an essential transcription factor for osteoblast differentiation in embryogenesis, has been identified in C2C12 cells stimulated with BMP-2 (Nakashima et al., 2002).

Bone morphogenetic protein signal transduction

Bone morphogenetic protein receptors

The members of the TGF-β family bind to transmembrane serine/threonine kinase receptors (Figure 2) (Rosen, 2006; Sieber et al., 2009; Miyazono et al., 2010; Mueller and Nickel, 2012). Although their structures are conserved overall, these proteins are classified into two subgroups, type I and type II receptors, based on the presence (type I) or absence (type II) of the GS domain, a glycine- and serine-rich sequence between the transmembrane and kinase domains (Figure 2) (Wrana et al., 1994). Osteogenic BMPs bind to four forms of type I receptors [ALK1, ALK2, BMPR-IA (also called ALK3) and BMPR-IB (also called ALK6)] and three forms of type II receptors (ActR-II, ActR-IIB and BMPR-II) (Figure 2) (Rosen, 2006; Miyazono et al., 2010; Mueller and Nickel, 2012). The BMP type I and II receptors exhibit >35% amino acid similarity with one another (Figure 2). The non-osteogenic members of the TGF-β family, such as TGF-β1 and activin, bind to the type I receptors ALK4 (also called ActR-IB), ALK5 (also called TβR-I) and ALK7, suggesting that the type I receptors and their downstream effectors determine the biological activity in response to these molecules (as discussed below).

A ligand dimer binds to a tetramer of two type I and two type II receptors (Greenwald et al., 2003; Keller et al., 2004; Ehrlich et al., 2012). The kinase of the type II receptor is constitutively active independent of ligand binding and phosphorylates the GS domain of the type I receptor (Figure 3). The receptor complex activates downstream signaling components including Smads, p38 mitogen-activated protein (MAP) kinase and phosphoinositide 3 kinase (Figure 3) (Hassel et al., 2003; Nohe et al., 2004). FKBP12, a binding partner of the immunosuppressant FK506, has been shown to suppress type I receptors by binding to unphosphorylated GS domains, and the phosphorylation of the GS domain by type II receptors releasing FKBP12 (Figure 3) (Nishinakamura et al., 1997; Huse et al., 1999, 2001).

It has been reported that BMP-3 does not induce heterotopic bone formation *in vivo* and that BMP-3 knockout mice showed higher bone mineral density than wild-type mice (Daluiski et al., 2001; Gamer et al., 2009). BMP-3 bound to ActR-IIB with high affinity, suggesting that it may

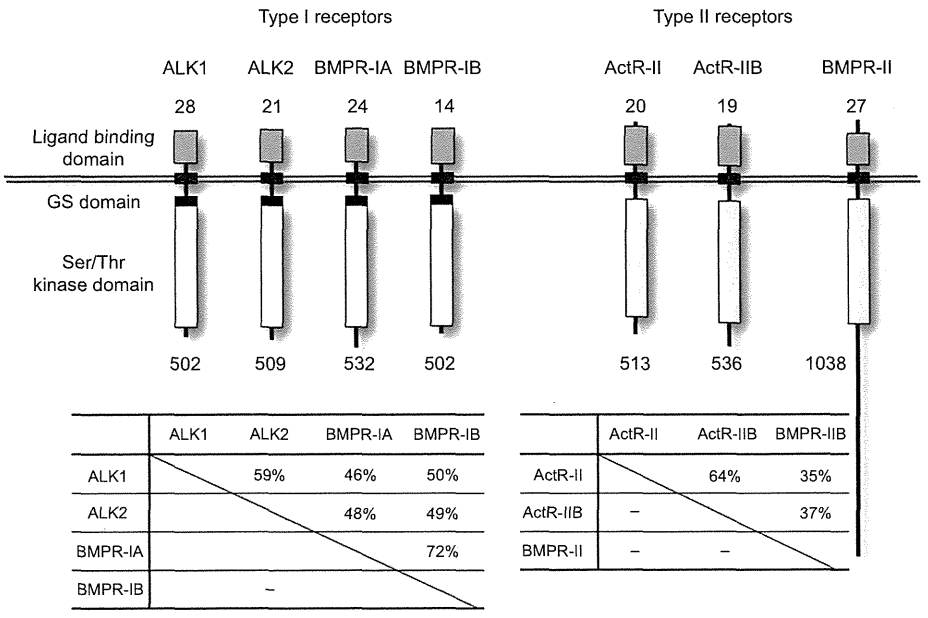


Figure 2 Type I and type II receptors for osteogenic BMPs.
Both type I and type II BMP receptors are transmembrane serine/threonine kinases, although only type I receptors have a GS domain in the juxtamembrane region. The type I and II receptors have high homology. The amino acid sequences of mouse ALK1 (accession #AAH14291), ALK2 (accession #NP001103675), BMPR-IA/ALK3 (accession #NP033888), BMPR-IB/ALK6 (accession #NP031586), ActR-II (accession #NP031422), ActR-IIB (accession #NP031423) and BMPR-II (accession #NP031587) were obtained from GenBank.

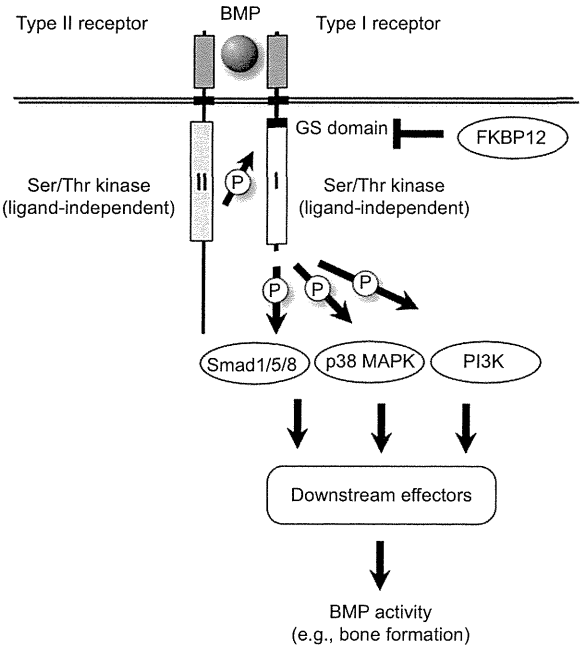


Figure 3 Intracellular signal transduction of BMPs.
A monomer of BMP binds to one type I and one type II receptor. The type II receptor phosphorylates the type I receptor at the GS domain, releasing the inhibitor, e.g., FKBP12. The activated type I receptor kinase then further activates the Smad1/5/8, p38 mitogen activated protein kinase and phosphoinositide 3 kinase pathways via phosphorylation. Their downstream effectors induce the unique activity of BMPs, including heterotopic bone formation.

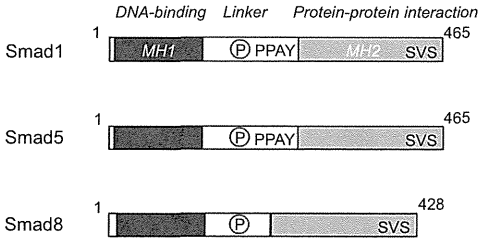
competitively inhibit other osteogenic BMPs (Kokabu et al., 2012a). BAMBI is a natural BMP receptor that lacks the kinase domain but still retains its ligand-binding domain and thereby blocks BMP activity in a dominant-negative fashion (Onichtchouk et al., 1999; Xavier et al., 2010). In addition, the substitution of a conserved asparagine residue in the GS domain has been shown to generate constitutively active type I receptor kinases, i.e., kinases that are active in the absence of ligand binding (Wieser et al., 1995; Akiyama et al., 1997; Aoki et al., 2001). These findings suggest that type II receptor-activated type I receptor kinases are critical for intracellular signal transduction.

