heterozygous missense mutations in *ACVR1* reveals a pharmaceutical target for the development of signal transduction inhibitors (STIs) such as dorsomorphin or its derivatives [Kaplan et al., 2007a; Yu et al., 2008] as well as other therapeutic strategies [Kaplan et al., 2007a]. However, in addition to treating FOP, postnatal inhibition of *ACVR1* could have a significant role in treating common acquired disorders of orthotopic and heterotopic ossification and, conversely, the mutation(s) of FOP and its variants could be harnessed for tissue engineering to form new bone for therapeutic applications. Genotype-phenotype correlations of the FOP *ACVR1* mutations will help elucidate *ACVR1* signaling mechanisms and in vivo functions to further these goals.

Acknowledgments

We thank the members of our research laboratory for their contributions, including George Feldman for his work related to Patient 11 and Bob Caron for figure preparation. We also thank Kathryn Ewens for helpful discussions. We dedicate this study to the memory of Dr. Victor McKusick for his lifelong guidance and contributions to the FOP community.

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