Bone morphogenetic protein-dependent Smad-signaling

Smad1/5/8s are substrates of bone morphogenetic protein type I receptors

Among the proteins that are phosphorylated by BMP type I receptor kinases, Smad1/5/8s appear to be critical substrates for the most unique activity of BMPs (Figure 3) (Kretzschmar et al., 1997; Hassel et al., 2003). Smads are the mammalian counterparts of Sma and Mad in *Caenorhabditis elegans* and *Drosophila*, respectively, that transduce the intracellular signaling downstream of the TGF-β family of ligands (Derynck et al., 1996). Smad1, Smad5 and Smad8 have a sequence homology of more than 78% (Figure 4). In contrast to the osteogenic BMPs, the non-osteogenic members of the TGF-β family induce the phosphorylation of Smad2 and Smad3 rather than Smad1, Smad5 and Smad8 (Massague et al., 2005; Wu and Hill, 2009; Zi et al., 2012).

The receptor-regulated Smads have conserved structures, including an N-terminal Mad homology 1 (MH1) domain and a C-terminal MH2 domain that are required for the DNA-binding activity of the protein and protein – protein interactions, respectively (Figure 4) (Shi and Massague, 2003). The MH1 and MH2 domains are connected by a linker region that has multiple phosphorylation sites and an E3 ubiquitin ligase recognition sequence, the PPAY motif (Figure 4) (Bruce and Sapkota, 2012). Mouse Smad8 lacks the PPAY motif and is shorter than Smad1 and Smad5 due to a deletion of the end of the linker region. Humans have both the short and long forms of Smad8. Smad1, Smad5 and Smad8 all have a conserved C-terminal SVS motif, which is the phosphorylation site for activated type I receptor kinases (Arnold et al., 2006).



	Smad1	Smad5	Smad8
Smad1		89%	80%
Smad5	–		78%
Smad8	–	–	

Figure 4 Schematic structures of Smad1, Smad5 and Smad8.
The amino acid sequences of mouse Smad1 (accession #AAG41407), Smad5 (accession #AAB39737) and Smad8 (accession #AAF77079) were obtained from GenBank. These proteins have conserved Mad homology 1 and Mad homology 2 domains that are required for their DNA binding activity and protein–protein interactions, respectively. The linker region contains multiple phosphorylation sites and the Smurf1 recognition sequence PPAY. The type I receptors of osteogenic BMPs phosphorylate a conserved SVS motif at the C-terminus. The sequences are more than 78% homologous.

Signaling pathways other than the Smad pathway are also induced by activated BMP type I receptors (Figure 3) (Hassel et al., 2003; Nohe et al., 2004). To manipulate the Smad pathway without affecting these other pathways, a constitutively activated form of Smad1 has been established. The receptor-phosphorylation of Smad1 in the C-terminal SVS motif introduces negative charges at the two serine residues. To mimic this condition, these serine residues in Smad1 have been replaced with aspartic acid, an acidic amino acid with an additional negative charge, termed Smad1(DVD) or Smad1(2SD), see Figure 5 (Qin et al., 2001; Nojima et al., 2010). Cells over-expressing Smad1(DVD), but not wild-type Smad1, exhibit various phenotypes in the absence of BMPs, such as positive recognition by an antibody specific to phosphorylated Smad1/5/8s, the activation of luciferase reporters driven by BMP-responsive elements (BREs) and osteoblastic differentiation in C2C12 myoblasts (Qin et al., 2001; Nojima et al., 2010). Moreover, an injection of Smad1(DVD) mRNA into *Xenopus* embryos induced ventralization, a typical BMP-mediated activity in this system (Nojima et al., 2010). In contrast to osteoblastic differentiation, the inhibition of myogenesis by BMP stimulation seemed to be dependent on nuclear Smad4, which might promote the expression of *Id* genes in cooperation with phosphorylated Smad1/5/8s (Nojima et al., 2010). These findings suggest that the type I receptor – Smad signaling axis plays a central role in the unique activity of BMPs.

Target genes of bone morphogenetic protein-regulated Smads

Phosphorylated Smad1/5/8s form complexes with Smad4 in the cytoplasm that then translocate to the nucleus to regulate the transcription of target genes, both positively and negatively. The *Id1*, *Id2* and *Id3* genes have been found to be induced within 1 hour after BMP treatment in various types of mammalian cells, suggesting that these genes have similar BREs in their regulatory regions (Katagiri et al., 1994; Hollnagel et al., 1999). The first BRE, a GC-rich short element that is recognized by Smad1 and Smad4 in electrophoresis mobility shift assays, was identified in the *Id1* gene (Katagiri et al., 2002; Korchynskyi and ten Dijke, 2002; Lopez-Rovira et al., 2002). Additional conserved BREs were later identified in the *Id2* and *Id3* genes (Shin et al., 2012). Recently, the novel BMP-inducible transcript-1 was identified in a search for BREs using the sequence conserved among the promoter regions of the *Id*

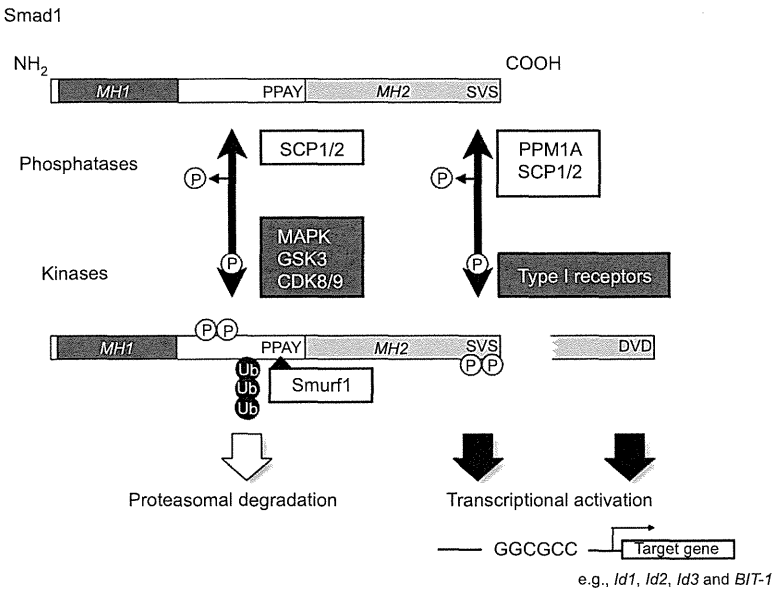


Figure 5 Positive and negative regulation of Smad1 activity by protein kinases and protein phosphatases.
The phosphorylation of a C-terminal SVS motif in Smad1 by BMP type I receptors or the mutation of this motif to DVD activates the transcription of early BMP-responsive genes such as *Id1*, *Id2*, *Id3* and *BMP-inducible transcript-1*. The protein phosphatases PPM1A and SCP1/2 have been shown to dephosphorylate this motif and inhibit BMP activity. Mitogen activated protein kinase and glycogen synthase kinase 3 phosphorylate multiple sites in the linker region to induce proteasomal degradation via Smurf1. SCP1/2 dephosphorylate residues within the linker region and stimulates the transcriptional activity of Smad1. Cyclin-dependent kinase 8/9 stimulate Smad1 through phosphorylation at the linker region.

genes (Shepherd et al., 2008; Nakahiro et al., 2010; Shin et al., 2012). The alignment of those BREs revealed a core sequence, GGCGCC, that is essential for the binding of Smad1 and Smad4 (Katagiri et al., 2002; Shin et al., 2012). An identical GGCGCC sequence was also found in human cells in a genome-wide chromatin immunoprecipitation sequence analysis using anti-phosphorylated Smad1/5/8 antibodies (Morikawa et al., 2011). Interestingly, the MDA-MD468 breast cancer cell line lacks the Smad4 locus, and *Id1* mRNA was not expressed in response to BMP-2 in these cells (Katagiri et al., 2002). However, the over-expression of exogenous Smad4 in these cells restored the induction of *Id1* mRNA by BMP-2, suggesting that Smad4 is an essential cofactor for BMP receptor-regulated Smads (Katagiri et al., 2002).

Roles of Smad post-translational modifications

Protein phosphatases for Smads

The phosphorylation of the SVS motif in Smads activates their transcriptional activity, therefore the dephosphorylation of this motif may have the opposite effect (Kokabu et al., 2012b). Some protein phosphatases that mediate this reaction have been identified (Figure 5) (Bruce and Sapkota, 2012). Protein phosphatase magnesium-dependent 1A (PPM1A) is one of the phosphatases that targets the phosphorylated SVS motif (Figure 5) (Duan et al., 2006; Lin et al., 2006), and the over-expression of PPM1A, but not an enzyme activity-deficient mutant, suppressed BMP activity, confirming that the phosphatase activity is essential for inhibition (Duan et al., 2006; Kokabu et al., 2010). However, PPM1A suppressed the activity of constitutively activated Smad1(DVD), even though the SVS motif of this protein cannot be dephosphorylated (Kokabu et al., 2010). Moreover, PPM1A decreased the protein levels of unphosphorylated and phosphorylated wild-type Smad1 and Smad1(DVD), suggesting that PPM1A may suppress Smad1 indirectly by dephosphorylating some additional substrate(s) (Kokabu et al., 2010).

Small C-terminal domain phosphatase 1 and 2 (SCP1 and SCP2) have also been shown to dephosphorylate the type I receptor-phosphorylated Smads (Knockaert et al., 2006; Sapkota et al., 2006). In addition to the C-terminus, SCP1 and -2 act on the Smad linker regions that are phosphorylated by mitogen activated protein (MAP) kinases to suppress the transcriptional activity of Smads (Figure 5) (Sapkota et al., 2006). The effects of the SCPs on Smads may therefore depend on a balance between phosphorylation and dephosphorylation at the linker region and the SVS motif. Interestingly, similar to PPM1A, SCP1 suppressed the constitutively active Smad1(DVD)-induced osteoblastic differentiation of C2C12 cells (Kokabu et al., 2011). In contrast to PPM1A, however, SCP1 did not change the protein levels of Smads and showed minimal effects on *Id1* expression, suggesting that SCP1 may target the downstream effector(s) of the BMP–Smad axis rather than the Smads themselves, at least in this model (Kokabu et al., 2011). The phosphorylation of the linker region by Cyclin-dependent kinase 8/9 has been shown to enhance Smad activity (Alarcon et al., 2009), although phosphorylation by MAPK and glycogen synthase kinase 3 (GSK3) suppresses Smad activity (Figure 5). The linker region of Smads has multiple sites that may be phosphorylated by protein kinases, therefore the combination of position-specific phosphorylation and/or the timing of phosphorylation may regulate Smad functions both positively and negatively in cooperation with other post-transcriptional modifications.

Smad ubiquitination

Smad1 and Smad5 contain PPAY motifs in their linker regions that are recognized by E3 ubiquitin ligases such as Smurf1 (Zhu et al., 1999; Sangadala et al., 2007). Smurf1 polyubiquitinates Smads to induce their degradation via the proteasome pathway, and this proteasomal degradation has been associated with linker phosphorylation (Zhu et al., 1999). In some cases, *Wnt* signaling cooperatively stimulates BMP activity (Nakashima et al., 2005; Fukuda et al., 2010). GSK3, a critical kinase in *Wnt* signaling, further phosphorylates the linker regions of Smads that have already been phosphorylated by MAP kinases, and phosphorylation by both kinases is required for the proteasomal degradation induced by Smurf1 via polyubiquitination (Sapkota et al., 2007; Verheyen, 2007).

USP15 has been identified as an enzyme that stimulates Smad deubiquitination (Inui et al., 2011). The expression of a siRNA targeting USP15 in C2C12 cells reduces the alkaline phosphatase activity induced by BMP-2 (Inui et al., 2011). Monoubiquitination at the MH1 domain of Smad3 prevents its DNA binding, while USP15 stimulates its transcriptional activity by removing ubiquitin (Inui et al., 2011). Thus, ubiquitination negatively regulates Smad activity.

Deregulated bone morphogenetic protein signaling in fibrodysplasia ossificans progressiva

Point mutations in ALK2 cause fibrodysplasia ossificans progressiva

Fibrodysplasia ossificans progressiva (FOP) is a rare autosomal dominant disorder that is characterized by progressive heterotopic bone formation in skeletal muscle tissue Kitterman et al., 2005; Katagiri, 2010, 2012; Pignolo et al., 2011). Currently, there is no effective treatment for preventing heterotopic bone formation in FOP. BMP signaling has been suggested to be involved in FOP because the process of heterotopic bone formation is similar to that induced by BMP implantation in skeletal muscle (Kaplan et al., 1990).

A recurrent heterozygous mutation in the *ACVR1* gene, which encodes the BMP type I receptor ALK2, was identified in familial and sporadic FOP patients (Shore et al., 2006). The mutation alters a single amino acid in the GS domain of ALK2 (Shore et al., 2006). Even in the absence of stimulation by BMPs, the mutant ALK2 found in FOP induces specific BMP signal transduction events, such as the phosphorylation of Smad1/5/8s and the activation of the Id1-luciferase reporter in C2C12 myoblasts (Fukuda et al., 2008, 2009). BMP stimulation of mutant ALK2-expressing cells further promoted these BMP activities, suggesting that the mutant ALK2 is mildly constitutively activated by the genetic mutation and hypersensitive to activation by type II receptors (Fukuda et al., 2009). A reduction in the affinity for FKBP12 at the GS domain was suggested to be involved in this activation (Shen et al., 2009). To date, 12 mutations have been identified in the GS or kinase domains of ALK2 in patients with typical and atypical FOP (Katagiri, 2012).

The role of the Smad pathway in fibrodysplasia ossificans progressiva

In FOP, muscle injury induces acute local heterotopic bone formation, suggesting that an inflammatory reaction synergistically activates the intracellular signaling of BMPs (Katagiri, 2010). Smad1 and Smad5 mRNA expression are transiently increased after muscle injury in mice (Fukuda et al., 2009). Co-transfection with mutant ALK2 and Smad1 or Smad5 induces osteoblastic differentiation in C2C12 cells, suggesting that the Smad-dependent pathway activated by mutant ALK2 plays a critical role in heterotopic bone formation in FOP (Fukuda et al., 2008, 2009; Ohte et al., 2011).

Based on these findings, the BMP receptor–Smad axis is being investigated as a potential target for treatments to prevent heterotopic bone formation in FOP. Some small chemical inhibitors have been generated for the BMP type I receptor kinases (Figure 6). Dorsomorphin is the first chemical inhibitor identified that is specific for the receptor-induced phosphorylation of Smad1/5/8s but not the MAP kinase pathway (Yu et al., 2008b). Dorsomorphin and its potent derivative, LDN-193189, block induction of the osteoblastic differentiation of C2C12 cells that is induced by the over-expression of the *ALK2* mutant (Fukuda et al., 2009; Ohte et al., 2011; Lowery and Rosen, 2012). In addition, LDN-193189 has been shown to reduce heterotopic bone formation in a transgenic mouse model carrying a mutant *ALK2* gene (Yu et al., 2008a). Using this model, all-*trans* retinoic acid (RA) and RARγ agonists have also been shown to inhibit heterotopic bone formation (Shimono et al., 2011). Both RA and RARγ agonists inhibited the BMP-induced Id1-luciferase reporter and reduced the protein levels of Smads, indicating that RA and RARγ inhibit BMP signaling via a different mechanism from that of dorsomorphin and LDN-193189 (Figure 6) (Shimono et al., 2011).

Recently, novel types of inhibitors for BMP signaling were reported in the development of treatments for FOP (Figure 6). NG-391 and NG-393, which are structurally-related compounds produced by a mold, were found in a screen for inhibitors of osteoblastic differentiation in C2C12 cells expressing the *ALK2* mutant found in FOP (Figure 6) (Fukuda et al., 2011). From another screen using 1040 US Food and Drug Administration-approved drugs, fendiline hydrochloride and perhexiline maleate were found to inhibit the Id1-luciferase activity induced by the *ALK2* mutant found in FOP (Yamamoto et al., 2012). Perhexiline reduced the phosphorylation levels of Smad1/5/8s *in vitro* and decreased the heterotopic bone volume induced by BMP implantation *in vivo* (Figure 6) (Yamamoto et al., 2012). Induced pluripotent stem cells provide a powerful system in which to study human diseases, particularly rare genetic disorders such as FOP. Recently, it was found that the generation of induced pluripotent stem cells from FOP skin fibroblasts was repressed due to the constitutive activation of *ALK2* (Hamasaki et al., 2012). The treatment of the cells with an *ALK2* inhibitor, LDN-193189, overcomes this repression, suggesting that phosphorylated Smad1/5/8s are involved in this repression (Hamasaki et al., 2012). RK-0071142 was also identified as a candidate drug for FOP using this system (Figure 6) (Hamasaki et al., 2012). Allele-specific RNA interference is a novel type of

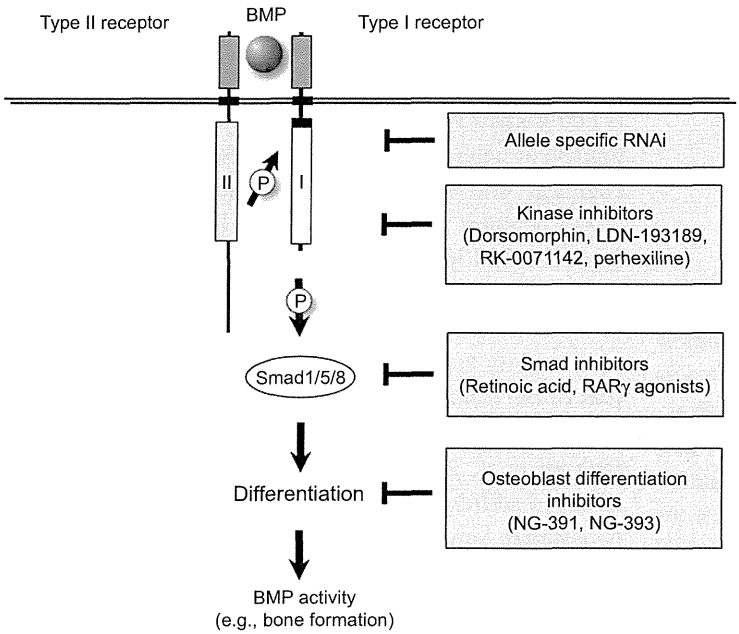


Figure 6 Specific inhibitors of the BMP type I receptor–Smad1/5/8 signaling axis at multiple steps.
Novel inhibitors have been developed for the specific suppression of the BMP type I receptor–Smad1/5/8 signaling axis. An allele-specific RNAi has been established for the mutant type I receptor, *ALK2*. Kinase inhibitors block the phosphorylation of Smad1/5/8 at the SVS motif. Smad inhibitors reduce the protein levels of Smad1/5/8 and also Smad4. Osteoblast differentiation inhibitors suppress the BMP signaling-induced pathway that promotes the differentiation of C2C12 myoblasts into osteoblast lineage cells. These inhibitors are all candidate therapeutic drugs for pathological BMP signaling diseases, including fibrodysplasia ossificans progressiva.

inhibition that is specific for the *ALK2* mutant (Lowery and Rosen, 2012). Although siRNAs targeting *ALK2* carrying one of the FOP mutations also reduced the levels of wild-type *ALK2* mRNA, the introduction of an additional mismatch in each siRNA conferred allele specificity for each mutant *ALK2* (Figure 6) (Takahashi et al., 2012).

Open questions regarding the bone morphogenetic protein signaling pathway

Although BMPs have been identified as bone-inducing factors in the bone matrix, their functions are not limited to skeletal tissue and have expanded to various tissues. It is still unclear how BMPs show these variable activities in tissues through the same receptor–Smad signaling system. It is possible that novel, tissue-specific non-Smad pathways and/or novel coactivators and corepressors of Smads are involved in the functions. The identification of such novel signaling pathways or factors will help us to understand the regulatory mechanisms of BMP functions in various tissues. Moreover, it may also help us to establish novel treatments for diseases caused by the deregulation of BMP signaling.

Conclusions

BMPs are able to induce heterotopic bone formation in skeletal muscle tissue. Their intracellular signaling is transduced by the ligand-bound type I and type II receptor kinases; among the proteins phosphorylated in response to type I receptor activation, Smad1/5/8s are critical effectors of BMP activity. Thus, inhibitors of this signaling pathway are potential candidates for therapeutic drugs for diseases caused by the pathological activation of BMP signaling, such as FOP.

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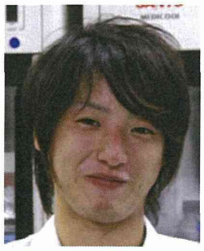

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RESEARCH Open Access

Perhexiline maleate in the treatment of fibrodysplasia ossificans progressiva: an open-labeled clinical trial

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Abstract

Background: Currently, there are no effective medical treatment options to prevent the formation of heterotopic bones in fibrodysplasia ossificans progressiva (FOP). By the drug repositioning strategy, we confirmed that perhexiline maleate (Pex) potentially ameliorates heterotopic ossification in model cells and mice. Here, we conducted a prospective study to assess the efficacy and safety of Pex in the treatment of FOP patients.

Methods: FOP patients in this open-label single-center study were treated with Pex for a total of 12 months, and followed up for 12 consecutive months after medication discontinuation. The safety of the treatment was assessed regularly by physical and blood examinations. The efficacy of Pex for preventing heterotopic ossifications was evaluated by the presence of flare-ups, measurements of serum bone markers, and changes in the total bone volume calculated by the three-dimensional computed tomography (3D-CT) images.

Results: Five patients with an average age of 23.4 years were enrolled. Within safe doses of Pex administration in each individual, there were no drug-induced adverse effects during the medication phase. Three patients showed no intense inflammatory reactions during the study period, while two patients had acute flare-ups around the hip joint without evidence of trauma during the medication phase. In addition, one of them became progressively incapable of opening her mouth over the discontinuation phase. Serum levels of alkaline phosphatase (ALP) and bone specific ALP (BAP) were significantly and synchronously increased with the occurrence of flare-ups. Volumetric 3D-CT analysis demonstrated a significant increase in the total bone volume of Case 2 (378 cm^3) and Case 3 (833 cm^3) during the two-year study period.

Conclusions: We could not prove the efficacy of oral Pex administration in the prevention of heterotopic ossifications in FOP. Serum levels of ALP and BAP appear to be promising biomarkers for monitoring the development of ectopic ossifications and efficacy of the therapy. Quantification of change in the total bone volume by whole body CT scanning could be a reliable evaluation tool for disease progression in forthcoming clinical trials of FOP.

Keywords: Fibrodysplasia ossificans progressiva, Perhexiline maleate, Clinical trial, Biomarker, Whole body CT

Background

Fibrodysplasia ossificans progressiva (FOP) (OMIM: 135100) is a severely disabling heritable disorder of connective tissue characterized by congenital malformations of the great toes and progressive heterotopic ossification in various extraskeletal sites. FOP is very rare with a worldwide prevalence of approximately 1/2,000,000 [1]. It is caused by a recurrent activating mutation (617G > A, R206H) in the gene encoding activin A receptor type I (*ACVR1*)/activin-like kinase 2 (*ALK2*), a bone morphogenetic protein (BMP) type I receptor [2]. In FOP, the mutant receptor causes up-regulation of a transcriptional factor, *Id1*. Typically, during the first decade of life, sporadic episodes of painful soft tissue swellings (flare-ups) occur, which can transform skeletal muscles, tendons, ligaments, fascia, and aponeuroses

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into heterotopic bone [3]. Progressive heterotopic ossifications span the joints, lock them in place, and render movement impossible [4]. Immobility is cumulative and most patients are wheelchair-bound by the end of second decade of life [5]. Attempts to remove heterotopic bones usually lead to explosive new bone formation.

At present, there is no definitive pharmacotherapy to prevent progressive heterotopic ossifications in FOP. Recently, dorsomorphin and LDN-193189, a selective inhibitor of BMP type I receptor kinases, have been reported to inhibit activation of the BMP signaling in cultures cells and mice [6,7]. Similarly, CD1530, an agonist of nuclear retinoic acid receptor-γ, prevented heterotopic ossification in FOP model mice [8]. None of these compounds, however, has been applied in clinical practice.

A promising alternative for orphan diseases is the drug repositioning strategy, in which a drug currently used for patients with a specific disease is applied to another disease [9]. The advantage of this strategy is that the identified drugs are readily available and the adverse effects are known. In order to search for clinically applicable drugs for FOP, we screened 1040 FDA-approved drugs for suppression of the *Id1* promoter activated by the mutant *ACVR1/ALK2* in mouse C2C12 myoblasts. We found that perhexiline maleate (Pex), which is a prophylactic antianginal drug widely used for stable angina but its use markedly declined in the early 1980s after reports of hepatotoxicity and peripheral neuropathy, suppressed the *Id1* promoter activity and mRNA expression of native *Id1* and alkaline phosphatase by down-regulating phosphorylation of Smad1/5/8. Pex also reduced the volume of heterotopic ossification in crude BMP-induced model mice [10]. Here, we conducted an open-labeled clinical trial of Pex administration in the management of FOP.

Methods

This study was a non-randomized, non-placebo-controlled investigation to prospectively estimate the effect of Pex treatment in FOP patients. Eligible for participation were the patients who presented classic features of FOP including congenital malformation of the great toes and progressive heterotopic ossification of soft tissues, and those who had R206H mutation in the *ACVR1/ALK2* gene [11]. Because safety of Pex administration in children has not been established, skeletally immature patients were excluded from the study. Since there is no known effective treatment in preventing heterotopic ossification of FOP, we did not exclude the patients who received concurrent use of other medications, such as nonsteroidal anti-inflammatory drugs (NSAIDs) or cyclooxygenase-2 (COX-2) inhibitors. After approval from the Institutional Review Boards of the Nagoya University, patients who provided written informed consent were enrolled in the study.

All patients continued to receive Pex administration for a total of 12 months. At the end of this period, they discontinued Pex pharmacotherapy and were monitored for 12 consecutive months of discontinuation follow-up phase. After two weeks administration of an initial dose of 100 mg/day, plasma concentration of Pex was measured to adjust the dosage in each individual. Therapeutic drug monitoring was then regularly performed during the medication phase by Drs. John D. Horowitz and Benedetta C. Sallustio (Queen Elizabeth Hospital, Woodville, Australia), and an optimal dose of oral Pex administration was individually determined based on a range for Pex of 0.15-0.60 mg/L. The Safety of treatment was assessed by a monthly physical examination and a complete blood count/serum chemistry evaluation every three months, with a special care for known adverse effects of Pex including peripheral neuropathy and drug induced hepatic dysfunction [12]. The efficacy of Pex for preventing heterotopic ossifications was evaluated clinically and biochemically, as well as by volumetric computed tomography (CT). Careful physical examination was performed on each patient to observe the presence of flare-ups and the development of new ectopic ossifications. Serum concentrations of non-specific alkaline phosphatase (ALP), bone-specific alkaline phosphatase (BAP) and osteocalcin (OC) were measured at baseline, after 1, 3, 6, 9, and 12 months of Pex treatment (M: medication phase), and after 1, 3, 6, 9, and 12 months of medication discontinuation (D: discontinuation phase), using the commercially available Japan Society of Clinical Chemistry (JSCC) method, enzyme immunoassay (EIA), and radioimmunoassay (RIA) respectively (SRL Inc, Japan). For quantitative evaluation of ectopic bones to be formed, whole body scanning by 16 slice multi-detector CT was performed before the intervention (baseline), at the end of Pex medication (M-12 m: 12 months after commencement of treatment), and at the end of the study (D-12 m: 12 months after medication discontinuation). Due to various degrees of contractures in the upper and lower extremities as well as in the trunk, the top of the skull or periphery of the limbs sometimes failed to be imaged in some patients. Thus, we defined structural regions of interest (ROI) as the maximum 3D-CT imaging ranges to be analyzable which was standardized in each individual. Based on 3D-CT images, total bone volume (expressed as cm^3) in each patient was calculated by quantitative density analysis. The volume of newly formed bones was quantified by change in the total bone volume during the medication and discontinuation phases.

Table 1 Patients' characteristics and clinical outcome

Case	Age (years)	Gender	Dose of Pex	Adverse events	Acute inflammatory reaction (site)
1	36	Male	150 mg/d	None	None
2	26	Male	200 mg/d	None	M-7 m (right proximal thigh)
3	18	Female	75 mg/d	None	M-8 m (left hip), D-2 m (right jaw)
4	18	Female	14 mg/d	None	None
5	19	Male	100 mg/d	None	None

Pex = Perhexiline maleate; M-7 m = Medication phase at 7 months.
M-8 m = Medication phase at 8 months;
D-2 m = Discontinuation phase at 2 months.

Result

Five FOP patients were enrolled in the study between July 2010 and July 2012 (Table 1). There were three males and two females with an average age of 23.4 years (range, 18–36 years). All patients had significant deformities associated with severely restricted mobility of the spine and limbs. Two patients were confined to a wheelchair and required assistance in performing activities of daily living. Two patients received concurrent treatment with COX-2 inhibitor on a regular basis, and three patients irregularly took fast-acting NSAIDs when they felt pain. Under strictly controlling plasma concentration of Pex within 0.15-0.60 mg/L, the steady dosage of Pex varied between individuals from 14 mg/day (100 mg/week) to 200 mg/day. No obvious drug-induced adverse effects were found and no patients discontinued Pex administration during the whole period of treatment.

In three of the five patients, there were no intense inflammatory reactions associated with flare-ups during the study period, although this could happen randomly (Table 1). On the other hand, acute flare-ups were observed in two patients (Cases 2 and 3) without evidence of trauma during the medication phase (M-7 m and M-8 m, respectively) and high-dose corticosteroid treatment was administered in each patient at the beginning of flare-ups according to the treatment guidelines of International Fibrodysplasia Ossificans Progressiva Association (IFOPA) [13]. These flare-ups occurred in the right proximal thigh in Case 2 and

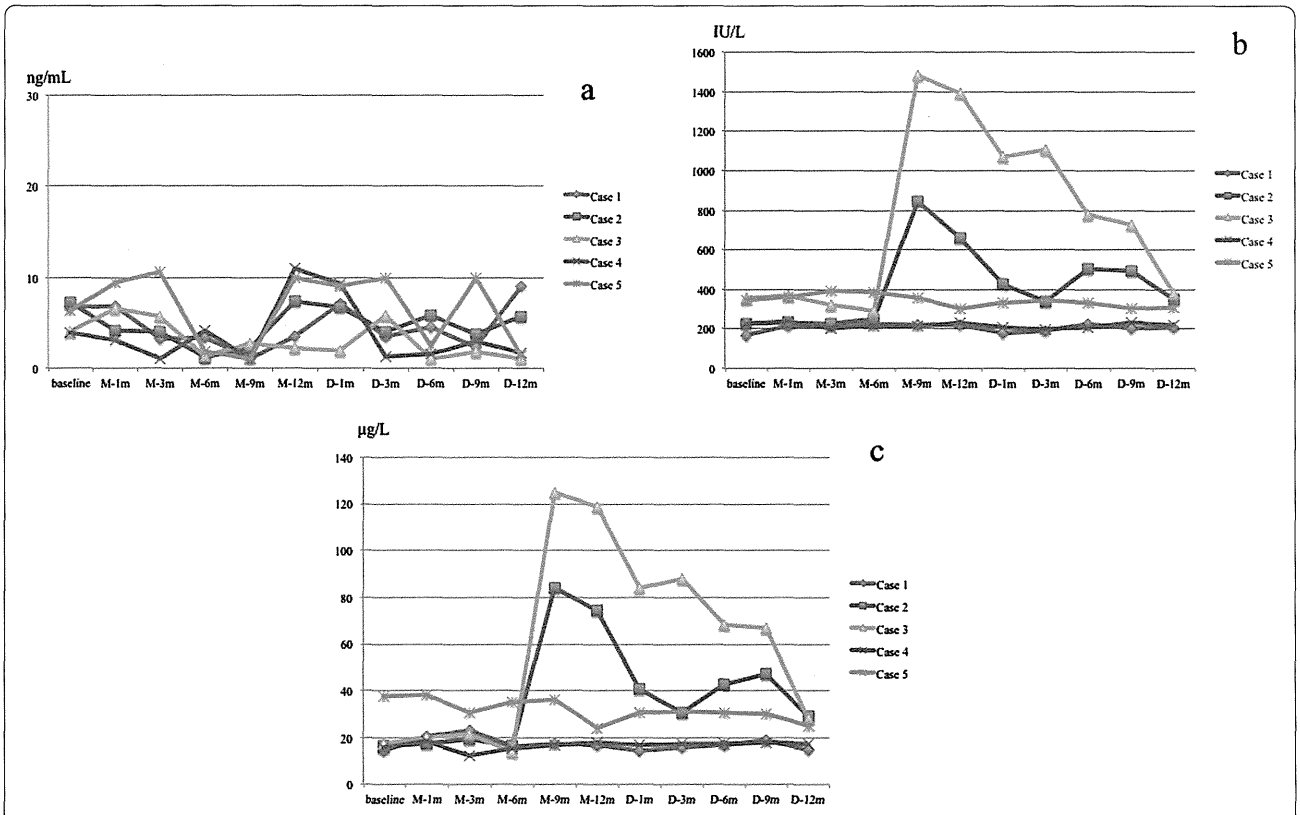

Figure 1 Serial measurements of serum osteocalcin (a), alkaline phosphatase (ALP) (b) and bone-specific alkaline phosphatase (BAP) (c) from 5 FOP patients. Serum levels of osteocalcin were maintained within the normal range during the study period in all patients. Serum levels of ALP rapidly elevated at M-9 m in Cases 2 and 3 who showed acute flare-ups at M-7 m and M-8 m, respectively. Case 3, who showed acute inflammation of her right jaw at D-2 m, had persistent higher ALP levels at D-3 m. Changes in serum BAP activity positively correlated to those in serum ALP levels.

around the left hip joint in Case 3. Following flare-ups, their hip joint mobility gradually deteriorated. In addition, Case 3 complained of severe right jaw pain and subsequent difficulty in mouth opening during the early discontinuation phase (D-2 m). Limited opening of the mouth resulted in interference with eating and oral hygiene.

Serum concentration of OC had no significant change in all patients (Figure 1a). During the whole study period, serum ALP and BAP levels were maintained at a normal range in the three patients who did not have inflammatory reactions. In the other two patients (Cases 2 and 3), on the other hand, these bone markers significantly and synchronously elevated following the occurrence of flare-ups during medication phase (Figure 1b,c). Elevated ALP and BAP levels were gradually reduced with time, but in Case 3, both bone markers rebounded with acute inflammation of her right jaw during the early discontinuation phase.

Quantitative 3D-CT analysis demonstrated that the total bone volume did not change in three patients (Cases 1, 4, and 5) during the study period, while a substantial increase in the total bone volume, both during the medication and discontinuation phases, was found in two patients (Figure 2a,b). In Case 2, increased bone volume of 223 cm^3 during the medication phase and that of 155 cm^3 during the discontinuation phase seemed to be associated with heterotopic bone formations in the right adductor muscle, and around the mid-femur, respectively (Figure 3a-g). In Case 3, there was an increase of 297 cm^3 in the total bone volume during the medication phase, which seemed to be related to intramuscular ossification in her left iliopsoas (Figure 4a-d). She also showed a maximal increased

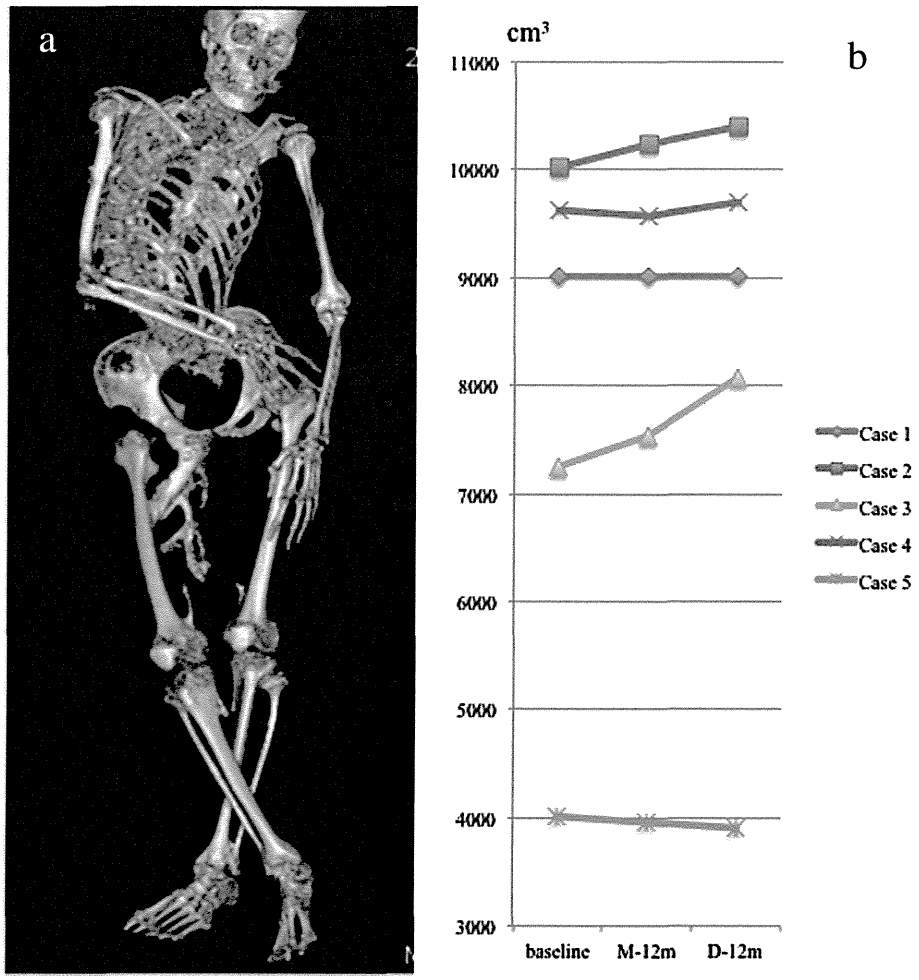

Figure 2 Volumetric computed tomography (CT) examination. Representative reconstructed 3D-CT image obtained by whole body scanning of Case 1 **(a)**, and changes in total bone volume within the region of interests (ROI) in each FOP patient **(b)**. In Case 2, total bone volume has increased 223 cm^3 and 155 cm^3 during the medication and discontinuation phases, respectively. In Case 3, there was an increase of 297 cm^3 and 536 cm^3 in the total bone volume during the medication and discontinuation phases, respectively.

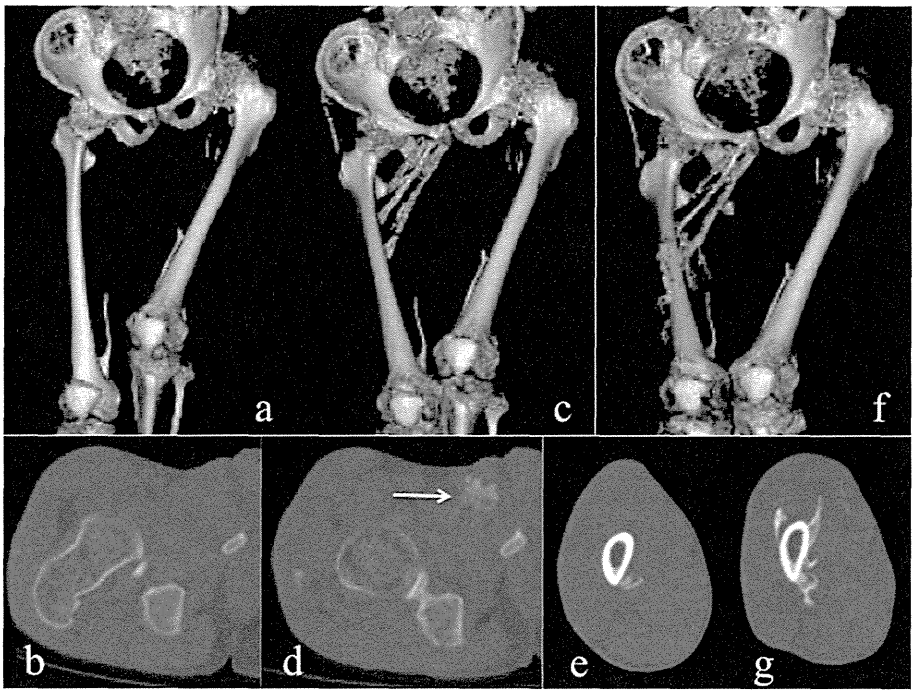

Figure 3 CT images from Case 2. Reconstructed 3D-CT images of the pelvis and bilateral femora at baseline **(a)**, at M-12 m **(c)**, and at D-12 m **(f)**. Axial CT images of the right hip at baseline **(b)**, and at M-12 m **(d)**, and those of the right mid-femur at M-12 m **(e)**, and at D-12 m **(g)**. Heterotopic ossifications in the right adductor muscle (arrow) developed during the medication phase and around mid-femur developed during the discontinuation phase ware clearly demonstrated.

bone volume of 536 cm^3 during the discontinuation phase, which appeared to reflect maturation of the iliopsoas ossification and newly developed bones in the gluteus medius (Figure 4e-g) and around the right jaw joints (Figure 4h,i).

Discussion

To date, there are few clinical trials in the treatment of FOP. Zasloff et al. [14] conducted a prospective study to assess the efficacy of isotretinoin (13-cis-retinoic acid) in the prevention of heterotopic ossification in FOP, and concluded that isotretinoin had no apparent effect in the prevention of new bone formation after surgery or after soft tissue trauma. Brantus and Meunier [15] evaluated the effects of intravenous administration of etidronate and oral corticosteroids for thirty-one FOP attacks in seven patients, and observed 10 new ossifications causing severe deterioration of joint mobility during the mean six years of follow up. These studies indicated that there is no proven efficacy with any therapy in changing the natural history of the disease. Despite the Pex treatment, heterotopic ossification developed rapidly in our two patients suggesting that oral administration of Pex within 0.15-0.60 mg/L seemed to be unsatisfactory in the inhibition of heterotopic ossifications in FOP. Moreover, there is a concerning possibility that Pex administration unexpectedly induced heterotopic ossification in these patients.

Abnormal biochemical measurements of bone mineral metabolism have rarely been reported in FOP [16]. Establishment of useful biomarkers as correlates of disease severity and clinical outcome is desirable to enable early proof-of-concept studies that can help screen potential drug candidates and identify therapeutic targets. Kaplan et al. [17] described that serum ALP activity may increase during disease flare-ups. Our serial clinical and biochemical evaluations demonstrated that elevation of serum ALP and BAP, which synchronized with acute flare-ups, preceded the heterotopic new bone formations. Serum levels of ALP or BAP could be useful biomarkers for monitoring the development of heterotopic ossifications and efficacy of the therapy in FOP.

Multi-detector-row CT has widely been used in clinical environments, and whole body scanning allows 3D structural characterization of entire bone segment at high resolution [18]. Despite the restricted movement and joint immobilization of our patients, standardization of ROI in each individual could allow for accurate volume calculating capabilities. To the best of our

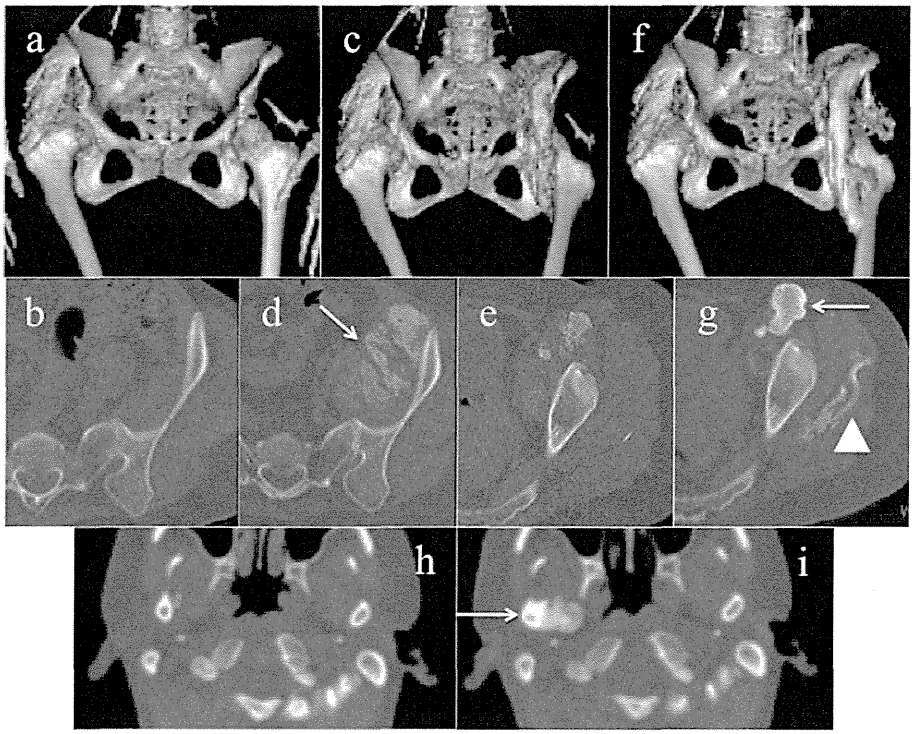

Figure 4 CT images form Case 3. Reconstructed 3D-CT images of the hip joints at baseline **(a)**, at M-12 m **(c)**, and at D-12 m **(f)**. Axial CT images of the left pelvis at baseline **(b)**, at M-12 m **(d, e)**, and at D-12 m **(g)**, and those of the bilateral jaws at M-12 m **(h)**, and at D-12 m **(i)**. Massive new bone formations in the left iliac muscle were developed during the medication phase **(d**, arrow**)**. During the discontinuation phase, the left intra-iliopsoas ossification matured **(g**, arrow**)**, and heterotopic boned in the left gluteus medius **(g**, arrow head**)** and the right jaw joint **(i**, arrow**)** were newly formed.

knowledge, no studies have presented quantitative assessment of ectopic bone formations in FOP. Volumetric 3D-CT analyses demonstrated that change in the total bone volume correlated with the clinical symptoms and laboratory examinations in two patients who showed active flare-ups. Our study highlights greater capabilities of whole body CT scanning as an evaluation tool for disease progression in FOP, especially in assessment of treatment efficacy during forthcoming clinical trials.

There are several major limitations in the present study. First, since there are no better natural history studies for FOP to date, it is difficult to design any clinical trial with meaningful endpoints. Furthermore, we do not know the natural evolution of ectopic bone formations in our patients. Second, the present study could not be designed for pediatric FOP patients because of uncertainty in safety, tolerability, and pharmacokinetics of Pex in the pediatric population. Third, heterotopic ossification in FOP is generally formed via an endochondral ossification process, but we did not confirm heterotopic cartilage formation in our patients. Quantification of total bone volume based on 3D-CT images could be a reliable evaluation tool for assessment of ectopic bone formations, but radiation exposure by CT examination may be a major issue for young patients. Besides, whole body scanning has always been uncomfortable for severely deformed FOP patients, although it takes less than five minutes. Without scanning the whole body, heterotopic ossification following flare-ups (if they occur) could be evaluated by a narrow scan around the flare-ups region. For future clinical trials, standardization of imaging protocol will be expected in evaluating heterotopic bones in FOP.

Conclusions

Although the number of patients is too small to draw reliable conclusions, oral administration of Pex within the safety dose seemed not to be effective in the inhibition of heterotopic ossifications in FOP, despite the absence of significant adverse effects.

Abbreviations

FOP: Fibrodysplasia ossificans progressiva; ACVR1: Activin A receptor type I; ALK2: Activin-like kinase 2; BMP: Bone morphogenetic protein; Pex: Perhexiline maleate; NSAIDs: Nonsteroidal anti-inflammatory drugs; COX-2: Cyclooxygenase-2; CT: Computed tomography; ALP: Alkaline phosphatase; BAP: Bone-specific alkaline phosphatase; OC: Osteocalcin; JSCC: Japan society of clinical chemistry; EIA: Enzyme immunoassay; RIA: Radioimmunoassay;

3D: Three-dimensional; ROI: Regions of interest; IFOPA: International Fibrodysplasia ossificans progressiva association.

Competing interests
The authors declare that they have no competing interests.

Authors' contribution
H Kitoh did most of the patients' follow up, participated in the whole study and drafted the manuscript. MA did the CT interpretations. H Kaneko, MK, MM, IK participated in the clinical trial. JDH and BCS measured plasma concentration of Pex and suggested an optimal dose of Pex administration. KO and NI participated in the design of the study. All authors contributed to elaborating the manuscript. All authors read and approved the final manuscript.

Authors' information
H Kitoh is a member of the Japanese Research Committee on Fibrodysplasia Ossificans Progressiva.

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ORIGINAL ARTICLE

A genome-wide sib-pair linkage analysis of ossification of the posterior longitudinal ligament of the spine

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Abstract Ossification of the posterior longitudinal ligament of the spine (OPLL) is a common musculoskeletal disease among people after middle age. The OPLL presents with serious neurological abnormalities due to compression of the spinal cord and nerve roots. The OPLL is caused by genetic and environment factors; however, its etiology and pathogenesis still remain to be elucidated. To determine the susceptibility loci for OPLL, we performed a genome-wide linkage study using 214 affected sib-pairs of Japanese. In stratification analyses for definite cervical OPLL, we found loci with suggestive linkage on 1p21, 2p22–2p24, 7q22, 16q24 and 20p12. Fine mapping using additional markers detected the highest non-parametric linkage score (3.43, $P = 0.00027$) at D20S894 on chromosome 20p12 in a

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subgroup that had no complication of diabetes mellitus. Our result would shed a new light on genetic aspects of OPLL.

Keywords OPLL · Sib-pair · Genome-wide linkage study

Introduction

Ossification of the posterior longitudinal ligament of the spine (OPLL; MIM 602475) is ectopic ossification due to abnormal endochondral ossification of the posterior longitudinal ligament. The incidence of OPLL is 1.9–4.3 % in Japan [1, 2]. Comparable incidence has been reported in other countries, especially in East Asia [3]. Its average age of onset is over 50 years with male predominance. The OPLL presents with neurological disturbances due to compression of the spinal cord and nerve roots, nociceptive and neuropathic pain, and stiffness of the trunk. These symptoms affect motility and quality of life of the patients.

Many reports on the underlying mechanism of OPLL have suggested that the pathogenesis of OPLL is linked to genetic and environmental determinants. Genetic factors have been implicated by the epidemiological studies [4–6]. Case–control association studies and affected sibling-pair linkage studies have shown a number of genes/loci that link to OPLL susceptibility, including COL11A2 [7], COL6A1 [8], NPPS [9], BMP2 [10], BMP4 [11], TGF-β1 [12], TGF-β3 [13], RUNX2 [14], HLA haplotype [15], retinoic X receptor β [16], VDR [17], ER [18], IL-1β [18], IL-15RA [19] and TLR5 [20]. However, none of them are undisputable. The genetic factors of OPLL remain mostly unclear. To examine the genetic factor(s) of OPLL, we performed a genome-wide linkage study using 214 affected sib pairs.

Materials and methods

Subjects

The OPLL was diagnosed by experienced spinal surgeons of the participating hospitals based on the examination of plain X-ray films of the spine. Ectopic bone formation of the posterior longitudinal ligament in the cervical, thoracic and lumbar regions of the spine was separately evaluated. The numbers of ossified vertebrae in the regions of the spine were recorded. The siblings of OPLL patients were screened for OPLL and identified affected siblings were

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Table 1 Result of linkage analysis with various stratification

Population	Whole	Definite cervical spine OPLL				
Stratification item:		OPLL severity		Age	DM	Obesity
Exclusion criteria		<2 cervical vertebrae	<3 cervical vertebrae	Age at Dx ≥65 years	History of DM	BMI ≥30
% male	55.1	56.9	59.8	55.6	53.5	57.4
Age (years)						
Mean	61.1	62.1	62.5	54.3	62.4	62.4
SD	10.1	9.6	9.7	5.8	10.0	9.3
BMI (kg/m^2)						
Mean	24.8	24.6	25.0	25.2	24.4	23.6
SD	4.2	4.0	4.1	4.4	4.1	2.8
Sib pair						
Number of pairs	214	154	89	74	103	115
Family structure						
Pair	181	135	82	66	89	102
Trio	12	5	2	4	4	5
Quartet	3	3	1	0	2	1
Markers showing suggestive linkage (NPL score)	(–)	D1S206 (2.55) D2S367 (2.23) D2S165 (2.20)	D2S165 (2.65) D2S305 (2.59)	D16S520 (2.31)	D1S206 (2.64) D7S515 (2.21) D20S186 (2.21)	(–)

BMI body mass index, *DM* diabetes mellitus

recruited to this study, irrespective of the presence of their symptoms related to OPLL. DNA samples and clinical data were collected for all sib pairs in 31 participating hospitals. All the participants provided written informed consent. This research project was approved by the ethical committees at the Center for Genomic Medicine (formerly, SNP Research Center), RIKEN and the participating institutions.

Microsatellite genotyping/genome scan

The ABI PRISM linkage mapping set version 2.5-MD10 (Applied Biosystems) was used for genotyping. Genetic mapping information on 400 markers of the mapping set was obtained from the Marshfield Medical Research Foundation [21]. Because 41 of the 400 markers were not polymorphic (heterozygosity <0.60), 47 additional markers were selected from the database to fill the gaps. Allele frequencies of the additional markers and their heterozygosity in Japanese have already been reported [22]. Their primer sequences were modified to facilitate genotyping [23]. Polymerase chain reaction (PCR) amplification was performed in 96-well plates in a volume of 5 μl, containing 5 ng of genomic DNA. Reaction components and programs of the GeneAmp 9700 thermal cyclers (Applied Biosystems) were set according to the manufacturers' protocols. The PCR products were analyzed on a ABI PRISM 3730xl DNA Analyser (Applied Biosystems), and genotyped using GeneMapper (version 4.0) software (Applied Biosystems). Insertion and deletion polymorphisms were genotyped by direct sequencing of PCR products using a ABI PRISM 3730xl DNA Analyser according to manufacturer's protocols. Mendelian inconsistencies in genotype data were checked and corrected using the Checkfam program [24].

Stratification analysis

For stratification, we first confined subjects only to those who had definite cervical OPLL. We examined linkage for sib pairs who had OPLL of more than two vertebral segments. We further divided the subjects with definite cervical OPLL by age at diagnosis of OPLL, presence/absence of diabetes mellitus (DM), obesity based on body-mass index (BMI) and OPLL severity based on the number of ossified vertebrae, and analyzed their linkage for the whole genome.

Fine mapping

We selected 25 known markers around the linkage peaks in 1p21, 2p22–2p24, 7q22, 16q24 and 20p12 based on information in the database (Heterozygosity of the Microsatellite Markers in the Japanese Population,